Epidemiological, immunological and virological aspects of acute and chronic hepatitis C virus infections

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"Science investigates; religion interprets. Science gives man knowledge, which is power; religion gives man wisdom, which is control. Science deals mainly with facts; religion deals mainly with values. The two are not rivals. They are complementary. Science keeps religion from sinking into the valley of crippling irrationalism and paralyzing obscurantism. Religion prevents science from falling into the marsh of obsolete materialism and moral nihilism."

Dr. Martin Luther King, Jr.

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Chapter 1

General Introduction

Chapter 1 includes sections of chapters published in:

- *Genotypering van hepatitis-C-virus ten tijde van nieuwe behandelingsmogelijkheden uitdagingen en oplossingen. J. Schinkel, X.V. Thomas, R. Molenkamp. Nederlandse Vereniging voor Medische Microbiologie 2012:20 34-83.*
- *Hepatitis C virus reinfection following treatment among people who use drugs. B.P.X. Grady, J. Schinkel, X.V. Thomas, O. Dalgard. Clinical Infectious Diseases 2013:57.*

Hepatitis C virus (HCV) is a single-stranded positive-sense RNA virus classified as a member of the *Hepacivirus* genus in the family *Flavirviridae*, and was first described by Choo et al. in 1989 as the causative agent of non-A-non-B post-transfusion hepatitis [1]. HCV is a major cause of blood-borne infections and chronic liver disease throughout the world with no vaccine available to prevent infections in individuals at risk.

Epidemiology

HCV is a major global health problem with an estimated 180 million chronically infected individuals, and this number is thought to be increasing with millions of new infections each year [2-5]. In Europe the estimated HCV prevalence ranges from 0.5% to ≥ 3.0%, with the highest prevalence in Southern and Eastern European countries [6]. The estimated seroprevalence of HCV in the Netherlands is 0.22% (range 0.07%-0.37%), which would correspond to 28100 (range 9600-48000) HCV-infected individuals. Of these individuals, the largest HCV-infected group with a prevalence of >2%, are from first-generation migrants from HCV-endemic countries and is followed by injecting drug users (IDUs) and human immunodeficiency virus (HIV)-infected men who have sex with men (MSM) [7].

Transmission

Any behaviour, occupation, or medical condition that results in frequent percutaneous exposure to blood presents an important risk for acquiring an HCV infection. The largest risk-groups are IDUs, recipients of blood products prior to 1992 and immigrants from HCV-endemic countries [5,8,9]. Vertical HCV transmission occurs in 3-5% of HCV-infected mothers and is increased if the mother is co-infected with HIV [10]. Even though sexual transmission of HCV is rare, the incidence of acute HCV infections among HIV-infected MSM has significantly increased since 2000 [11–14]. The increased incidence of HCV infections among these men suggests that sexual transmission is possible and might be enhanced by certain sexual techniques and co-morbidities [15–17].

Molecular epidemiology

To date seven genotypes and >100 subtypes have been identified. Sequence similarity between genotypes is 65-70%, whereas within genotypes there is a 70%-85% nucleotide similarity [18,19]. Within subtypes, especially in epidemiologically related individuals with high-risk behaviour, the genetic similarity between variants is even greater [20]. The distribution of HCV genotypes depends on geographical area, route of transmission and the epidemic history [21,22]. The prevalence of HCV genotypes differs between geographic areas and does not follow the same epidemiologic pattern [23]. Worldwide, the predominant genotype is HCV genotype 1, followed by HCV genotypes 2 and 3. The remaining genotypes are more restricted to specific geographic areas [23]. In the Netherlands, HCV genotype 1 is the most prevalent, followed by genotypes 3, 2 and 4 respectively [24].

Besides geographical determinants, transmission route is another important factor for distribution of genotypes. Within IDU populations, HCV genotypes 1a and 3a are the most dominant while genotype 1b is more prevalent in patients who acquired HCV through blood transfusion [23,25–27]. Among HIV-infected MSM, HCV genotypes 1a and 4d are the most common [28]. HCV variability makes it possible to investigate both old and new transmission-networks in detail. This is exemplified by studies on the introduction of HCV among HIV-infected MSM in the late nineties. Phylogenetic analysis showed that

new HCV infections in this population were part of a great international transmission network of HIV-infected MSM [12].

HCV infection

Clinical course of infection

Acute HCV infections are rarely diagnosed because of the asymptomatic nature of the infection. In the acute phase only 20% of infected individuals experience mild aspecific symptoms like fatigue, nausea, flu-like symptoms, abdominal pain and occasionally jaundice [29,30]. HCV infections are usually not detected until alanine aminotransferase (ALT) levels rise [31]. T-cells and HCV-antibodies do not become detectable until 8-12 weeks after exposure [32–36]. Within this period of time approximately 10-15% of individuals develop a strong immune response and are able to spontaneously eradicate the HCV infection. The ability to naturally clear this infection has been associated with age at time of infection, female sex, favourable IL28B single-nucleotide polymorphism rs12979860 (i.e. genotype "CC"), hepatitis B virus (HBV) co-infection, HIV-negative status and high CD4+ T-cell counts in HIV-infected individuals [37–39]. Unfortunately 70-80% of HCV infected individuals are not able to spontaneously clear their infection and become chronically infected [40].

During the chronic phase of the infection, that is when HCV RNA measurements have been positive for more than 6 months [41], clinical presentation remains similar as during the acute phase of the infection. Chronically infected patients may develop chronic inflammation and progressive liver fibrosis, leading to liver cirrhosis in approximate 20% of individuals within 20-25 years [42,43]. Of those, at least 1-4% of individuals develop hepatocellular carcinoma (HCC) [44]. If the individual is of female sex or young age at time of infection, the disease progression is slower [39]. Accelerated disease progression has been associated with non-alcoholic fatty liver disease, alcohol consumption and co-infection with HBV or HIV [45–47]. Although end-stage liver disease occurs only in a minority of individuals, chronic HCV is the main indication for liver transplantation in Western Europe and the USA [42,43].

Genome organization and features of the viral proteins

HCV is a small enveloped RNA virus of about 50 nm in diameter. The HCV genome consists of a 9.6 kb single-stranded positive-sense RNA, which contains an open reading frame encoding a polyprotein of approximately 3000 amino acids flanked by untranslated regions (UTRs) at both the 5' and 3' termini. The 5' UTR together with part of the first structural gene that forms the nucleocapsid (core) constitutes an internal ribosome entry site (IRES), allowing a cap-independent translation [48,49]. The two other structural genes encode two envelope glycoproteins (E1 and E2) that are important for viral entry and fusion with hepatocytes [50–52]. E2 harbours two hyper variable regions (HVR1 and HVR2) which exhibit extreme sequence variability resulting from the selective pressure of the immune system combined with the error-prone RNA-dependent RNA polymerase (RdRp) that lacks proofreading activity [53,54]. E2 also contains the binding site for CD81, a major HCV (co)receptor [55–57].

Downstream of the E2 glycoprotein the small p7 protein [58] is encoded, followed by the non-structural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (Figure 1). These proteins are part of the replication complex and have proteases, helicase and RdRp functions [53,59–62]. NS2 is a cysteine protease that, besides its proteolytic ability, coordinates virus particle assembly through physical interactions with the E1 and E2 glycoproteins and NS3-NS4A enzyme complexes [59,63]. NS3 contains a

180 amino acid serine protease with a His-57, Asp-81, and Ser-139 catalytic triad [64–66] and is activated by interaction with the NS4A cofactor located at the N-terminus of the NS3 protein [60]. When NS3 is co-expressed with NS4A, they are localized to endoplasmic reticulum (ER) and ER-like membranes. Thereafter, NS3 cleaves NS4A to separate itself from the polyprotein [67,68]. Once released from the polyprotein, NS3 cleaves each of the remaining four polyproteins. NS4B, in conjunction with NS5A, is primarily responsible for the synthesis of RNA negative-strands and subsequently of RNA positive-strands. NS4B induces the formation of ER-derived membranous vesicles, referred to as a membranous web, that harbours all HCV proteins [69]. It is thought that this membranous web serves as a temporary platform for the viral replication complex [62,70]. As described, NS5B RdRp is a crucial viral component for positive and negative-strand HCV RNA synthesis. The HCV regulatory proteins (NS5A, NS3 and NS4B) together with cellular components, like cyclophilin A and fatty acid synthase (FASN), form a complex mechanism to bind, activate and catalyse the NS5B RdRp [71,72]. Two sites in the 5' UTR of the viral RNA directly bind to another cellular component, the liver-specific microRNA-122 (miR-122), and exert a positive effect on translation and replication of HCV [73,74].

Life cycle

HCV binding and entry occurs through receptor-mediated endocytosis using receptors found on the surface of hepatocytes, namely: low-density lipoprotein (LDL) receptor, the tetraspinin CD81, scavenger receptor B type I (SR-BI), Claudin I (CLDN), Ocludin (OCLN) and Niemann-Pick C1-like 1 (NPC1L1). Once attached, a low pH-mediated fusion process occurs between the envelope glycoproteins and along with the first non-structural protein, the ion channel P7, RNA is released into cell cytoplasm [57,58,75–79]. The IRES mediates cap-independent initiation of the HCV polyprotein translation using ribosomes found on the outer surface of the ER. Here, the HCV polyprotein is processed into the mature 10 structural and non-structural proteins (Figure 1) [80]. NS4B together with the ER, form a membranous web as mentioned above [62,69,70]. Within this membranous web the NS5B RdRp produces a negative-sense RNA from the positive-sense RNA which is combined to form a double-stranded intermediate. This intermediate serves as a template for the production of excess amounts of positive-sense RNA [81]. Subsequently, viral RNAs are packaged into new viral particles by budding through the ER membrane and leave the cell via the secretory pathway to infect neighbouring cells. All steps of the HCV life cycle are summarized in Figure 2.

HCV has a high turnover rate and produces approximately 10e12 virions per day in an infected individual [82]. The NS5B RdRp lacks a proofreading function and has an estimated error frequency of approximately 10e-4 to 10e-5 mutations per nucleotide [83,84]. This constant proliferation of slightly mutated HCV copies results in a great number of viral variants. Even though the majority of variants produced result in defective virus, a great number of variants do survive and a diverse but genetically related viral population often referred to as quasispecies is formed [83,85]. The rapid evolution of diverse but related viruses within an infected person presents a major challenge with respect to the immune-mediated control and development of directly acting antiviral agents.

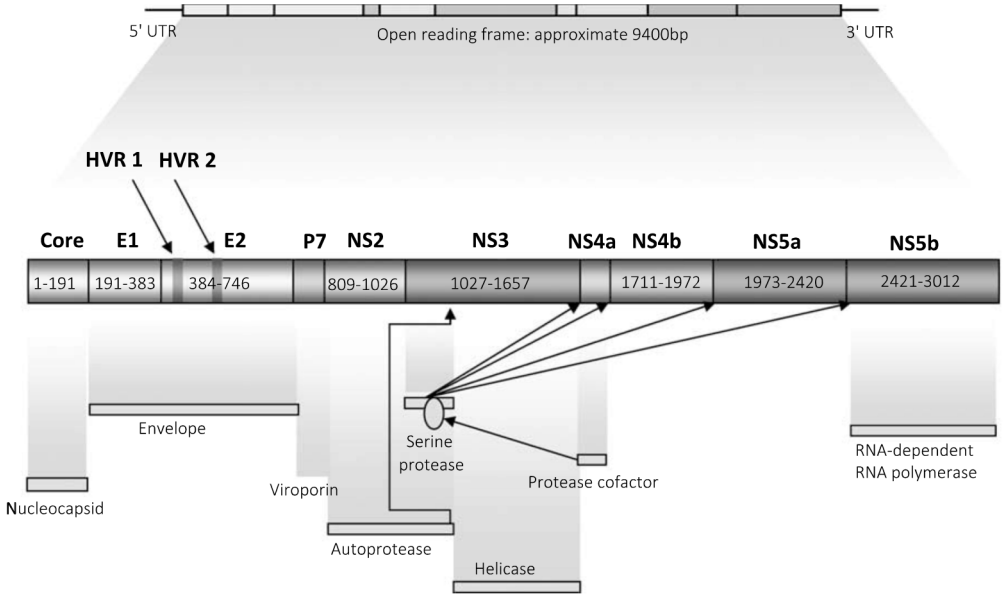

Figure 1. HCV genome, polypeptide structure and protein functions. *The HCV genome consists of an open reading frame of approximately 9400 nucleotides, which is translated as a single polypeptide of approximately 3000 amino acids. The polypeptide is cleaved to produce 10 mature proteins as indicated (Core, E1, E2, P7, NS2, NS3, NS4a, NS4b, NS5a, NS5b). Protein functions products and position of the two hyper variable regions 1 and 2 in E2 are shown. Reprinted from A.R. Lloyd et al. Immunol.Cell.Biol.2007 with permission from the authors, ©2006 Nature Publishing Group.*

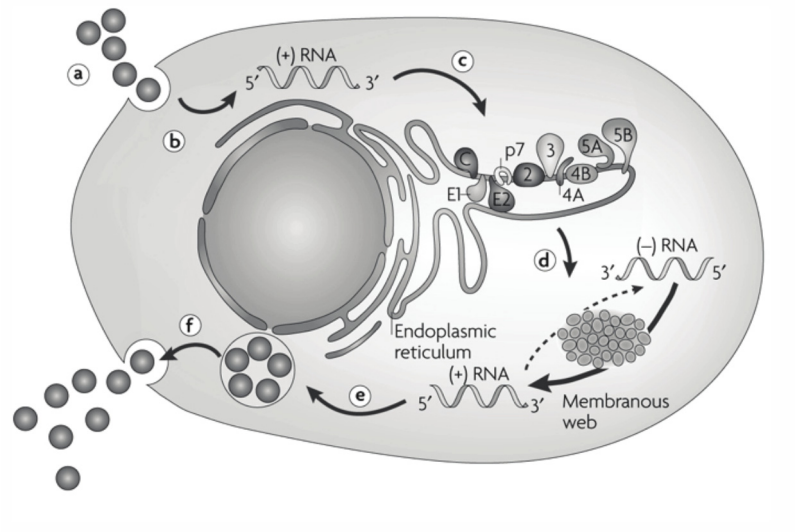

Figure 2. Life cycle of HCV. *HCV life cycle: a) viral entry, b) uncoating, c) replication, d) assembly, e) maturation and f) release. Reprinted from D. Moradpour et al. Nat Rev Microbiol. with permission from the authors, ©2007 Nature Publishing Group.*

Immune-mediated control of hepatitis C infection

Innate immune response

For many pathogens the first line of defence, which limits viral replication and spread, is the innate immune response which gives rise to the induction of type I, type II and III interferons (IFN) [88]. Viruses, including HCV, have developed counteracting strategies to antagonize the immune system such as blocking of IFN function, interference with signalling triggered by IFNs or inhibition of the action of one or several IFN-stimulated genes (ISGs) [89].

Another critical response to viral pathogens as part of the innate immune response are large granular lymphocytes called natural killer (NK) cells [90]. The majority of innate immune human cells are NK cells and play an important role in the control of viral infection by regulatory and direct-antiviral effects [91,92]. Indeed, a sustained NK cell activation contributes to protection against HCV infection [93].

Despite an important role for the innate immune system in clearance of HCV, the adaptive immune system, including both T- and B-cell mediated immunity, also appear to play a critical role in clearance of HCV [94].

Adaptive immune response

HCV-specific T-cells are detectable within 5-9 weeks after infection [95]. A strong and sustained broad HCV-specific CD4+ T-cell response appears to contribute to HCV clearance [36,96–98]. Patients with an active proliferation of CD4+ T-cells in the acute phase of hepatitis C infection subsequently clear their infection, whereas patients with low CD4+ T-cell counts develop a poor immune response leading to viral persistence [99,100]. Unfortunately, with viral persistence, rapid exhaustion and deletion of HCV-specific CD4+ T-cells occurs in the majority of patients [101]. In contrast to CD4+ T-cells, the CD8+ T-cell response during the acute phase of HCV infection does not appear to determine viral clearance [102]. During the acute phase, stunned HCV-specific CD8+ T-cells are present that are unable to secrete antiviral cytokines [103,104]. Eventual viral clearance occurs when CD8+ T-cells regain their ability to secrete antiviral cytokines during the later phases of infection [105,106].

HCV-specific antibodies are usually detected within 8-20 weeks after infection [33]. A fraction of antibodies, referred to as neutralizing antibodies (nAbs) is able to inhibit virus binding, entry and/or uncoating [107,108]. Studies have shown that clearance of infection is strongly associated with an early nAb response, whereas patients with a late nAb responses are more likely to develop a chronic infection [109,110]. It has been demonstrated by *in vitro* neutralizing assays that nAbs increase in both titre and breath during infection [109]. Once chronic infection is established, these nAbs exhibit cross-reactivity against multiple HCV genotypes [109–111].

Most nAbs are directed at the E2 glycoprotein that contains the tetraspinin CD81 binding site. Interaction with the CD81 receptor is necessary for viral entry [52,54,56,112–116]. NAbs are principally directed at two different domains of E2 [113,117]. Not surprisingly, strain-specific antibodies are targeted at the HVR1 [54,118]. Antibodies with a broader affinity target different linear and conformational epitopes that overlap with the CD81 binding site [110,113,115,119–123]. Antibodies target linear epitopes at amino acid positions 412-423 of the envelope glycoprotein - also referred to as epitope I [119,120] -, conformational epitopes at the CD81 binding site in E2 at amino acid positions 529-535 - also referred to as the antigenic domain B or epitope III [110,113,117,121]-, and a third group of nAbs that target epitopes overlapping both linear and conformational epitopes [110,115,123].

NAbs have been shown to exert selective pressure on the envelope glycoproteins with ongoing escape during the chronic phase of infection [124,125]. To date, a substantial number of sites have been reported to be associated with escape from nAbs. One region that conveys immunogenic epitopes in E2 at amino acid positions 384-410, designated as HVR1, induces nAbs but these are strain-specific [54,118]. Through another mechanism of viral escape, called glycan shifting on the E2 glycoprotein, shifts in glycosylation sites at amino acid positions N415 and N417 decrease the efficacy of nAbs [126]. Mutations at amino acid position L413, N415, G418, S419, N434 K610, E655 have been associated with escape from monoclonal antibodies HC33.1 and/or AP33 targeting linear epitopes in E2 [127–129]. Mutations at amino acid positions D431, A439 N434, T435, L438, S501, V506 decreased CBH-2 and/or HC-11 binding targeting conformational epitopes in domain B [55,130,131]. Domain B is a very conserved target of nAbs where apparently no escape is allowed, as it probably disrupts binding to the CD81 receptor [57,110,117,131].

Protective immunity

Despite described innate immune and adaptive immune responses directed at HCV, there seems to be no broad long-term immunity following viral clearance as HCV reinfections and superinfections have been described [28,132–136]. However, spontaneous clearance of such reinfections may occur [107,137,138]. Among patients who spontaneously clear a primary infection, partial non-sterilizing immunity may occur as lower HCV RNA loads, reduced periods of viremia, reduced ALT levels, as well as earlier production and higher levels of antiviral cytokines IFN-gamma and TFN-alpha are observed upon reinfection compared to the primary infection [28,132–136].

Longitudinal studies on the long-term outcome of HCV infection in populations where ongoing transmissions occur, like HIV-infected MSMs and IDUs, will provide valuable insights into HCV immunity.

The conflicting results on protective immunity *in vivo* to date might be explained by differences in study-designs. In studies investigating protective immunity, reinfection incidence estimates and the probability of clearing reinfections are likely to be biased by differences in HCV testing-interval. Indeed, the more frequent one tests, the more one finds [139]. Also, for individuals in older IDU cohorts the likelihood of detecting a reinfection is smaller as they might have controlled multiple infections in the past, leading to progressively shorter durations of viremia [135]. Moreover, if viral sequencing is not performed, incidence of new infections with the same genotype might be underestimated [20,28,140].

Treatment and prevention

The past, current and future treatment perspectives

The first approved treatment for HCV was recombinant IFN-alpha, which achieved low sustained viral response (SVR) rates in HCV genotype 1 infected patients and had significant side effects [141–143]. In the late 1990's the IFN molecule was pegylated (pegIFN), making the treatment more tolerable, and treatment was combined with ribavirin (RBV) which increased the SVR rate to approximately 50% [143,144]. Therefore, during the past ten years pegIFN combined with RBV has been the standard of care for infection with HCV. Patients received this combination for 24-48 weeks depending on tolerability and response [145,146]. Response rates and consequently treatment periods varied by genotype: HCV genotypes 1 and 4 infected patients (treatment period 48 weeks) achieved lower SVR rates compared to HCV genotypes 2 and 3 infected patients (treatment period 24 weeks) of whom up to

80% of patients achieved a SVR [147–149]. Given the poor response rates in HCV genotype 1 and 4 infected patients and poor response rates in certain types of populations such as cirrhotic and black patients [150,151], new antivirals have been developed and their development is still ongoing.
A better understanding of the HCV life cycle identified potential new therapeutic targets [152,153]. Currently new antivirals are being developed for the treatment of HCV-infected patients. These antivirals can be dived into two classes: direct-acting antivirals (DAA) and host targeting agents (HTA) [154–156].

Direct-acting antivirals

DAAs inhibit or interfere with important steps in the viral life cycle. DAAs of which some are already licensed are NS3/4A protease inhibitors, NS5A inhibitors and NS5B polymerase inhibitors [157–161]. Interference of these specific steps in the viral life cycle results in failure of polyprotein processing, post-translational processes, and primary transcript RNA production, hence failing to generate mature viral proteins [154].

NS3/4A protease inhibitors

The class of NS3/4A protease inhibitors consist of first-generation first-wave, first-generation second-wave and second-generation NS3/4A protease inhibitors [162,163].
The first-generation first-wave of NS3/4A protease inhibitors belong to the linear class of protease inhibitors. These are alpha-ketoamide protease inhibitors that form a reversible covalent bond with serine in the active site [164,165]. NS3/4A protease inhibitors telaprevir and boceprevir are the first generation of DAAs approved for treating chronic HCV genotype 1 infected patients in combination with pegIFN and RBV [157,159]. In early-phase clinical-trials both telaprevir and boceprevir achieved high SVR rates as mono-therapy or in combination with pegIFN [163]. In these trials SVR rates as high as 70% were achieved in HCV genotype 1 infected patients [167–170]. However, this wave of protease inhibitors exhibit a low resistance barrier and has significant side effects [171–173].
The first-generation second-wave of NS3/4A protease inhibitors belong to the macrocyclic class of protease inhibitors and are competitive, non-covalent protease inhibitors [174–177]. This generation of inhibitors bind stronger to the active site through non-covalent interactions and have a higher resistance barrier [178]. These protease inhibitors offer higher efficacy and improved pharmacokinetic and safety profiles [179,180]. Also, compared with the first-wave, the second-wave first-generation protease inhibitors have been proven to be effective against all genotypes except for HCV genotype 3 [174]. Simeprevir is the first approved first-generation second-wave NS3/4A protease inhibitor in combination with pegIFN and RBV [158,181]. Faldaprevir, danoprevir, vaniprevir and asunaprevir are in late stage of clinical trials and will soon be approved for HCV treatment [175,177,182,183].
With the second generation of NS3/4A protease inhibitors, SVR rates >90% are achieved with or without pegIFN and RBV [184]. These DAAs are also macrocyclic competitive, non-covalent protease inhibitors. They target multiple HCV genotypes and have a higher barrier for the emergence of resistant variants compared with the first-generation protease inhibitors [184]. MK-5172 and ACH-2684, are both second-generation protease inhibitors that are currently in phase II clinical trials [162,184,185].

NS5A inhibitors

NS5A inhibitors have high-potency with picomolar half-maximal effective concentrations together with multi-genotypic coverage. These agents disrupt signalling and block viral replication by inhibiting the viral protein release [186–190]. As these inhibitors bind to domain 1 within NS5A, viral assembly and release is blocked [191]. The first-generation NS5A inhibitors are currently in different phases of clinical development and include daclatasvir, ledipasvir, ACH-2928 and samatasvir. They are mainly directed at genotypes 1-4 [187,188,190,192]. Observed resistant variants selected after treatment with this generation of NS5A inhibitors seem to persist in the long-term [189,192]. These compounds have been shown to be well-tolerated and to achieve high SVR rates within 4 weeks in treatment-naïve genotype 1 patients [189,192].

The second generation NS5A inhibitors (MK-8742, ACH-3102, GS-5816) are improved versions of the previous generation as they target all genotypes and have an even higher genetic barrier to resistance [193–195].

NS5B polymerase inhibitors

NS5B polymerase inhibitors are divided into nucleotide or nucleoside analogues and non-nucleotide analogues [196]. Nucleotide or nucleoside analogues (sofosbuvir, mericitabine, VX-135/ALS-2200) act as substitute substrates for NS5B polymerases and harbour the same activity across all HCV genotypes [197–199]. Currently, sofosbuvir is already approved for the treatment of chronic HCV infection [160]. Resistant variants have been described *in vitro*, but these variants have shown to have low viral fitness *in vitro* and have never been associated with viral breakthrough [200,201].

Non-nucleotide inhibitors (ABT-333, deleobuvir, lomibuvir, BMS-791325) bind to an allosteric region of the NS5B. They harbour a significantly better activity against HCV genotype 1 and harbour a low resistance barrier [202–205]. These compounds are well tolerated with limited severe side effects to treatment. Also, SVR rates as high as 100% have been reported for genotype 1 infected patients [196].

Resistance to direct-acting antivirals

As also has been learned from the HIV-field, a major concern using substances that directly interact with the virus is the a priori presence or emergence of drug-resistant variants [206]. The substantial diversity of the virus population during infection creates viral subpopulations which might have survival advantages under changing circumstances. Selection pressure by the host-immune system and/or treatment with agents like DAAs might lead to outgrowth of resistant viruses that have an advantage and become the dominant strain [207–209]. These dominant strains harbour amino acid changes that affect interaction with the antiviral and may lead to treatment failure [210,211]. Resistance associated mutations often result in reduced viral fitness compared to the wild-type variant. It has been shown that the higher the level of resistance, the more compromised viral fitness will be [171]. Resistant variants are likely always present prior to treatment given the high error-rate of the viral polymerase, but this does not always lead to treatment failure [212,213]. The question remains to what extent pre-existing variants are selected during treatment and persist in the long term [211,213].

For protease inhibitors, fourteen resistance mutations have been described with different levels of resistance to either linear (NS3 amino acid positions 36, 54, 55, 109, 158, 170), macrocyclic (NS3 amino acid positions 80, 138, 168) or both classes of protease inhibitors (NS3 amino acid positions 41, 43, 54, 155, 156) [211,214–220]. Within the NS5B polymerase region, fifteen resistance mutations (NS5B amino acid positions 96, 282, 316, 365, 414, 419, 423, 448, 482, 494, 495, 496, 499, 554, 559) have been

reported and five NS5A resistant mutations (NS5B amino acid positions 28, 30, 31, 58 and 93) have been observed, all of which have been associated with different levels of resistance [221]. Resistant variants to protease inhibitors, NS5B inhibitors and non-nucleotide polymerase inhibitors emerge rapidly during mono-therapy. In contrast, variants resistant to nucleotide analogue polymerase inhibitors have been shown to have low viral fitness *in vitro* and have never been associated with viral breakthrough in patients treated with these agents [200,201,222].

Host targeting agents

There are two strategies known for host targeting agents (HTAs). The first one is targeted at boosting the host immune response by administration of IFN-lambda or Toll Like Receptor agonists resulting in better antiviral activity [223–225]. The second approach interferes with key host enzymes, like cyclophilin inhibitors, FASN inhibitors and miR-122, preventing these enzymes to contribute to the HCV life cycle [226–230]. HTAs have a high genetic barrier to resistance and a pan-genotypic coverage to all HCV genotypes and subtypes. Also, these compounds may act synergistically with one other or with approved DAAs, which might be useful for difficult to treat patients [156].

Methods for studying hepatitis C

HCV variant analysis

As mentioned earlier, the rapid evolution of diverse but related variants within an infected person presents a major challenge with respect to the immune-mediated control and development of DAAs and vaccines [83,108,209,210]. Population or direct-sequencing provides insights into the major genetic variant present, but will not provide information about viral variants that might be present at very low levels. These variants can carry resistance-associated mutations and could be selected during treatment [172,211]. For this purpose it is important to get a better understanding of intra-host dynamics of the genetic variation at quasispecies level. For quasispecies or variant analysis, especially for the detection of low frequency drug-resistance mutations, either molecular cloning or next generation sequencing can be used. The major difference using these techniques is their sensitivity. HCV variant analysis is usually followed by molecular phylogenetic sequence analysis.

Molecular clonal analysis

A widely used technique for the study of variant analysis is molecular clonal analysis [231]. Clonal sequencing allows amplification of individual DNA molecules, which are cloned into a vector and transformed into a bacterial host cell. The cells are cultured and plated on a selective medium, where each cell will give rise to a colony and each distinct colony will have the original DNA molecule as the genetic ancestor [231]. Clonal sequencing is a labour-intensive process and is often limited to 100 clones at which a minority variant present at 3% can be detected with 95% confidence [213]. Unfortunately, given the vast amount of virions produced per day in infected individuals, together with the error-prone RdRp, part of the viral variation could remain undetected if a small number of clones are analysed [220].

Next generation sequencing

Next-generation sequencing (NGS) or massive parallel sequencing technologies generate a great number of sequences at considerable less time and costs, as compared to clonal sequencing [232].

One of the NGS platforms is 454 sequencing, which uses pyrosequencing to sequence individual DNA molecules in parallel, thereby generating up to a million sequences, also called reads, per run [233]. Reads can be generated by two approaches, 1) from polymerase chain reaction (PCR) amplicons in which the DNA is identical in length and in genomic region covered and 2) by shotgun sequencing in which the DNA is nebulized and randomly fragmented. The amplicon approach is often preferred if a specific region of the viral genome is of interest. The shotgun approach is usually considered when the region is large, displays a high heterogeneity or when the region of interest is to be determined [234]. However, with either approach the sequence length is significantly shorter compared to clonal sequencing. In addition, typical errors of 454 sequencing platform are insertions or deletions after a homopolymeric region. Reduction of the NGS platform errors is important for the downstream analysis, as these artefacts could lead to an overestimation of the sequence variation or lead to frame shifts in protein coding areas [235].

Molecular phylogenetic sequence analysis

Molecular phylogeny studies the evolutionary relationships between organisms through molecular sequencing of (part of) their genome [12,236–239]. A molecular phylogenetic analysis requires different important stages. First, individual sequences are obtained which are referred to as taxa (Figure 3)[236,240]. Second, these taxa are aligned by homologous nucleotide or amino acid positions. Correct manual editing of alignments, inspecting for sequence errors, is important because alignments form the basis for reconstruction of phylogenies [240]. In addition, when working on a coding region, the reading frame should be corrected. Constructing the actual evolutionary relationship between taxa require distance or nucleotide substitution methods which vary in complexity. For HCV, a nucleotide based method, also known as the Maximum Likelihood method is often used [241]. This approach searches for the most probable tree -a graphical way of presenting divergence between taxa-, for a set of taxa based on the nucleotides at each position of the sequence alignment and a model of evolution [241].

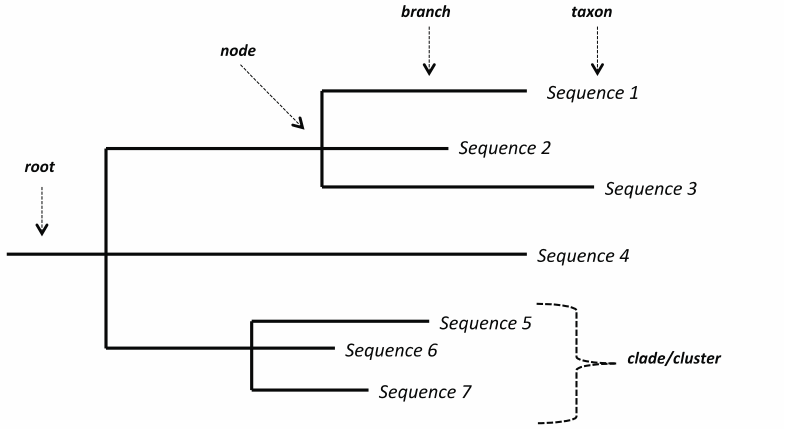

Figure 3. Molecular phylogenetic tree. *Example of a molecular phylogenetic tree illustrating parts of the tree like the root, branch, node, taxa and clades.*

Models of evolution play a significant role in molecular phylogenetics. It is known that the use of particular models may affect many stages of phylogenetic interference [242]. For HCV, dependent on the fragment used, the Hasegawa Kishino Yano and generalised time reversible models of evolution are the ones more often used [243,244]. These models correct for the probability of certain multiple substitutions, reversals, and convergent or parallel evolution steps to occur. Moreover, gamma distribution which models the rates of variation across different sites together with the proportion of invariant sites across the taxa are also used [245]. The best evolutionary model for each fragment can be determined using a Model test [246], which calculates which model fits the dataset best and calculates parameters that can be used. Thus, next to handling the sequence alignment with great care, it is also important to choose an appropriate model of evolution for each dataset [247]. Finally, a tree is constructed which is a graphical way of presenting divergence between taxa referred to as genetic distances. In the phylogenetic tree, viral strains that share a common ancestor are connected through a node. When viral strains are closely related they form a clade or cluster, which might suggest a common source of infection and or transmission route (Figure 3).

Molecular clock analysis

The molecular clock is a useful method for estimating evolutionary timescales. It hypothesises that sequences evolve at a relatively constant rate over time and among different species since they last shared a common ancestor [250,251]. Molecular clock analysis using sequences has been shown to be very powerful in e.g. dating epidemics. A nice example is the recent paper of Faria et al. who showed that the transmission of HIV started in the 1920s in Central-Africa [252]. Another example is a study where we describe the origin of HCV genotype 2 in West-Africa, and its subsequent spread via slave trade to Suriname and Indonesia, and from there to the Netherlands [238]. Molecular clock analysis can also be used for modelling intra-host sequences, providing detailed insight in intra-host evolutionary dynamics [253].

Time-measured phylogenies can be inferred using strict or relaxed molecular evolutionary clock models and can be constructed using a Bayesian Markov Chain Monte Carlo approach [254]. The strict molecular clock assumes that evolutionary rates across lineages are constant whereas relaxed molecular clock allow the evolutionary rate to vary among lineages [255]. Relaxed-clock models are further divided into two types. The first type assumes that the rate varies around an average value over time and among organisms whereas for the second type it is assumed that the rate is influenced by other biological characteristics [250,255].

When using either a strict- or relaxed-clock method of genetic analysis the molecular clock needs to be calibrated as the molecular clock alone cannot assign concrete dates. For viral sequences the dates at which the samples were taken can be used for calibration [237,238,256].

HCV Genotyping

Knowledge about the infecting HCV genotype is important for clinical practice. Aside from response to treatment, the duration of standard pegIFN/RBV treatment is also dependent on the genotype. Easy-to-treat genotypes are treated for 24 weeks whereas difficult-to-treat genotypes are treated for 48 weeks. Therefore, as the genotype is a marker for the likelihood of response to treatment and treatment duration, a good genotyping method is of great importance to clinicians.

With currently approved DAAs and those under development, knowledge of the genotype remain important. Approved first-generation protease inhibitors are licensed for HCV genotype 1 infections.

Treatment with these protease inhibitors present different genetic barriers between HCV genotypes 1a and 1b, where resistant variants are more likely to occur with HCV genotype 1a as only 1 nucleotide change is needed for resistance to occur (R155K), whereas HCV genotype 1b needs two nucleotide changes. With the first generation of protease inhibitors, this lower genetic barrier leads to approximately 10% less SVR rates if patients are infected with HCV genotype 1a. Therefore not only knowledge of the genotype, but also of the subtype is of great importance for clinical management.
HCV strains exhibit widespread genome heterogeneity, although the variation is not uniform throughout [83]. The most accurate method for identifying HCV genotypes is to use an appropriate coding region that is divergent enough to allow the discrimination of types and subtypes [19]. Well-conserved regions such as core, E1 and NS5B, have led to the classification of 7 genotypes and over >100 subtypes [18]. For HCV genotyping in this thesis, sequences from a 389-nucleotide fragment from the NS5B region as described by Murphy et al. were used [248]. Murphy et al. designed and extensively validated a PCR/sequencing assay that targets a 389-nucleotide fragment of NS5B. This assay has been shown to result in good-quality sequences allowing for correct identification of viral genotype and subtype, using a single primer set for all genotypes except for genotype 6 for which an additional primer set has to be used. This genotyping assay, or any other reliable genotyping assay based on viral sequencing, can also be used to investigate the possibility of reinfection or coinfection, in which case the presence of another genotype or subtype suggests a new or concurrent different HCV infection. Another similar and often used genotyping assay, which targets the core/E1 region, is described by Corbet et al. [249], which involves two consecutive rounds of amplification in a nested protocol. Sequences derived from these genotyping assays may also be used to identify the presence of new variants from the same viral subtype. In this patient population, the NS5B region provides sufficient phylogenetic signal for discriminating reinfection with the same subtype from relapse after cessation of treatment.

Genotyping in emerging epidemics

The most straightforward indication of a reinfection is the detection of HCV viremia in someone who had previously cleared the virus, either by treatment or spontaneously. However, in cases with a (late) relapse following treatment or rebound following primary infection, it may be unclear whether the recurrent viremia is caused by the primary infection or by infection with a new viral strain. No clear "cut-off" has been defined for the duration of the HCV RNA–negative interval followed by recurrent viremia, which unambiguously indicates reinfection, although an interval of 60 days has been used [137]. However, with the arrival of DAAs, relapses occurring as late as 1 year after end of treatment (EOT) have been documented [210]. Therefore, whenever HCV reinfection is suspected, for example in IDUs with continuing risk behaviour, or when treatment outcome needs to be assessed in patients with a suspected relapse after EOT, sequencing is necessary to distinguish true relapse from reinfection. For this purpose, different regions of the viral genome can be used. However, given the high variability of HCV, universal assays targeting all genotypes with a single set of primers are not easily designed.
Although NS5B proved to be divergent enough to allow the discrimination of types and subtypes, it proved to be less appropriate in the setting of a recent outbreak. In the latter case, as is the case for the epidemic of HCV in HIV-infected MSM, genetic diversity may be limited with a few, often highly similar clades circulating in that specific population. Here NS5B sequences from different patients are, in some cases, 100% identical, demonstrating that the phylogenetic signal is insufficient to discriminate reinfection from relapse in this particular setting. In such epidemic settings, where similar viruses are circulating, it may be necessary to sequence a fragment of the viral envelope that contains a more

variable region, such as the HVR1 in E2 glycoprotein. This is the region with the greatest genetic variability, allowing discrimination of homologous strains from heterologous strains from the same viral subtype. As described above, diagnosing reinfection is primarily based on population sequencing, which generates a consensus sequence averaging the genomic variation present.

In vitro systems for the study of neutralizing antibodies

As most acute HCV infections are asymptomatic and small animal models for HCV infection have been limited, most comprehensive immunological and virological data have been obtained from chimpanzees [54,257–259]. Although chimpanzees serve as a good model for investigating the immune response to HCV, study results cannot be extrapolated to humans as chimpanzees have a higher rate of viral clearance. Also, the likelihood of human HCV infections becoming chronic and develop cirrhosis of the liver is greater compared to chimpanzees [260]. The most recent and promising *in vivo* system consist of humanized mouse models that generate a specific immune response against HCV, and develop liver diseases that include hepatitis and fibrosis [261].

Although HCV cannot be cultured, several surrogate systems enabling *in vitro* viral replication have been developed during the past ten years which have significantly advanced our understanding of the immune response to HCV infection [262]. These systems include an infection system, sub-genomic replicon, retroviral pseudo-typed virus (HCVpp) and cell culture-derived HCV (HCVcc) systems [262]. HCVpp and HCVcc systems are mostly used for the study of nAbs [137,263,264].

HCV pseudo-particles

The HCVpp system was developed to study the early stages of the viral life cycle [51]. In this system particles are produced by transfecting three DNA vectors in Human Embryo Kidney (HEK) 293T cells [51,265]. The first vector is a retroviral packaging vector containing the Gag and Pol genes from Murine leukemia virus which is responsible for RNA encapsidation and particle budding at the plasma membrane [266]. Second, a transfer vector encoding a reporter protein like Green fluorescent protein (GFP) or luciferase is included. This vector contains the RNA signals that are required for packaging the RNA into retroviral nucleocapsids. The third vector encodes the HCV envelope glycoproteins which are necessary for interaction with the receptors and fusion of the HCVpp with the target cell membrane [51,265]. HCVpp secreted by HEK 293T cells can be used for a single-round infection in human hepatoma-7 (Huh-7) cells and infectivity can be measured by the amount of luciferase activity or GFP expressed in Huh-7 cells.

The major disadvantages using the HCVpp system are that the system does not use the normal assembly route and not all E1E2 glycoproteins can be used to produce infectious HCVpp. It has been shown that HCVpp are infectious when CD81, SRBI, CLDN I and OCLN are expressed by the target cell [51,265]. In contrast, for *in vivo* infectivity of native HCV the additional NPC1L1 receptor is also required, illustrating the limitations of the HCVpp system [75]. After transfection, particles can be used to investigate the presence of nAbs in patient sera (this thesis). Moreover, HCVpp can also be used to identify inhibitors that block HCV entry and to identify fusion mechanisms of HCV [267,268].

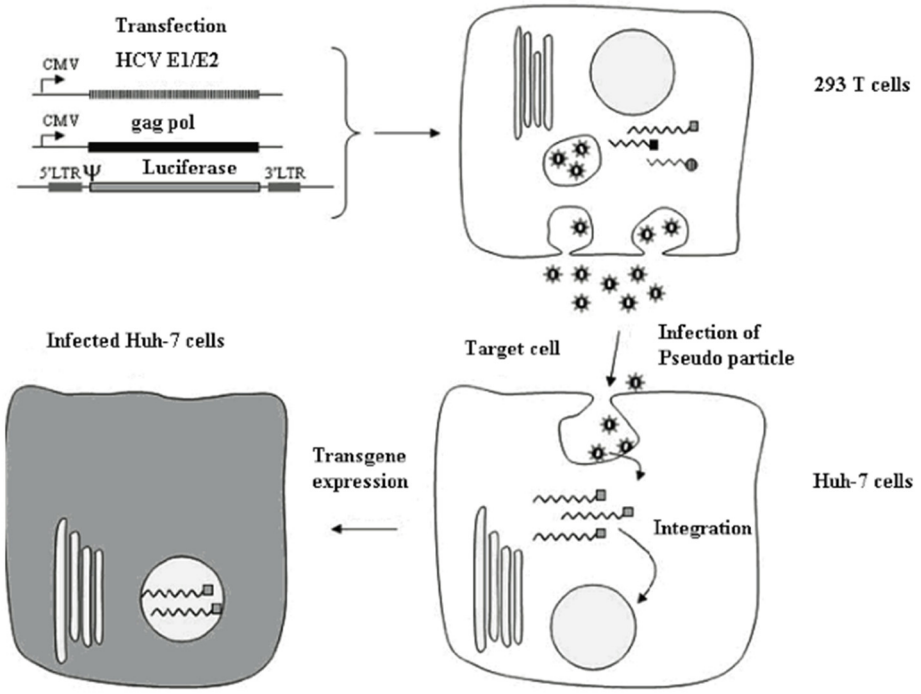

Figure 4. Scheme of HCVpp production and infection. *Reprinted from U.A. Ashfaq et al. Genet Vaccines Ther. 2011; 9: 7 with permission from the authors Copyright ©2011 Ashfaq et al; licensee BioMed Central Ltd.*

Cell culture-derived HCV

A full-length HCV genome replicating efficiently in Huh-7 cells was developed from a HCV genotype 2a full-length replicon (JFH-1) isolated from a Japanese patient with fulminant hepatitis [269]. Its infectivity was improved by the engineering of chimeric constructs of JFH-1 with the structural region of genotype 2a J6 clone [264]. For reasons still not completely understood, replicons derived from this isolate replicate to exceptionally high levels without requiring adaptive mutations [270]. Further optimization has led to the development of Huh-7 derived cell lines such as Huh-7.5 which significantly enhanced HCV replication [252]. Huh-7.5 cells have a defective retinoic acid-inducible gene-I pathway, which is essential for an antiviral immune response [272]. Huh-7.5.1 cells which are derived from Huh-7.5 are even more permissive for HCV replication [254].

This system can be used to study all aspects of the HCV life cycle, such as viral attachment, entry, transfer and replication. Like HCVpp, HCVcc can also be neutralized with (monoclonal) antibodies directed against E1E2 glycoproteins. But unlike HCVpp, all structural and non-structural proteins are present. Therefore the HCVcc replicates with similar kinetics to the wild-type virus without cell culture adaptation. Unfortunately the use of a backbone with other genotypes show poor replication and acquire cell-culture adaptive mutations [274,275].

MOSAIC

The MSM Observational Study of Acute Infection with hepatitis C (MOSAIC) study was initiated in December 2008. MOSAIC is a cohort of MSM with chronic HIV infection who have contracted an acute HCV infection. It is an open, prospective observational cohort in multiple centres including Academic Medical Center, Onze Lieve Vrouwe Gasthuis, Slotervaartziekenhuis, Universitair Medisch Centrum Utrecht and Erasmus Medisch Centrum. Patients are prospectively and retrospectively included if the time interval between the first RNA positive measurement and the last RNA negative measurement or last anti-HCV negative or ALT elevation is 6 months or less. During follow-up, blood samples are regularly collected and questionnaires concerning quality of life and sexual risk behaviour are taken. Control participants are HIV-infected MSM without HCV infection. By the end of November 2014, MOSAIC included 151 HCV-infected MSMs and 144 controls who are currently prospectively followed.
Researchers involved in this study investigate the contribution of HIV-infected MSM to the transmission of HIV, the driving factors of the HCV epidemic and the role of HIV in this, and the impact of acute HCV infection, reinfection and treatment on disease progression.

Outline of Thesis

This thesis combines different aspects of HCV, both during the acute and chronic phase of the disease.
The first part of the thesis addresses the genetic diversity of HCV. **Chapter 2** evaluates the persistence of resistant variants using next generation sequencing, in chronically infected patients treated with the licensed protease inhibitor telaprevir. In **Chapter 3** the evolutionary dynamics of the HCV protease domain during and following treatment with another protease inhibitor, narlaprevir, is investigated using clonal sequencing. By genotyping two regions of the viral genome of HCV genotype 2 infected patients in Amsterdam recombinant viral strains were found. Using a Bayesian phylogenetic approach we described the origin and evolution of these rare circulating recombinants in **Chapter 4**.
The second part of this thesis describes HCV reinfections in HIV-infected MSM. **Chapter 5** describes the incidence of HCV reinfections after treatment-induced viral clearance in this population. **Chapter 6** describes the evolutionary dynamics of HCV in HIV-infected MSM and provides a unique method that was used to describe the incidence of reinfections in HIV-infected MSM in both treated and untreated HIV-infected MSM. The moment of HCV infection is important for the outcome of treatment, therefore we investigated whether the genetic diversity of HCV could help estimate the moment of infection and this is described in **Chapter 7**. **Chapter 8** describes humoral responses against HCV infection in HIV-infected MSM following clearance of acute HCV infection and following reinfection. The speculation in this chapter about the occurrence of partial immunity against HCV in HIV-infected MSM led to further studies on the presence of nAbs against HCV in this population as described in **Chapter 9**.
In the third part of this thesis the summary of all chapters is provided in **Chapter 10** and in **Chapter 11** some of the implications of the findings described in this thesis are discussed.

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Part I

Genetic Diversity of Hepatitis C Virus during Chronic Infection

Chapter 2

Evaluation of Persistence of Resistant Variants with Ultra-Deep Pyrosequencing in Chronic Hepatitis C Patients Treated with Telaprevir

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Abstract

Background & Aims: Telaprevir, a hepatitis C virus NS3/4A protease inhibitor has significantly improved sustained viral response rates when given in combination with pegylated interferon alpha-2a and ribavirin, compared with current standard of care in hepatitis C virus genotype 1 infected patients. In patients with a failed sustained response, the emergence of drug-resistant variants during treatment has been reported. It is unclear to what extent these variants persist in untreated patients. The aim of this study was to assess using ultra-deep pyrosequencing, whether after 4 years follow-up, the frequency of resistant variants is increased compared to pre-treatment frequencies following 14 days of telaprevir treatment.

Methods: Fifteen patients from 2 previous telaprevir phase 1 clinical studies (VX04-950-101 and VX05-950-103) were included. These patients all received telaprevir monotherapy for 14 days, and 2 patients subsequently received standard of care. Variants at previously well-characterized NS3 protease positions V36, T54, R155 and A156 were assessed at baseline and after a follow-up of 4±1.2 years by ultra-deep pyrosequencing. The prevalence of resistant variants at follow-up was compared to baseline.

Results: Resistance associated mutations were detectable at low frequency at baseline. In general, prevalence of resistance mutations at follow-up was not increased compared to baseline. Only one patient had a small, but statistically significant, increase in the number of V36M and T54S variants 4 years after telaprevir-dosing.

Conclusion: In patients treated for 14 days with telaprevir monotherapy, ultra-deep pyrosequencing indicates that long-term persistence of resistant variants is rare.

Introduction

Worldwide, an estimated 170 million people are chronically infected with hepatitis C virus (HCV) [1]. Chronic hepatitis C is a major cause of liver cirrhosis and hepatocellular carcinoma. HCV-related end-stage liver disease is now the main indication for liver transplantation in North America and Western Europe [2]. The current standard of care, pegylated interferon-alpha-2a/b (PEG-IFN) combined with ribavirin (RBV), has limited efficacy and causes significant side effects. In patients infected with HCV genotype 1, the most prevalent genotype in developed countries, treatment for 48 weeks results in rates of sustained virologic response (SVR) of only 40-50%.

Efforts to improve patients' outcomes have resulted in the development of direct-acting antiviral agents (DAAs) such as non-structural protein 3/4A (NS3/4A) serine protease inhibitors. The NS3/4A protease mediates the cleavage of the HCV polyprotein into functional viral proteins essential for viral replication [3]. NS3/4A serine protease inhibitors block this NS3/4A protease-dependent cleavage [4–6]. Two of those protease inhibitors, telaprevir and boceprevir, are now licensed in several countries for clinical use in combination with PEG-IFN and RBV, after extensive preclinical and clinical evaluation [7,8]. Telaprevir (TVR) is a selective, reversible, orally bio-available NS3/4A protease inhibitor that has demonstrated potent antiviral activity in patients infected with HCV genotype 1 [9,10]. Phase 3 clinical studies investigating TVR, PEG-IFN and RBV combination therapy demonstrated significant improvement of SVR rates compared to standard treatment in both treatment-naive and prior treatment-experienced patients infected with HCV genotype 1 [11,12].

However, the flexibility of the HCV genome, caused by the high error rate of its polymerase, allows the virus to adapt rapidly to the presence of an antiviral drug through the selection of minor variants with drug resistant mutations [13,14]. Both clinical and replicon studies have demonstrated that resistant variants are characterized by mutations at positions V36, T54, R155 or A156 [15,16]. Indeed, in 74% of patients who failed to respond to TVR combination treatment in phase 3 clinical TVR trials, the virus population was dominated by resistant variants immediately after treatment [Sullivan et al. Unpublished]. The abundant presence of resistant variants in patients who failed treatment is cause for concern, as this may limit the options for future retreatment in these patients and ultimately may also result in the spread of resistant viruses. Whether the virus population returns to baseline with respect to frequency of resistant variants is therefore an important issue to address. Using population and clonal sequencing, a few studies have monitored the frequency of resistant variants at different time points following TVR treatment in phase 1 and phase 3 clinical trials. These studies suggest that after termination of TVR treatment, the resistant virus population is gradually replaced by wild-type (WT) virus [17], [Sullivan et al. Unpublished]. The observed decline in frequency of resistant variants is not surprising as their fitness is impaired compared to WT virus [14,15,17].

The aim of the study presented here was to study the frequency of resistant variants in patients 4 years after 14-days of monotherapy with TVR using the novel ultra-deep pyrosequencing (UDPS) technique. The extreme sensitivity of UDPS enables a comparison of changes in frequency of minor variants compared to baseline far beyond the limit of detection of conventional techniques. In addition, the large number of sequences that are generated also allows for a robust statistical analysis of observed changes in the virus population.

Materials and Methods

Study Design and Patient Characteristics

The VX04-950-101 and VX05-950-103 clinical phase 1 studies investigated the safety and antiviral activity of TVR [9,10]. Both studies were conducted at 2 collaborative sites in The Netherlands and one site in Germany in 2005 and 2006. These studies were conducted in full compliance with the guidelines of Good Clinical Practice and of the World Medical Assembly Declaration of Helsinki. Prior to study initiation, the protocol and informed consent form were reviewed and approved by the institutional review boards at each site. All patients provided written informed consent before participating in any study-related activity.

For the 101-study, patients naïve or experienced to an interferon-based regimen were enrolled, whereas for the 103-study only treatment-naïve patients were eligible. All patients were chronically infected with HCV genotype 1. In the 101-study, 34 patients were randomized to receive placebo or TVR at doses of 450 mg or 750 mg every 8 hours or 1250 mg every 12 hours for 14 days. In the 103-study, 20 patients were randomized to receive TVR monotherapy, TVR with PEG-IFN or PEG-IFN with placebo for 14 days. At the completion of the 14-day study dosing, off-study standard of care with PEG-IFN and RBV was offered to all patients. The complete study-design is shown in Figure 1. During these studies, plasma samples for viral sequencing were collected at baseline, during, and after dosing.

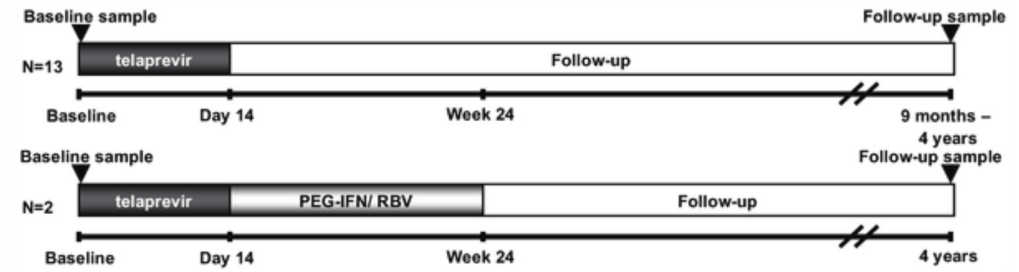

Figure 1. 101 and 103 Study design*. All 15 patients received TVR monotherapy for 14 days. Off-study treatment with PEG-IFN and RBV was offered to all study participants after day 14. Only 2 patients received the off-study standard of care.*

In the study presented here, 15 patients from The Netherlands who received 14 days of TVR monotherapy in either the 101- or 103-study were included. Selection was based on availability of both a baseline and a long-term follow-up sample. A total of 12 and 3 patients were included from the 101- and 103-study, respectively. Patient characteristics including TVR-dosing are summarized in Table 1. Eight patients were infected with HCV genotype 1a and 7 patients with genotype 1b. Two patients (patient 14 and 15) subsequently received PEG-IFN and RBV, but failed to achieve a SVR. The interval between last TVR-dose and the follow-up time point ranged from 0.8 to 4.8 years, with a mean interval of 4 (±1.2) years. Mutations that were present at the end of treatment (EOT) at frequency ≥10% as determined by clonal sequencing are also summarized in Table 1 [15].

Genotyping and Viral Load

HCV RNA levels were determined using the Roche COBAS TaqMan HCV/HPS assay. Genotyping was performed according to Murphy et al.[18].

HCV NS3 UDPS Sample Preparation, Sequencing and Mutation Analysis

HCV RNA for UDPS purposes was isolated from 100 µl plasma using the method described by Boom et al.[19]. Complementary DNA (cDNA) was synthesized from 9.4µl isolated RNA with the Transcriptor High Fidelity cDNA synthesis kit (Roche Applied Science). The amount of starting RNA was not normalized for each patient, as viral loads were all in the same range.

For UDPS, the 454 GS FLX titanium platform was used (Roche 454 Life Sciences, Branford, CT). The HCV NS3 protease region was amplified in 2 separate fragments. Forward primers comprised the 454 GS FLX titanium sequence primer A, followed by a patient-specific multiplex identifier sequence (MID) and a HCV-specific sequence. Reverse primers comprised the 454 GS FLX titanium sequence primer B followed by a patient-specific MID and a HCV-specific sequence as shown in Table 2.

Patient ID (age in years, sex)	TVR dose (mg tid)	EOT resistance mutations[a]	Previous Treatment Outcome	GT	Baseline HCV RNA (IU/mL)	Follow-up HCV RNA (IU/mL)	Years between baseline and follow-up
1 (53, M)	450	V36M+R155K(75%)	NR	1a	1.0E+06	1.3E+06	2.0
2 (43, M)	450	V36M(20%),V36A(13%), R155K(16%),R155T(16%),R155I(11%), A156T(11%)	NR	1a	1.2E+06	3.2E+05	4.8
3 (47,M)	450	V36M(22%), V36A(13%), R155K(10%), R155T(18%)	N	1a	8.2E+06	6.7E+05	4.7
4 (52,M)	450	None ≥ 10%	NR	1b	2.6E+07	3.8E+06	3.4
5 (49,M)	450	T54A(22%), A156V(35%)	NR	1b	9.4E+06	2.2E+05	4.7
6 (33,M)	450	V36A(47%),T54A(22%), A156S(21%)	NR	1b	1.3E+06	1.5E+05	4.7
7[b] (48,F)	750	T54A(26%)	NR	1b	5.6E+06	3.2E+06	4.6
8 (50,M)	750	V36M(25%),V36A(16%), R155K(26%), R155T(13%), R155I (16%)	NR	1a	9.2E+05	1.2E+06	4.5
9 (62,M)	1250	V36A(35%),T54A(27%), A156T(12%)	R	1b	2.2E+06	1.9E+06	1.9
10 (37,M)	1250	V36M(13%)	R	1a	3.1E+05	3.2E+05	4.5
11 (46,M)	1250	V36M(29%), V36M+R155K(49%)	N	1a	6.8E+06	1.8E+05	0.8
12 (44,M)	1250	T54A(67%), A156V(30%)	NR	1b	2.9E+06	2.3E+06	3.8
13 (52,F)	750	R155K(22%), V36M+R155K(63%), V36M+A156T(12%)	N	1a	1.1E+07	6.1E+05	3.9
14 (51,M)	750	A156V/T(100%)	N	1b	4.2E+06	4.9E+05	3.8
15 (61,M)	750	R155K(19%), V36M+R155K(57%)	N	1a	3.9E+07	4.8E+06	3.8

Table 1. Baseline characteristics of patients and previously published resistance mutations found at EOT.

[a] Resistance mutations found at ≥10% of clonal population at EOT [15]. Viral variants were cloned at EOT and the proportion of resistant variants was calculated based on the total number of clones sequenced. [b] EOT sequence data were not available for patient 7, short-term follow-up (7 to 10 days post dosing) data are provided here. GT,genotype; F, female; M, male, NR, nonresponder; N, naïve; R, relapse.

Sensitivity of UDPS

Using basic calculation of probabilities, the chance of detecting at least one specific minor variant (P[m]) with 95% certainty is: P[m] = 1-0.05 (1/N), where N is the number of individual reads. Thus, in theory, when analysing 1000 reads, a minor variant present at 1-0.051/1000 = 0.3% can be detected with 95% certainty.

Genomic region and primer	Sequence	Position*	Amplicon coverage (bp)
Amplicon NS3-I: V36/T54			
VX1-Sense	5'- 454A-MID- AGCYTIACYGGCCGRGA - 3'	3477 - 3493	181
VX1-Antisense	5'- 454B-MID- TGGTCYACATTGGTRTACATYTG - 3'	3636 - 3658	
Amplicon NS3-II: R155/A156			
VX2-Sense	5'- 454A-MID- CAYGCYGATGTCATYCC -3'	3747 - 3763	298
VX2-Antisense	5'- 454B-MID- CCRCTICCIGTRGGRGC - 3'	4029 - 4045	

Table 2. HCV NS3-specific UDPS-primers. **Relative to H77 (GenBank accession number AF00960).bp, base pairs.*

Analysis of Genetic Variability

To identify changes in quasispecies diversity, genetic variability was determined at both time points using average pairwise distances (APD) and average Shannon entropy (ASE) values. Genetic diversity of viral populations at both time points was determined by calculating the APD for each time point using a p-distance model [20] as implemented in MEGA 5.05 software [21]. The APD between baseline and follow-up were compared for both amplicons and separately for each genotype with a paired T-Test. The APD between genotypes were compared with the independent sample T-test.

The Shannon entropy was calculated with a script implemented in the software package ANDES [22]. The average Shannon entropy (ASE) per amplicon between time points and genotypes were also statistically compared using the same methods described for APD. In addition, Shannon entropy per positions of NS3-I and NS3-II were calculated for both time points.

Statistical Analysis

Resistant variants were tabulated at each time point and compared statistically between time points at each position, evaluating the null hypothesis of equivalence between time points (baseline and follow-up) in the proportion of resistant variants. This was approached in 2 ways: (i) in an evaluation of the hypothesis of equivalence across all patients, and (ii) in an evaluation of the hypothesis of equivalence between time points independently for each patient and at each position.

(i) To test for the equivalence of the proportion of resistant variants between time points across patients, the arcsine-square root transformed percentage of resistant variants present at each time point was compared using least squares estimation with variation between subjects controlled by considering the 'patient' to be a random variable and considering 'time point' a fixed variable in a mixed model (JMP, v8.0.1). In this way, the effect of the fixed variable of time was tested while controlling for patient-level variation. The hypothesis of equivalence between time points in the proportion of resistant variants was evaluated independently for each position.

(ii) The count of resistant (V36A/M, T54A/S, R155K/T/M/I, and A156S/T/V) and 'non-resistant' variants was tabulated at each position independently for each patient. The null hypothesis of equivalence was evaluated independently at each position (4 positions; NS3-36, 54, 155, and 156) and for each patient (n = 20) using a two-sided Fisher's Exact test (R, v.2.8.1), for a total of 80 tests of hypothesis (4×20 = 80). Type I error was controlled using a Bonferonni correction.

Phylogenetic Analysis

For one patient (patient 15) NS3 clonal analysis of the EOT [17] and follow-up time point was performed. For clonal sequencing of the follow-up time point, viral RNA was extracted from plasma using the QIAamp BioRobot 9604 (Qiagen, Valencia, CA; Kit 965662). A cDNA fragment was synthesized from viral RNA and amplified by nested PCR. Agarose gel purified amplicons containing the entire NS3 protease coding region were cloned using the TOPO® XL PCR Cloning Kit (Invitrogen Corp). Cloning plates were sent to Beckman-Coulter (Agencourt® Biosciences; Danvers, MA), where 96 clones were amplified and sequenced.

For this patient the evolutionary history of resistant variants detected at follow-up was reconstructed through time using the Bayesian inference framework implemented in the program BEAST 1.6.1 [23–25]. Given an alignment of sequences sampled at different points in time, the rate of viral evolution and the phylogenetic history of infection were estimated on the observed time scale [26,27]. Sequences were analysed using the Hasegawa, Kishino & Yano substitution model with gamma distribution under a relaxed molecular clock. A constant-size coalescent model was applied. Using this model structure, the dates of the most common recent ancestor (tMRCA) of the resistant variants present at follow-up were estimated using Bayesian Markov Chain Monte Carlos (MCMC) sampling. The MCMC algorithm was run for 5.0e7 states sampling every 5.0e3 states. Seven independent runs were combined using Logcombiner v1.6.1. Chain convergence and posterior distributions were investigated using Tracer v1.5.

Results

UDPS Results

Amplification of fragments NS3-I and NS3-II succeeded in 13 out of 15 patients at both baseline and follow-up. For the 2 remaining patients (patient 9 and patient 10) amplification of fragment NS3-I failed at both baseline and follow-up. UDPS of fragment NS3-I succeeded in 12 out of 13 amplicons at both baseline and follow-up and in 14 out of 15 amplicons for fragment NS3-II. UDPS of one patient (patient 6) failed in both fragments at both baseline and follow-up.

UDPS of fragment NS3-I, spanning positions V36 and T54, resulted in a median of 14840 sequence reads (range 8687–20588) and 13717 sequence reads (range 8687–19968), per position in baseline samples. UDPS of fragment NS3-II, spanning positions R155 and A156, resulted in a median of 4017 (range 545–7363) and 3994 (range 545–7361) sequence reads, respectively, per position as shown in Tables 3 and 4. In follow-up samples, UDPS of fragment NS3-I, resulted in a median of 9628 (range 3927–88567) and 10009 (range 3869–86402) sequence reads at positions V36 and T54 respectively. A median of 4930 reads (range 1253–20478) and 6074 reads (range 1253-20466) for positions R155 and A156 respectively were analysed for fragment NS3-II.

	HCV-1a Amplicon NS3-I				HCV-1a Amplicon NS3-II							
	V36				T54				R155				A156			
ID	Baseline		Follow-up		Baseline		Follow-up		Baseline		Follow-up		Baseline		Follow-up	
1	0	*(13817)*	0.05 M	*(10541)*	0	*(13113)*	0	*(9386)*	0	*(2977)*	0	*(4854)*	0	*(2977)*	0	*(4853)*
2	0.28 L; 0.04 A	*(12481)*	0	*(5383)*	0	*(12305)*	0.04 S	*(5327)*	0	*(545)*	0	*(2780)*	0	*(545)*	0	*(2757)*
3	0.03 A	*(18294)*	0	*(15074)*	0	*(17191)*	0.02 A	*(13650)*	0	*(3534)*	0	*(2249)*	0	*(3532)*	0	*(2248)*
8	0.08 L	*(16263)*	0	*(4791)*	0	*(14215)*	0	*(4517)*	0	*(5453)*	0	*(8700)*	0	*(4456)*	0	*(8696)*
10		*NA*		*NA*		*NA*		*NA*	0	*(5379)*	**0.55 G**	*(8154)*	0	*(5379)*	0	*(8146)*
11	0	*(8687)*	0	*(6448)*	0	*(8687)*	0.01 I	*(10631)*	**0.14 S**	*(2873)*	0	*(2986)*	0	*(2873*	0	*(8116)*
13	0	*(20588)*	0	*(17458)*	0	*(19968)*	0	*(17201)*	0	*(5398)*	0	*(7147)*	0	*(5396)*	0	*(7144)*
15	0	*(18993)*	**4.53 M**	*(12836)*	0	*(18320)*	0.35 S	*(11769)*	0	*(5291)*	0	*(9032)*	0	*(5722)*	0.01 T	*(9032)*

Table 3. HCV-1a NS3 UDPS mutation analysis. *Percentage of UDPS reads with mutations. The parenthetical values indicate the number of reads analysed. The bold numbers indicate variation present at a frequency above 0.1%. Underlined amino acids indicate resistant variants that were also observed by clonal sequencing at EOT.*

	HCV-1b Amplicon NS3-I								HCV-1b Amplicon NS3-II							
	V36				T54				R155				A156			
ID	Baseline		Follow-up		Baseline		Follow-up		Baseline		Follow-up		Baseline		Follow-up	
4	0.02 A	*(16899)*	0	*(7312)*	0	*(16564)*	0	*(7224)*	0.02 S	*(4500)*	0	*(2459)*	0.02 G	*(4523)*	0.04 D; 0.04 V	*(2459)*
5	**0.39 I**	*(15804)*	0	*(13911)*	0.02 I	*(15191)*	0	*(13333)*	0	*(7363)*	0	*(5005)*	0.04 T	*(7361)*	0	*(5004)*
7	0	*(10184)*	0	*(88567)*	0	*(10068)*	A 0.02	*(86402)*	0	*(3030)*	0.03 W	*(7924)*	0	*(3029)*	0.05 D	*(7916)*
9		*NA*		*NA*		*NA*		*NA*	0	*(988)*	0	*(3046)*	0	*(988)*	0	*(3045)*
12	0	*(10433)*	0.03 G	*(3927)*	**0.54 A**	*(10257)*	0	*(3869)*	**0.72 P**	*(2340)*	0	*(1253)*	**0.68 G**	*(2340)*	0	*(1253)*
14	0	*(13875)*	0	*(8714)*	0	*(13218)*	0	*(8129)*	0.03 W	*(7180)*	0	*(20478)*	0	*(7180)*	0	*(20466)*

Table 4. HCV-1b NS3 UDPS mutation analysis. *Percentage of UDPS reads with mutations. The parenthetical values indicate the number of reads analysed. The bold numbers indicate variation present at a frequency above 0.1%. Underlined amino acids indicate resistant variants that were also observed by clonal sequencing at EOT. UDPS of both fragments (NS3-I, NS3-II) failed for patient 6 at both baseline and follow-up.*

Frequency of Resistant Variants

Resistant variants, if present at either the baseline or follow-up time points, constituted a small fraction of the viral population (Figure 2, Table 3 and Table 4). Resistant variants were detected at baseline in 5 out of 12 patients. In 4 of these 5 patients, the baseline resistant variants were present at 0.04% of the population or less. In the remaining patient (patient 12) the baseline variant T54A was present at 0.54%. Together, resistant variants were detected at ~0.5% or less of the viral population in 12 out of 14 patients.

As described before, at the end of the 14-day TVR-dosing period, all patients had TVR-selected variants as summarized in Table 1. After cessation of TVR-dosing, the proportion of the viral population comprising these TVR-selected variants decreased with a commensurate increase in the frequency of WT virus as published before [15,17]. At the long-term follow-up assessment, the distribution of resistant variants was comparable to the baseline state. With the exception of 2 patients (patient 12 and 15; discussed below), resistant variants were detected at follow-up in 4 out of 11 patients with data from both amplicons, with a prevalence of 0.05% or less.

In one patient (patient 15) the combination of V36+T54S was observed in one variant. In all other patients no combination of resistance mutations was observed.

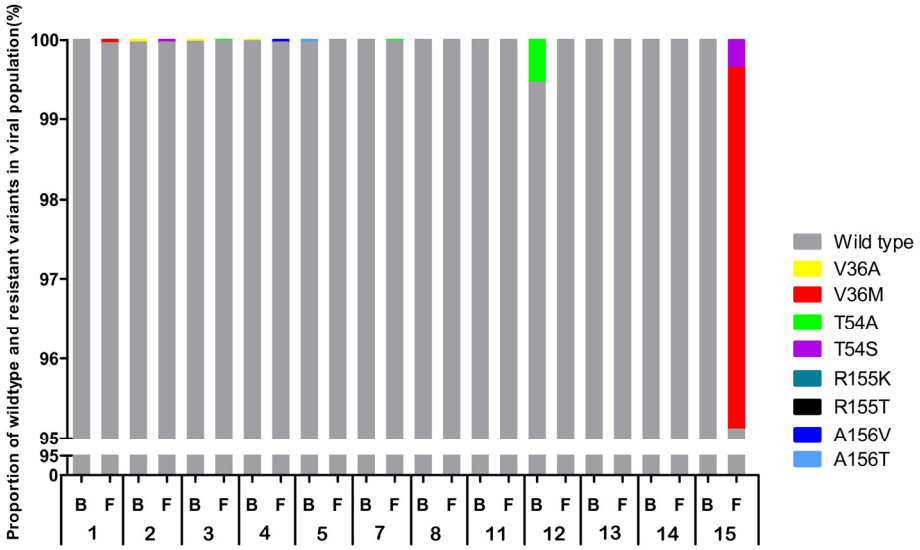

Figure 2. Prevalence of TVR resistance mutations at baseline and long-term follow-up. *The percentage of resistant variants in the viral population at baseline (B) and at long-term follow-up time point (F) is depicted for each patient. For display purposes, only the segment from 95–100% is displayed; the portion of the viral population from 0–95% for all patients is WT. For comparison, viral population composition at the EOT time point is provided in Table 1. Note that amplification of amplicon NS3-I for UDPS failed for patients 9 and 10 (B, F). Furthermore UDPS of both fragments (NS3-I, NS3-II) failed for patient 6 (B, F).*

Genetic Diversity at Baseline and Follow-up

Both APD and ASE values were used to study changes in viral diversity between follow-up and baseline. No significant difference in the APD and ASE values were observed between the two time points as seen in Figure 3A and 3B. Subanalysis per genotype also did not indicate a differential diversity of the virus population between genotype 1a and 1b (data not shown).

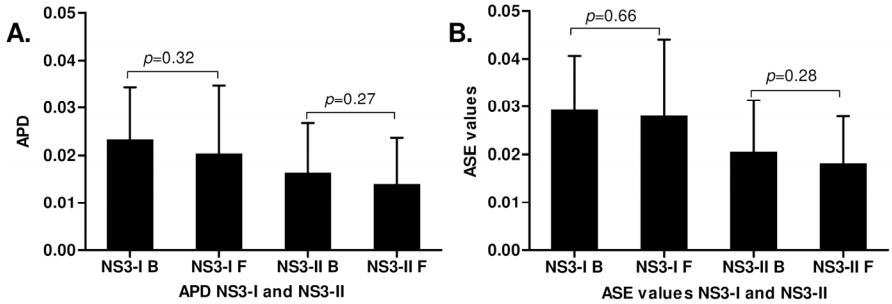

Figure 3. Genetic diversity at baseline and long-term follow-up*. APD and ASE values are plotted in graph 3A and 3B respectively. No significant difference in the APD and ASE values were observed between baseline and follow-up. P-values are depicted in the graphs.*

In Figure 4 the Shannon entropy per position is plotted for both amplicons and time points averaged for all patients. No statistical significant difference was observed comparing baseline Shannon entropies to follow up.

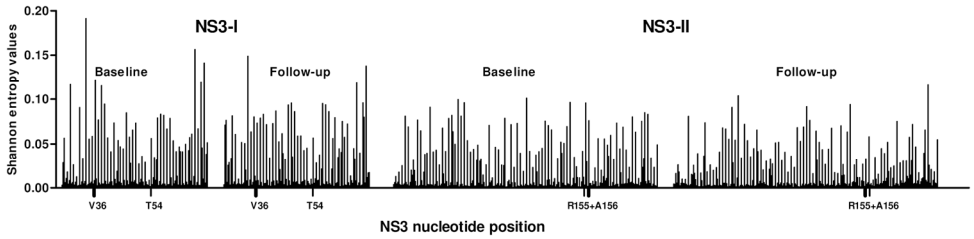

Figure 4. Shannon entropy per nucleotide position at baseline and follow-up*. Shannon entropy per nucleotide position (averaged for all patients) is plotted for both amplicons and time points. Resistance associated amino acid positions are indicated on the X-axis.*

Test for Resistant Variant Enrichment: Baseline vs. Follow-up

The null-hypothesis of equivalent proportions of resistant variants between baseline and follow-up was evaluated independently at NS3 amino acid positions 36, 54, 155, and 156. Results suggested that, across patients, there was no difference in the frequency of resistant variants at baseline and follow-up. P-values were 0.53, 0.80, and 0.74 for NS3-36, 54, and 156. A comparison between time points in the case of NS3-155 variants was not possible, as no resistant variants were observed at this position at either time point (indicating equivalence between the time points).

To evaluate the same hypothesis individually (i.e., to test for enrichment in the proportion of resistant variants independently for each patient), a Fisher Exact Test was performed for each patient and at each position. For 13 out of 14 patients, there was no suggestion of a significant difference between time points. For patient 12, there was a statistically greater prevalence of T54A at baseline (baseline; 0.54%) than at follow-up (0%; p = 4.21e-8). This mutation was also found at the EOT time point in 67% of clones [15]. For patient 15, a statistically significant enrichment of both V36M and T54S (follow-up; 4.53% and 0.35%) variants at follow-up relative to baseline (both 0%; p = <2.2e-16) was observed. Neither V36M nor T54S were detected at baseline in this patient but were present as 4.53% and 0.35% of the viral population at follow-up, respectively.

Phylogenetic Analysis of Clonal Sequences of Patient 15

At the EOT time point, the majority of clones sequenced from patient 15 were resistant, with V36M+R155K present in 57% of clones [17]. If the clones sequenced at the long-term follow-up time point had persisted for 4 years as a remnant of the resistant clones present immediately after dosing, it would be expected that in the phylogeny these resistant clones would cluster with the resistant clones present at the EOT. Instead, these clones formed part of the monophyletic clade that comprises the long-term follow-up viral population (Figure 5). In addition, calibrating the tree using BEAST, the MCMC analysis yielded tMRCA estimates for the resistant variants present at long term follow-up of 126 (95% highest posterior density (HPD) interval 1-360) and 63 (95% HPD 1-195) days for the V36M and T54A/S mutants respectively, suggesting de novo generation, rather than persistence, of the V36M and T54A/S clones observed at long-term follow-up.

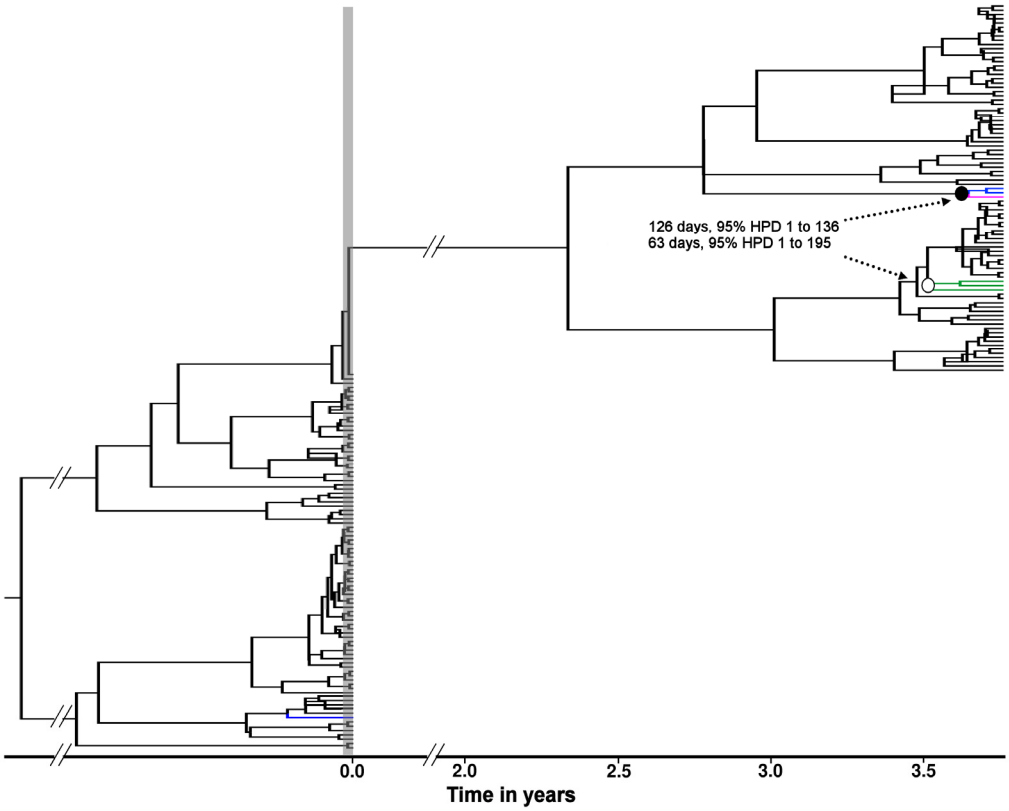

Figure 5. Molecular clock phylogeny from patient 15. *The tree represents the maximum clade credibility tree from the Bayesian MCMC analysis. The bullets at the nodes indicate the tMRCA estimates for the V36M (• = 126 days, 95% HPD 1 - 360) and T54A/S (○ = 63 days, 95% HPD 1 - 195) resistant variants. The V36M, T54A and T54S mutations are indicated in green, blue and pink respectively. The vertical grey shaded bar indicates the TVR-treatment period. Time scale in years relative to the EOT period is indicated on the X-axis.*

Discussion

In early phase 1 studies of TVR, it has been shown that resistant variants are rapidly selected in patients who received 14 days of TVR monotherapy. Assessment of the viral population 3-7 months after the end of TVR-dosing by clonal sequencing showed the predominance of WT virus in the majority of patients [17]. However, whether the viral population in these patients eventually returned to baseline state or whether resistant variants persisted at low levels is unknown.

This study was designed to investigate whether the selection of resistant variants after short-term TVR monotherapy results in long-term persistence of these variants. To address this question, a highly sensitive UDPS analysis of resistance was carried out on plasma samples taken after a median follow-up of 4 years after participation in the clinical phase 1 trial of 14-day TVR monotherapy [9,10]. At this follow-up time point, frequency of resistance mutations was low and in general not increased compared to baseline frequencies. In addition, no significant overall change in quasispecies diversity, expressed by genetic distance or Shannon entropy, was present comparing the follow-up time point to baseline.

To our knowledge, this is the first study that investigated the frequency of protease inhibitor-resistant variants at baseline and after treatment using UDPS. Using UDPS, clonal and population sequencing, the sporadic presence of naturally occurring resistance mutations at low frequencies have been reported by others [17,28–30]. In the present study, mutations associated with resistance were detected in baseline samples of naïve patients in 6 out of 12 patients, with frequencies of less than 0.1% in 4 of these 6 patients.

Of note, the observed baseline resistant variants were not predictive of the presence of resistance at the end of the 14 days dosing period as in all but one patient resistant variants were detected at a level exceeding 10% post-dosing. Furthermore, the low level presence of resistance mutations at baseline did not necessarily result in selection of that variant during treatment as shown by the baseline A156T mutation in patient 5. Interestingly, mutations R155K or R155T, which are key mutations for resistance to both linear and macrocyclic protease inhibitors in genotype 1a, were not detected in any of the baseline or follow-up samples. At the follow-up time point, frequency of resistant variants was in general not increased.

Only patient 15 had a small but statistically significant increase in the low-level resistant variants, V36M and T54S. Interestingly, the T54S mutant, that was considered enriched in patient 15 relative to baseline, was not observed by clonal sequencing of 88 clones at EOT [15]. In addition, the phylogenetic analysis of clonal sequences of this patient suggests that the most recent common ancestors of the two clusters with resistant variants present at the follow-up time point are estimated to have an origin of 126 and 63 days before the long-term follow-up time point for the V36M and the T54S respectively. This suggests that in this patient the variants with the T54S and V36M mutations sequenced at follow-up are naturally occurring variants that arose after treatment.

Using conventional population or clonal sequencing others have reported a gradual decline to WT virus population after treatment discontinuation of either short term monotherapy or combination treatment with interferon for longer treatment periods [17,31]. In other viral infections treated with DAAs, such as human immunodeficiency virus or hepatitis B virus infections, mechanisms to improve the fitness of resistant variants, such as selection of compensatory mutations, enable the resistant variants to persist. The short dosing period of 14 days was perhaps insufficient for the development of adaptive mutations that may restore fitness to WT levels. It is possible that with longer treatment durations, the fitness of resistant variants could be compensated by additional mutations that enhance the replication efficiency

[17]. However, the evolution of resistant variants may be limited by the implementation of stopping rules that instruct to discontinue use of TVR in patients who are likely to have virologic failure. Furthermore, the currently approved TVR combination regimen includes PEG-IFN and RBV, which synergistically suppress virus replication thereby reducing the likelihood of occurrence and persistence of mutations.

There are some limitations to this study. First, there is a theoretical possibility of oversampling but as viral loads of all samples exceeded 10e5 IU/ml (or 5.0e5 copies/ml), viral RNA input was at least 10e4 virus copies per test, demonstrating that redundancy or oversampling was not a problem in the UDPS test set up. Second, while at EOT resistant variants were detected in all patients [15], at follow-up the results from three patients (patient 6, 9 and 10) were missing due to unsuccessful amplification or UDPS. However it is unlikely that this has affected the conclusion of our study, as these sequence failures were random. Third, the intrinsic error rate of the UDPS technique may have caused some of the variability that was observed. A cut off of 0.1% or even 0.5% is often used for reliable detection of mutants based on plasmid controls [32]. If we would have implemented such a cut off in this study, observed variation at resistant sites would have been even less than the limited variation already present, as most of the observed variation at resistance associated sites was present at a level of less than 0.5%. Instead, sequencing errors in our system set up seem to occur at a much lower level than 0.5%. This can be inferred from the fact that observed variation at the resistance associated sites was very limited with 100% WT amino acid residues and nucleotide conservation in most samples, as shown in Tables 3 and 4. The little variation that was observed resulted in amino acid changes that have been described as polymorphisms of resistance associated mutations, indicating that these mutations do not result in non-viable virus and could well be true variation.

Results from a previous study by Susser et al. [31] who used clonal sequencing indicate that at long-term follow-up after initial TVR-monotherapy the majority of the viral population consisted of wild-type variants. Our study confirms and extends the results from this study as we demonstrate that in most patients, frequencies of resistant NS3 variants after 4 years of a 14 day monotherapy course measured by an extremely sensitive sequence analysis technique are comparable to baseline. Since HCV is not known to be archived, patients could potentially be retreated in the future with more expanded combination therapy regimens that still contain TVR or other protease inhibitors from the same class. Indeed, in a recent study, such quadruple combination regimens, consisting of PEG-IFN, RBV, a protease- and a polymerase inhibitor was very powerful [33]. However, re-treatment clinical trials are necessary to fully understand the implications of resistance.

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Chapter 3

Evolutionary Dynamics of Hepatitis C Virus NS3 Protease Domain during and following Treatment with Narlaprevir, a Potent NS3 Protease Inhibitor

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Abstract

Narlaprevir, a hepatitis C virus (HCV) NS3/4A serine protease inhibitor, has demonstrated robust antiviral activity in a placebo-controlled phase 1 study. To study evolutionary dynamics of resistant variants, the NS3 protease sequence was clonally analysed in thirty-two HCV genotype 1–infected patients following treatment with narlaprevir. Narlaprevir monotherapy was administered for one week (period 1) followed by narlaprevir/pegylated interferon-alpha-2b combination therapy with or without ritonavir (period 2) during two weeks, interrupted by a washout period of one month. Thereafter, all patients initiated pegylated interferon-alpha-2b/ribavirin combination therapy. Longitudinal clonal analysis was performed in those patients with NS3 mutations. After narlaprevir re-exposure, resistance-associated mutations at position V36, T54, R155 and A156 were detected in five patients in >95% of the clones. Narlaprevir retreatment resulted in a 2.58 and 5.06 log10 IU/mL viral load decline in patients with and without mutations, respectively (P = <0.01). After treatment, resistant variants were replaced with wild-type virus within 2–24 weeks in three patients. However, the R155K mutation was still observed 3.1 years after narlaprevir dosing in two patients in 5% and 45% of the viral population. Resistant variants could be detected early during treatment with narlaprevir. A slower viral load decline was observed in those patients with resistance-associated mutations detectable by direct population sequencing. These mutations disappeared within six months following treatment with the exception of R155K mutation, which persisted in two patients.

Introduction

Narlaprevir (SCH-900518) is a potent oral linear NS3 serine protease inhibitor with a 90% maximal effective concentration for hepatitis C virus (HCV) genotype 1b suppression of approximately 40 nM (approximately 28 ng/mL) in a replicon system [1]. In a previous phase 1b study, narlaprevir administration resulted in a robust HCV RNA decline and high sustained viral response (SVR) rates when followed by treatment with pegylated interferon (Peg-IFN) and ribavirin (RBV) in both treatment-experienced and treatment-naive HCV genotype 1–infected patients [2].

Phase 3 registration trials showed that triple therapy with Peg-IFN, RBV and NS3/4A protease inhibitors telaprevir or boceprevir significantly improves SVR rates from approximately 45–70% in treatment-naïve patients [3,4]. These impressive treatment improvements are challenged by the potential emergence of drug-resistant variants in patients who do not achieve an SVR. Variants with reduced susceptibility to protease inhibitors may be present naturally before treatment [5], usually at low levels, and can be rapidly selected upon treatment with a NS3 protease inhibitor [6–8]. In addition, even using a highly effective antiviral agent able to inhibit viral replication with 99.9%, in theory, resistance variants can be generated given the high number of virions that are produced each day [9].

In the presence of a direct-acting antiviral (DAA), resistant-associated variants that are less susceptible to DAAs have a fitness advantage over wild-type virus and become the dominant viral species. The number of nucleotide changes required for a virus to become resistant to a protease inhibitor determines the genetic barrier to resistance. The genetic barrier to resistance for first-generation NS3 protease inhibitors is low, and only one or two nucleotide changes are required for high-level resistance to occur [10,11]. Different drug resistance substitutions have been shown to develop *in vitro* and *in vivo* in the presence of linear (V36A/M, T54S/A, R155K/T/Q, A156S/T/V and V170A/T) or macrocyclic NS3 protease inhibitors (Q80R/K, R155K/T/Q, A156T/V, D168A/V/T/H and V170A/T) within a few days [6,7]. The appearance of resistant variants at high frequencies such a short time after administration of protease inhibitors is strikingly faster than observed after monotherapy for human immunodeficiency virus (HIV) or hepatitis B virus (HBV) [12]. Selection of resistance-associated variants is generally followed by a viral breakthrough or nonresponse leading to therapy failure. Moreover, in HIV and influenza virus infection, it has been shown that if a variant with reduced susceptibility to a drug is allowed to replicate in the presence of a drug, it can acquire additional mutations (e.g. compensatory mutations) to increase its replication capacity [13,14]. Whether low frequencies of resistant variants can persist over longer time periods in the absence of drug-selective pressure and potentially affect the virological response upon retreatment is currently unknown.

Here, we describe the evolutionary dynamics of resistance-associated variants and additional mutations in patients treated with narlaprevir during two consecutive dosing periods, followed by standard of care (SOC) with Peg-IFN and RBV and long-term follow-up.

Materials and Methods

Study design and patient population

Forty HCV genotype 1–infected patients were enrolled into a randomized, placebo-controlled, double-blind phase 1b trial. The study was conducted as a two-period, fixed sequence study (Figure 1). During period 1, patients received one of the following doses of narlaprevir: 800-mg narlaprevir three times daily (16 patients), 400-mg narlaprevir in combination with 200-mg ritonavir twice daily (16 patients) or placebo (eight patients) for seven consecutive days. A 4-week washout period was applied between period 1 and period 2. Period 2 consisted of 14 consecutive days of dosing with the same dosing regimen as in period 1 in combination with weekly administration of 1.5 ug/kg/week PEG-IFN-alpha-2b. Upon completion of the second treatment period, patients were offered 1.5 ug/kg/week Peg-IFN-alpha-2b and daily weight-based ribavirin (800–1,400 mg). The clinical results of the trial have been reported in detail elsewhere.

All patients had a chronic infection with HCV genotype 1 (29 genotype 1a and 11 genotype 1b) with plasma HCV RNA levels >10e5 IU/mL and negative HBV surface antigen and antibodies to HIV. Patients were either treatment-naïve or had a non-SVR after previous Peg-IFN-based treatment. Written informed consent was obtained from each patient in accordance with the 1975 Declaration of Helsinki.

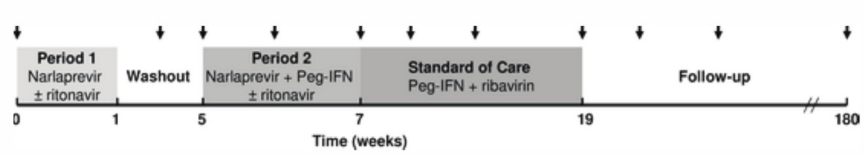

Figure 1. Study design. *The arrows indicate the timepoints of clonal sequencing in patients with viral resistance. Twenty clones were generated at each timepoint if hepatitis C virus (HCV) RNA was >1000 IU/mL. Peg-IFN, pegylated interferon-alpha-2b.*

Viral response and sample selection

A total of 40 patients completed the phase 1 study and initiated treatment with Peg-IFN and RBV immediately after period 2. SVR was achieved in 19 narlaprevir-treated patients (59%) upon SOC. The remaining 13 patients experienced a viral breakthrough (n = 10), nonresponse (n = 2) or viral relapse (n = 1). Seven patients with plasma viral load >1000 IU/mL at the end of narlaprevir dosing were analysed for known NS3/4A resistance mutations at amino acid (aa) position 36, 54, 155 and 156 by population sequencing. Treatment-emergent variants known to be associated with resistance to narlaprevir were observed in five patients, whom were selected for extensive clonal analysis (Figure 2). Longitudinal clonal analysis was performed if plasma viral load was >1000 IU/mL throughout the phase 1 study including SOC and follow-up (see Figure 1). Twenty clones were generated for each time point and patient. A total of 1200 NS3 clonal sequences (with a mean of 240 clones per patient) were generated and analysed. Demographic and other baseline characteristics are shown in Table 1.

Patient	Age (years)	Gender	Race	HCV RNA at baseline (log_{10}IU/mL)	Genotype	Response to prior Peg-IFN based treatment	SOC duration (weeks)
101	56	Male	Caucasian	6.76	1a	Naïve	12
202	43	Female	Caucasian	7.07	1a	Nonresponse	12
309	42	Male	Caucasian	6.88	1a	Naïve	12
409	36	Male	Asian	6.54	1a	Nonresponse	4
411	56	Male	Caucasian	6.07	1a	Nonresponse	12

Table 1. Patient characteristics. *HCV, hepatitis C virus; Peg-IFN, pegylated interferon; SOC, standard of care.*

Viral assessments

Hepatitis C virus RNA levels during the narlaprevir treatment phase of the study were measured using the Roche Cobas TaqMan HCV/HPS assay version 2.0 (Covance, Switzerland) with a lower limit of quantification of 25 IU/mL and a lower limit of detection of 9.3 IU/mL. Plasma HCV RNA levels during SOC were assessed at the Academic Medical Center (Amsterdam, The Netherlands) using the Roche Cobas Ampliprep/Cobas TaqMan assay version 1.0 with a lower limit of detection of 15 IU/mL. Genotyping of patients was performed as described by Murphy et al.[15] by sequence analysis of the NS5B region.

Amplification, cloning and sequencing of the gene encoding the NS3 protease domain

Direct amplification of the NS3 protease domain was performed in a 25 µL volume using a single-round amplification protocol with either forward primer HCV3302deg (5'- CCAAGCTCATYACRTGGGG-3', position 3283–3301) or HCV3355deg (5'– GGCTTGCCCGTCTCYGCYCG-3', position 3336–3355) in combination with reverse primer HCV4050R-ecoRI (5'-ACATGAATTCTYTTICCRCTRCCIGTRGGRGC-3', position 4029–4050). FastStart Taq DNA Polymerase was added with additional ready-to-use PCR Grade PCR Mix kit (ROCHE Diagnostics GmbH, Mannheim, Germany). Final concentrations of 0.9 um NS3 genotype 1a-specific primer, 0.8 mm/L deoxynucleoside triphosphate, 0.1 ug/uL bovine serum albumin and 2.5 mm MgCl2 were used. The amplification cycling conditions were as follows: 5 min at 95 °C, followed by 45 cycles each consisting of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C. Purified amplicons containing the appropriately sized NS3 fragment (approximately 750 base pairs) were cloned using the TOPO® TA PCR Cloning Kit (Invitrogen Corp, Carlsbad, CA, USA). A total of 20 clones per time point from each patient were analysed according to the manufacturer's instructions using the M13 forward or M13 reverse primers. Subsequent Sanger sequencing of these clones was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA).

Sequence alignment and putative NS3 resistant variants analysis

The first 540 nucleotides (aa 1–180) of the NS3 protease domain were analysed in CodonCode Aligner, version 3.6.1 (CodonCode Corporation, Centerville, MA, USA). Previous *in vitro* work identified changes at aa positions 36, 54, 155, 156 and 170 within NS3 as being involved in decreased susceptibility to narlaprevir, which is consistent with the observed resistance mutations for boceprevir and/or telaprevir, which belong to the same class of linear alpha-ketoamide protease inhibitors [1]. In addition, as replicon assays have suggested that aa changes at positions 41, 43, 55, 87, 109, 117, 138 and 174 reduce the susceptibility to boceprevir and telaprevir, these positions were also included in our analysis [16–21]. All clones with resistant variants at aa position 36, 54, 155 and 156 were compared with the individual

baseline consensus sequence to detect additional polymorphic sites, which potentially could enhance the viral fitness of the resistant variant. Aa changes within the NS3 sequence for each clone were defined as changes from reference sequence H77 [AF009606] for genotype 1a. Sequencing 20 clones allowed a lower limit of resistant variant detection of 13.9% with 95% confidence. Resistant variants were divided into high-level (>100-fold), intermediate-level (11- to 100-fold) and low-level (≤10-fold) resistance, according to the fold change in half-maximal effective concentration (EC50) relative to wild type using the replicon system [1].

Statistical analysis

The Student's t-test was used for analysis of differences between groups. P values <0.05 were considered indicative of significant difference between groups.

Results

Genotypic Analysis of resistant variants at NS3 amino acid positions 36, 54, 155 and 156

Resistant variants were detected by population sequencing in five genotype 1a–infected patients at the end of narlaprevir dosing. Clonal sequencing was performed on plasma samples for these five patients throughout the study period. The resistance mutations at four positions (36, 54, 155 and 156) in the HCV protease catalytic domain identified in clinical trials with telaprevir and boceprevir were also observed in the present study. Plasma HCV RNA levels and frequency of viral variants are depicted in Figure 2.

At baseline, wild-type viral population without any resistant variants at aa positions 36, 54, 155 and 156 was observed in all patients. After one-week narlaprevir dosing, resistant variants were detected in three of five patients. Both single mutations at aa positions 54, 155 and 156 and double mutations at aa position 36 + 155 or 36 + 156 were observed. During the washout period, almost all resistant variants disappeared with more than 95% of all clones being wild-type virus.

At baseline of period 2, after the 4-week washout period, T54A and R155T variants were still present in patients 202 and 309, respectively. Upon re-exposure to narlaprevir with Peg-IFN during period 2, the majority of wild-type virus was replaced by high-level (A156T, V36M+R155K/T, V36M+A156T/S, T54S+R155K), intermediate-level (R155K) and low-level (V36M) resistance variants. The R155K substitution was present in different combinations in all patients for a prolonged period after narlaprevir dosing (median 14 weeks). In patient 409, we observed one clone with a V36M+R155T+A156S triple-resistant variant, which was present four weeks after narlaprevir dosing was stopped. Resistant variants with double mutations were replaced by single mutation variants during SOC and follow-up. Over time, resistant variants gradually disappeared in all patients and were replaced by wild-type virus in three of five patients. Time of reversion to wild type after the second narlaprevir dosing period was two, 16 and 24 weeks in patients 411, 309 and 101, respectively. Strikingly, sequence analysis of the long-term follow-up sample, approximately 3.1 years after the last narlaprevir dose, revealed that R155K variants remained detectable in patients 202 and 409. In patient 202, R155K was present in 2/20 clones and in patient 409 in 9/20 clones.

Putative NS3 resistant variants

Additional NS3 loci 41, 43, 55, 87, 109, 117, 138, 170 and 174 were investigated as aa changes at these positions reduced susceptibility in the replicon assay to telaprevir, boceprevir or narlaprevir (Table 2) [16–21]. *In vitro* resistance mutations Q41R, F43S, V55A and R117H, which showed at least a twofold decrease in susceptibility to telaprevir and/or boceprevir in the replicon assay, were observed in at least one clone. Of these, Q41R (patient 411), F43S (patient 101) and R117H (patient 309) were present at baseline of period 1. These variants were scarcely detected over time in one or two clones suggesting an incidental detection rather than a fixed mutation or selection. V55A, a mutation conferring an intermediate level of resistance against boceprevir [22], was observed in three patients after narlaprevir dosing. This mutation was detected in four single clones at four different timepoints. F43C and V170A variants are known to moderately reduce susceptibility to narlaprevir *in vitro* [1]. However, these variants were not detected throughout this study in any patient.

Additional polymorphic sites in the NS3 domain

Multiple clones with aa changes were observed in all patients at polymorphic and conserved sites demonstrating the high degree of genetic variability of HCV. These aa changes were almost exclusively found shortly (<15 weeks) after narlaprevir dosing in a viral population consisting of mostly resistant variants, predominantly R155K variants with or without V36M. The variability of the NS3 domain was greatest in patient 101. Interestingly, H57L and S139P changes were observed in this patient, both key residues of the catalytic triad. These two sporadic mutations coexisted in two clones together with an R155K resistant variant at weeks 2 and 14, suggesting viability of variants harbouring these mutations. C16R was observed in patients 202, 309 and 409 in multiple clones at several timepoints after narlaprevir dosing. This mutation was exclusively found in viral populations, which harboured NS3 mutations at known resistant sites. Clonal sequences of patient 409 with R155K resistant variants in 9/20 clones at week 180 also had a T89Q+V170I switch in all clones. P89L mutation enhanced the viral fitness in the presence of SCH6, a linear NS3/4A protease inhibitor, in the replicon assay [23]. However, aa position 89 is a polymorphic site, and Q89 and I170 is found in the H77 reference sequence. Mutations providing a potential enhancement of the viral fitness observed in ≥ two clones or time points are listed in Table 3.

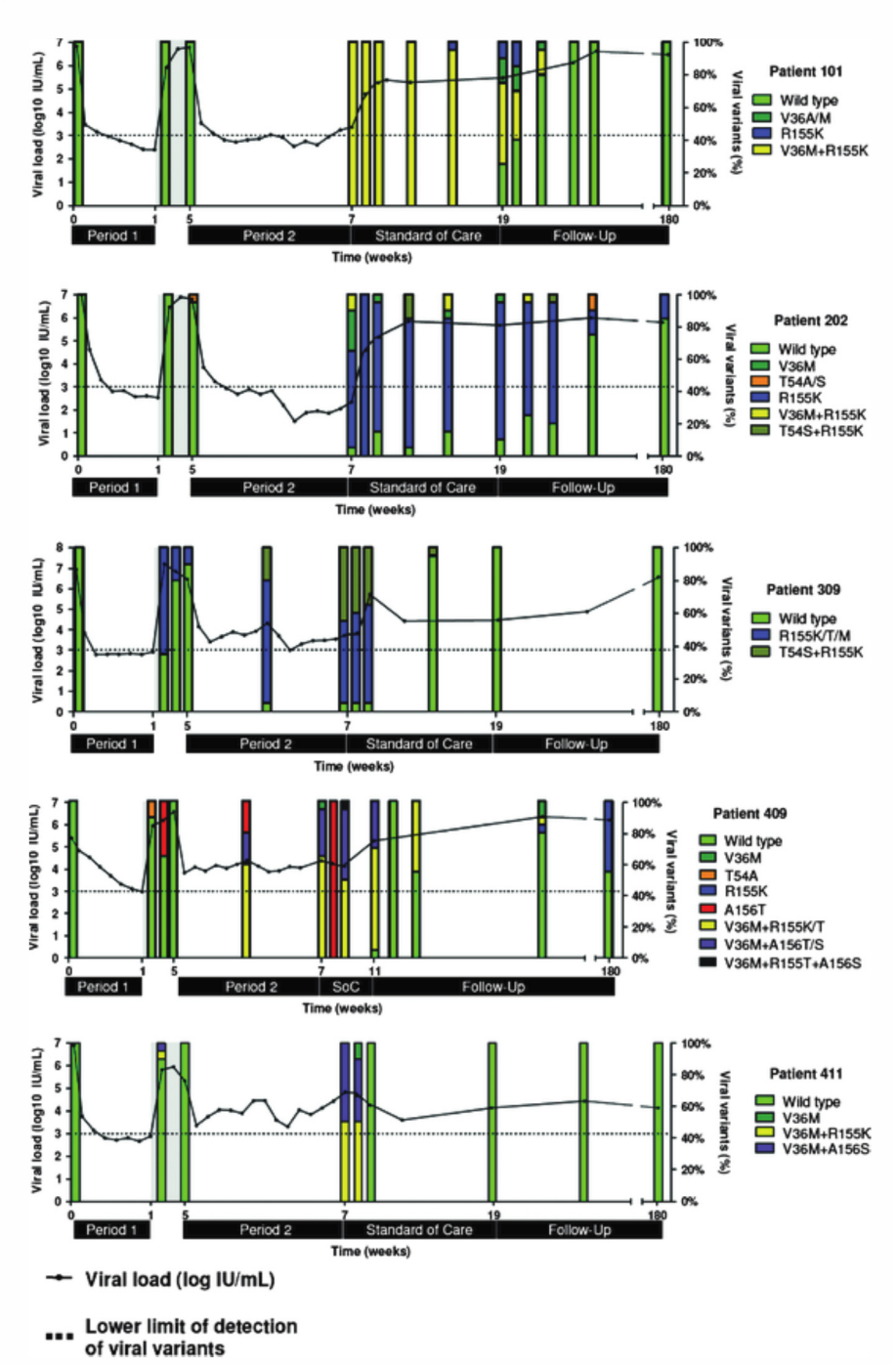

Figure 2. Plasma hepatitis C virus (HCV) RNA levels and frequency of viral variants at NS3 amino acid positions 36, 54, 155 and 156.

Patient	Time point	Q41	F43	V55	A87	R109	R117	S138	V/I170	S174
101	Baseline Period 1	–	**S**	–	–	–	–	–	T	–
	Washout	–	–	–	–	–	–	–	–	–
	Baseline Period 2	–	–	–	–	–	**S**	–	–	–
	SOC									
	Week 7–18	–	–	–	–	–	–	–	–	–
	Week 19	–	–	–	–	–	C*	–	–	–
	Follow-up									
	Week 20–29	–	–	–	–	–	–	–	–	–
	Week 30	–	L	–	–	–	–	–	L	–
	Week 31–39	–	–	–	–	–	–	–	–	–
	Week 40	–	–	–	–	–	–	P*	–	–
	Week 41–180	–	–	–	–	–	–	–	–	–
202	Baseline Period 1	–	–	–	–	–	–	–	–	–
	Washout	–	–	–	–	–	**H**	–	–	–
	Baseline Period 2	–	–	–	–	–	–	–	–	–
	SOC									
	Week 7–18	–	–	–	–	–	–	–	–	–
	Week 19	–	–	–	–	–	**H**	–	–	–
	Follow-up									
	Week 20–29	–	–	–	–	–	–	–	–	–
	Week 30	–	–	**A**	–	–	–	–	–	–
	Week 31–179	–	–	–	–	–	–	–	–	–
	Week 180	–	**S**	–	–	G	–	–	–	G
309	Baseline Period 1	–	–	–	–	–	**H**	–	–	–
	Washout	–	–	–	–	–	–	–	–	–
	Baseline Period 2	–	–	–	–	–	–	–	–	–
	SOC									
	Week 7–14	–	–	–	–	–	–	–	–	–
	Week 15	**R**	–	–	–	–	–	–	–	–
	Week 16–19	–	–	–	–	–	–	–	–	–
	Follow-up									
	Week 20–180	–	–	–	–	–	–	–	–	–
409	Baseline Period 1	–	–	–	–	–	–	–	–	–
	Washout	P	–	–	–	G	–	–	G	G
	Baseline Period 2	–	–	–	–	–	–	–	–	–
	SOC									
	Week 7	–	–	**A**	–	–	–	–	–	–
	Week 8	–	–	**A**	–	–	C	–	–	–
	Week 10	**R**	–	–	–	–	–	–	–	–
	Follow-up									
	Week 11–179	–	–	–	–	–	–	–	–	–
	Week 180	**R**	–	–	–	–	–	–	–	–
411	Baseline Period 1	**R***	–	–	–	–	–	–	–	–
	Washout	–	–	–	–	–	–	–	–	–
	Baseline Period 2	–	–	**A**	–	–	–	–	–	–
	SOC									
	Week 7–19	–	–	–	–	–	–	–	–	–
	Follow-up									
	Week 20–180	–	–	–	–	–	–	–	–	–

Table 2. Mutations at NS3 amino acid positions associated with viral resistance to linear protease inhibitors in the replicon assay. *Changes at NS3 amino acid positions Q41, F43, V55, A87, R109, R117, S138, V170 and S174 have been described to confer resistance to telaprevir, boceprevir or narlaprevir. Mutations in bold showed at least a twofold increase with telaprevir and/or boceprevir in the replicon assay [6, 22]. Mutations were defined as changes from reference sequence H77 [AF009606]. All aa changes were detected in 1/20 clones except those indicated with an asterisk (*), which were present in 2/20 clones. Q, glutamine; F, phenylalanine; V, valine; A, alanine; R, arginine; S, serine; T, threonine; L, leucine; C, cysteine; P, proline; G, glycine; H, histidine; SOC, standard of care.*

Patient	Mutation	Time point (weeks)	# of clones/ # of resistant clones	Mutation at aa positions 36, 54, 155, and 156
101	Y6N	9	2/20	V36M+R155K
	Y6D	9	3/20	V36M+R155K
	Q8R	9	5/20	V36M+R155K
	Q8P	9	1/20	V36M+R155K
		16	1/20	V36M+R155K
	H57L+S139P	21	2/12	R155K
	Y75C	20	1/15	V36M+R155K
		22	2/4	V36M+R155K
	Y75H	21	1/12	V36M
	L127P	12	1/20	V36M+R155K
		21	1/12	V36M+R155K
		22	1/4	V36M+R155K
202	**C16R**	9	1/20	R155K
		20	1/19	R155K
	T76A	9	1/20	V36M+R155K
		10	1/17	V36M+R155K
	R92C	9	5/20	V36M+R155K
	R118Q	9	1/20	V36M+R155K
		180	1/3	R155K
	C145R	8	1/19	V36M+R155K
		20	1/19	V36M
309	**C16R**	6	1/18	R155K
		10	1/19	R155K
409	**C16R**	7	2/20	V36M+A156S

Table 3. Additional mutations at conserved positions in the NS3 domain in clones with resistant mutations at amino acid positions 36, 54, 155 and 156. *Mutations in bold were detected in clones with viral resistance in 3 patients. WT, wild type; aa, amino acid. Patient 411 harboured no additional mutations at conserved positions in the NS3 domain.*

Viral kinetics and resistance mutations

Patients who received narlaprevir during the phase 1 study were divided into two subgroups depending on the presence of resistance mutation to detect potential differences in viral load decline. The subgroups consisted of patients with mutations (n = 5) and patients without NS3 viral variants (n = 27) based on presence of viral resistance at the end of the second narlaprevir dosing period. Mean HCV RNA levels at baseline of period 1 were 6.66 $\log_{10}$ (range, 6.07 to 7.07) and 6.51 $\log_{10}$ (range, 5.42–7.06) in patients with and without mutations, respectively. Differences in first phase (HCV RNA decline after 48 h narlaprevir dosing), maximal decline (nadir) and end of treatment (period 1, day 7; period 2, day 14) were compared to detect whether resistant variants affected HCV RNA decline in period 1 or period 2. First phase, maximal and end of treatment HCV RNA decline was not statistically different in patients with and without resistance after period 1, although a clear trend in differential maximum decline could be observed that reached borderline significance (P = 0.07) (Figure 3a–d). Both groups demonstrated a similar return of viral load to baseline during the 4-week washout period, with the exception of patient 411. In this patient, HCV RNA level at baseline of period 2 was 1.04 $\log_{10}$ lower compared to baseline of period 1.

The first phase decline upon re-exposure to narlaprevir did not differ between both groups although again a trend could be observed for a greater first phase decline in the group without resistance mutations (P = 0.11). However, the maximal HCV RNA decline was reduced (P = 0.04), and end of treatment HCV RNA levels were significantly higher (P < 0.01) in patients with viral resistance (Figure 3). No patient achieved undetectable HCV RNA levels (<9.3 IU/mL) at the end of the 14-day narlaprevir dosing period. The presence of T54A and R155T variants in patients 202 and 309 at baseline of period 2 did not impair the viral decline upon narlaprevir retreatment. SOC in the patients with resistant variants

resulted in virological breakthrough in four patients, and one previous nonresponder appeared to be a nonresponder again. Twelve weeks after treatment cessation, HCV RNA levels returned to pretreatment levels in patients 101, 202 and 411. A 2 $\log_{10}$ difference in HCV RNA levels was observed in patients 309 and 411 at follow-up week 12 compared to baseline level. In patient 411, HCV RNA levels remained low (1.36*10e4 IU/mL) at week 180 compared to baseline (7.93*10e6 IU/mL). The lower viral loads during follow-up in patients 409 and 411 were not related to a mutation in the NS3 region studied. HCV RNA levels returned to pretreatment levels in the remaining patients.

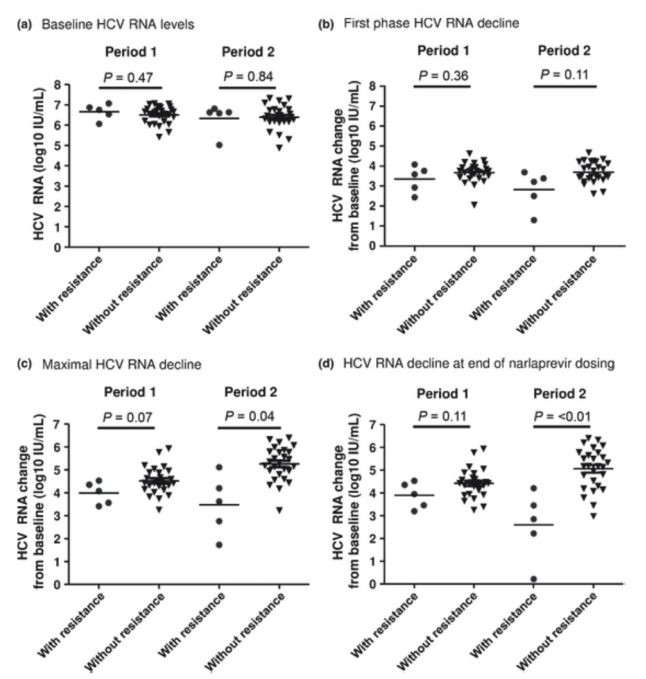

Figure 3. Antiviral response observed during the narlaprevir treatment phases in patients with and without treatment-emergent mutations at the NS3 domain. *(a–d) shows the differences in viral load in patients with and without resistance. At baseline, hepatitis C virus (HCV) RNA levels were comparable between both groups (a). A similar first-phase HCV RNA decline (48 h after narlaprevir dosing) was observed (b). Maximal viral load decline (c) and HCV RNA levels at the end of the second narlaprevir dosing period (d), period 2, was significantly less in patients in whom NS3 mutations were observed.*

Discussion

The addition of NS3 protease inhibitors to the current SOC has improved the SVR rates for the treatment of chronic hepatitis C patients tremendously. One of the major issues in development of DAA therapy is the emergence of drug-resistant variants. Previous phase 1b studies with telaprevir and boceprevir reported the development of resistance-associated variants after monotherapy with various NS3/4A inhibitors [6,7,24]. The boceprevir and telaprevir phase 1 study designs consisted of a single dosing period with protease inhibitor monotherapy or in combination with Peg-IFN for several days or weeks. The two-period phase 1 study, designed to investigate safety, tolerability and antiviral activity of narlaprevir, was considerably different. Here, all patients received two separate periods of narlaprevir

monotherapy and combination therapy with Peg-IFN, interrupted by a one-month washout period followed by SOC. Although the development of narlaprevir is on hold and this treatment regimen will not be used in daily clinical practice, this unique study design provided the opportunity to study the emergence of viral resistance upon (re)treatment with the same linear protease inhibitor within a short period of time.

In vitro data have shown cross-resistance exists for linear NS3/4A protease inhibitors, including telaprevir, boceprevir and narlaprevir [1,6,25]. In this study, resistant variants at loci 36, 54, 155 and 156 were selected rapidly after narlaprevir dosing with and without Peg-IFN. Selection of resistant variants was only observed in genotype 1a–infected patients, which is consistent with the lower frequency of resistant variants observed in genotype 1b patients in other studies [6,7,26]. This is most likely due to the intrinsic lower genetic resistance barrier of genotype 1a strains. For instance, R155K/T variants require only one nucleotide change in the triplet codon of HCV genotype 1a compared with two changes required in genotype 1b [9,10]. The mean viral load decline after the first week of treatment was -3.90 $\log_{10}$ IU/mL and -4.42 $\log_{10}$ IU/mL (P = 0.11) in narlaprevir-treated patients with and without resistance-associated variants, respectively. This slower decline in viral load in patients who develop resistance mutations, although not statistical significant at the early timepoints, becomes more pronounced during period 2, with a decline of 5.06 log IU/mL in patients without resistance compared to 2.58 log IU/mL in patients with resistance at the end of treatment. It is therefore tempting to speculate that early viral load decline kinetics may be used to predict subsequent treatment failure as a result of the occurrence of resistance mutations.

During the second narlaprevir treatment period, the antiviral pressure was increased by adding Peg-IFN and prolonging the narlaprevir dosing time to 14 days. Nevertheless, at the end of period 2, resistant variants were observed in five patients in >95% of the clones. The striking increase in the frequency of resistant variants during period 2 compared to period 1 could be explained by an incomplete return of the viral population to wild-type virus in the washout period.

Unlike HIV infection, in which resistant variants generated during unsuccessful antiretroviral therapy are archived as latently integrated proviruses in resting memory T cells, no such long-lived reservoir has been shown for HCV. The persistence of viral resistance in HIV infection is still the principal challenge during long-term antiretroviral therapy, leading to treatment failure, disease progression and death. For HCV infection, little is known about the persistence of resistant variants over time and the potential impact on retreatment with a cross-resistant DAA. As shown in other studies, wild-type viral population increases only slowly after DAA discontinuation and generally takes several weeks or months to regain dominance [7]. After the second narlaprevir dosing period, resistant variants were replaced by wild-type virus after a median of 14 weeks in three patients.

Surprisingly, in two of the five patients, the R155K mutation could still be detected more than three years after treatment discontinuation. The R155K mutation appears to be a key mutation in the resistance pathway of genotype 1a, as this variant was present in all five patients at some point during and following treatment with narlaprevir. The prolonged persistence of variants with the R155K mutation is in line with recent reports showing a relatively high fitness of these variants [27]. The scarce data on persistence of resistance in general have shown that the virus population tends to return to wild type in weeks or months upon cessation of treatment [7] [Sullivan et al. unpublished]. The longer persistence of resistance in two of the five patients could be caused by the design of this study consisting of two consecutive periods of narlaprevir treatment with a relative short washout period.

Besides the well-documented mutations at positions V36, T54, R155 and A156, replicon assays have suggested that mutations at position 41, 43, 55, 87, 109, 117, 138, 170 and 174 may be associated with resistance. In this study, these mutations were only scarcely observed and not selected during treatment, demonstrating that *in vivo* these mutations do not result in a clinically relevant degree of resistance.

Thus far, no compensatory fitness mutations in the NS3 region have been observed in patients who did not respond to treatment with telaprevir-containing regimens in phase II trials [28]. In this study, we observed a few mutations at conserved aa positions in variants harbouring resistance mutations, which may indicate that these mutations compensate for the loss of fitness caused by the resistance mutations. Multiple viral variants with C16R changes were observed in several patients and only in association with resistant variants. To investigate whether this aa change truly is a compensatory mutation enhancing the viral fitness, further *in vitro* studies are needed.

An unexpected finding in this study was presence of a S139P and H57L mutation, which both belong to the catalytic triad, in patient 101 in two clones at two timepoints. Given the highly conserved nature of the catalytic triad and its essential contribution to the NS3 protease activity, this is a remarkable finding, as these mutations probably lead to a loss of protease function and result in nonviable virus. Nevertheless, the presence of the S139P mutation has been reported before in untreated patients [29].

It is unclear to which extent mutations within the NS4A cofactor, the NS3/4A protease cleavage sites or other parts of the HCV genome may contribute to confer resistance or to impair the NS3/4A function. Sequencing other regions outside the NS3 serine protease domain might reveal additional mutations, which could result in enhanced viral fitness and could explain the long-term persistence of a relatively fit R155K variant in patients 202 and 409.

Although the results of this study clearly demonstrated the changes within the NS3 domain during and after narlaprevir administration, several limitations should be considered. The HCV RNA detection limit for the clonal analysis was 1000 IU/mL. This limited the timepoints analysed during narlaprevir treatment due to the rapid HCV RNA decline below 1000 IU/mL. Future resistance studies could improve the sensitivity of the clonal analysis by developing a patient-specific assay, analyse more clones to enhance the detection level of resistant variants and more sensitive techniques, such as ultra-deep pyrosequencing, and could be used to detect the presence of minor resistant variants.

In conclusion, this study demonstrated that narlaprevir rapidly selected (high level) resistant variants in genotype 1a–infected patients leading to therapy failure. No additional mutations were fixed over time, although C16R was identified in three of five patients at multiple timepoints in one or two clones together with either the R155K or the V36M/A156S mutation. We showed that wild-type virus slowly regained its dominance within four months after discontinuation of treatment although R155K, which appeared to be a key mutation for resistance to narlaprevir, persisted up to three years after narlaprevir discontinuation in two patients. Larger studies and more sensitive techniques are needed to investigate possible resistance persistence after treatment with protease inhibitors and the clinical implications for retreatment with (cross-resistant) NS3/4A protease inhibitors.

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Chapter 4

Origin and Evolution of the Unique Hepatitis C Virus Circulating Recombinant Form 2k/1b

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Abstract

Since its initial identification in St. Petersburg, Russia, the recombinant hepatitis C virus (HCV) 2k/1b has been isolated from several countries throughout Eurasia. The 2k/1b strain is the only recombinant HCV to have spread widely, raising questions about the epidemiological background in which it first appeared. In order to further understand the circumstances by which HCV recombinants might be formed and spread, we estimated the date of the recombination event that generated the 2k/1b strain using a Bayesian phylogenetic approach. Our study incorporates newly isolated 2k/1b strains from Amsterdam, The Netherlands, and has employed a hierarchical Bayesian framework to combine information from different genomic regions. We estimate that 2k/1b originated sometime between 1923 and 1956, substantially before the first detection of the strain in 1999. The timescale and the geographic spread of 2k/1b suggest that it originated in the former Soviet Union at about the time that the world's first centralized national blood transfusion and storage service was being established. We also reconstructed the epidemic history of 2k/1b using coalescent theory-based methods, matching patterns previously reported for other epidemic HCV subtypes. This study demonstrates the practicality of jointly estimating dates of recombination from flanking regions of the breakpoint and further illustrates that rare genetic-exchange events can be particularly informative about the underlying epidemiological processes.

Introduction

Hepatitis C virus (HCV) infection presents a major global health burden, with the WHO estimating that 170 million chronic carriers are at risk of developing severe clinical outcomes such as cirrhosis and hepatic cellular carcinoma [1,2]. The virus belongs to the single-stranded positive-sense RNA virus family *Flaviviridae* and is characterized by considerable genetic diversity. HCV diversity is classified into six main genotypes (genotypes 1 to 6), each of which is further divided into numerous subtypes, and the virus exhibits nucleotide sequence divergences of 30 and 20% at the genotype and subtype levels, respectively [3]. The high genomic heterogeneity of HCV is a result of both its high rate of evolution and its long-term association with human populations [4]. Although there is no indication for a zoonotic virus reservoir, a related virus has recently been discovered in dogs [5].

The greatest diversity of HCV is found in West and Central Africa and in Southeast Asia, where the virus appears to have persisted endemically for at least several centuries [4,6]. The current distribution of HCV genotypes and subtypes is geographically structured, reflecting differences in the rates and routes of transmission of the various subtypes and genotypes. Epidemic strains, exemplified by subtypes 1a, 1b, and 3a, are characterized by high prevalence, low genetic diversity, and a global distribution and are typically associated with transmission via infected blood products and injecting drug use (IDU) during the 20th century [7–12]. In contrast, endemic strains are more spatially restricted but harbour greater genetic diversity than epidemic strains, and it is currently thought that this endemic diversity provided the source of the epidemic strains that constitute the majority of HCV infections worldwide [4,13].

Recombination is thought to play a comparatively minor role in shaping the genetic diversity of HCV; however, an increasing number of reports suggests that it is not entirely insignificant in HCV evolution. Most notable of these was the initial discovery of a natural recombinant form of HCV circulating in injecting drug users resident in St. Petersburg, Russia [14]. This recombinant, labelled 2k/1b, has a 5' genome region that is most closely related to subtype 2k and a 3' genome region that is most closely related to the global epidemic subtype 1b, with a single recombination breakpoint located at genomic position 3175 or 3176 in the NS2 gene [14]. Since the discovery of 2k/1b, several other studies have reported both inter- and intragenotypic HCV recombinants in natural populations, although the evidence presented for recombination varies in strength; the weakest studies report only discordant genotyping results between genome regions (which could also result from coinfection), whereas the most convincing studies repeatedly sequence the same recombination breakpoint from independent extractions (thereby excluding the possibility of *in vitro* genetic exchange). Thus far there have been nine descriptions of HCV recombinant forms, although only in six cases have the breakpoints been sequenced [15–21].

Inspection of the recombination breakpoint positions within the HCV genome reveals a difference between inter- and intragenotypic recombinants. Breakpoints in the intrasubtypic recombinants (1a/1c and 1b/1a) are located in the E1/E2 region, while in the intergenotypic recombinants (including 2k/1b), the breakpoints are consistently found in the NS2-NS3 region [17–22]. Interestingly, naturally occurring intergenotypic HCV recombinants have more often than not involved genotype 2 in the 5' genome region [18–21,23]. This may reflect some inherent yet unknown biological or ecological properties of this genotype to produce viable recombinant viruses.

Nevertheless, the low rate of discovery of novel recombinant forms suggests that although recombination does occur in HCV, it is an uncommon event, at least in comparison to HIV-1 [24]. Indeed, the 2k/1b strain discovered in St. Petersburg is the only known circulating recombinant form (CRF) of

HCV and has therefore been designated CRF01_1b2k, following a naming scheme similar to that developed for HIV-1 recombinants [25]. A CRF is a recombinant form that is found repeatedly in different patients. Since its discovery, CRF01_1b2k has been isolated from patients in many countries, including Ireland, France, Cyprus, Azerbaijan, Uzbekistan, and Russia [20,22,23,26–29]. CRF01_1b2k is the only recombinant strain of HCV to have transmitted widely; therefore, it is important to investigate its genesis and dissemination in order to understand why it might be unique and to evaluate the likelihood that other HCV recombinant forms could increase in prevalence in the future.

Evolutionary analysis of viral genomes using methods based on molecular clocks and coalescent theory has previously proved useful in reconstructing the epidemic history of various HCV strains, including subtypes 1a and 1b worldwide [10,30] and subtype 1b in Japan [31]. Similar analyses of HCV genotype 4 in Egypt have estimated the timescale of the large HCV epidemic in that country and have confirmed its iatrogenic cause [32,33]. Very little is known about the evolutionary history of HCV subtype 2k, most likely because of the lack of sequence data for the strain.

In order to address the lack of information about the epidemiological and transmission history of HCV CRF01_1b2k, we have conducted a comprehensive evolutionary analysis of all available viral genome sequences using a well-established Bayesian framework [34]. Since previously published sequence data on CRF01_1b2k are limited, we sought to increase the sample size by isolating and sequencing a panel of new recombinant isolates from patients of Russian origin resident in Amsterdam, The Netherlands.

By combining information from different genomic regions in a single analysis, we provide the first estimate of the date of the recombination event that generated CRF01_1b2k. The date that we obtained considerably predates the discovery of the strain and requires a re-evaluation of the circumstances surrounding its origin. This is the first time that a recombination event has been dated for any virus other than HIV-1 [35–37] or influenza A virus [38]. Further, we estimate the CRF's past rate of transmission and its pattern of global geographic spread. To obtain more precise parameter estimates when dating recombination events, we employed a joint phylogenetic approach that improves on methods previously applied to HIV-1 CRFs [36,37]. The methods introduced here should serve as a model for future phylogenetic investigations of genetic-exchange events in RNA virus populations.

Materials and Methods

Identification and sequencing of new HCV 2k/1b isolates from Amsterdam

In the course of a study of HCV-infected patients resident in Amsterdam (unpublished data), it was found that HCV genotyping results from the 5' untranslated and NS5B regions were discordant for 6 (out of 200) patients. Of these, five were male and one was female, and their mean age was 34 years (Table 1). For this study, the 5'-end sequences were not used.

HCV RNA was isolated from 200 µL plasma using the purification method described by Boom et al. [40]. cDNA was generated using random hexamer primers as described before [40]. The amplification was performed using a conventional PCR with the following cycling conditions: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles, each consisting of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C.

Amplicons were purified from a 1% agarose gel as described by Boom et al.[40]. Amplification of a 724-nucleotide (nt) fragment (unpublished data) of the NS5B region was performed as previously described [41]. Amplification of core/E1 was performed in a 25 µL volume using HCV1b/2F (GCGTGAGRGTCCTGGAG) as the forward primer and HCV1b/2R (TGCCARCARTANGGCYTCAT) (positions

292 to 312; all primer locations are indicated relative to the H77 reference genome) as the reverse primer, using the same amplification conditions mentioned above. Amplicons were also purified from a 1% agarose gel as described by Boom et al. [40]. Sequencing was performed using HCV1b/2F seq. (CTTCYTACTAGCTCTYTTGTCTT; positions 128 to 112) as the forward sequencing primer and HCV1b/2R seq. (TGCCAACTGCCRTTGGTGT; positions 2386 to 2410) as the reverse sequencing primer.

To confirm that the viral variants were recombinants, a 234-nt fragment harbouring the known breakpoint in NS2 was also amplified and sequenced. Amplification and sequencing of the NS2 breakpoint were performed using HCV2K_F (GCACGCCATACTTCGTCAGAG) as the forward primer and HCV1B_R (CAGGTAATGATCTTGGTCTCCATGT) as the reverse primer, also using the same cycling conditions mentioned above.

In addition to new CRF01_1b2k sequences from Amsterdam, 6 recombinant isolates from an unpublished study were also included. These sequences were sampled from IDUs in Azerbaijan (Table 1). Most of the remaining CRF01_1b2k isolates came from a study of IDUs in Uzbekistan [25] and from a cohort study of HCV-positive patients from seven countries [26]; detailed demographic information on isolates from these two studies is provided here (Table 1). In the latter study, all individuals infected with CRF01_1b2k came from either Russia or Uzbekistan, and 6 of these patients were linked to high-risk groups, namely, blood transfusion recipients or intravenous drug users.

Isolate name	Age	Gender	Sampling	Risk	Country of	NS5B	Core/E1	NS2	Reference
1b2k_AZ_01AZ051_2000	34	M	Azerbaijan	IDU	Azerbaijan	FJ435529	FJ435462	NA	Unpublished
1b2k_AZ_01AZ082_2000	38	M	Azerbaijan	IDU	Azerbaijan	FJ435544	FJ435480	NA	Unpublished
1b2k_AZ_02AZ105_2001	32	M	Azerbaijan	IDU	Azerbaijan	FJ435550	FJ435490	NA	Unpublished
1b2k_AZ_02AZ114_2001	41	M	Azerbaijan	IDU	Azerbaijan	FJ435556	FJ435497	NA	Unpublished
1b2k_AZ_02AZ129_2001	30	M	Azerbaijan	IDU	Azerbaijan	FJ435564	FJ435505	NA	Unpublished
1b2k_AZ_02AZ139_2001	33	M	Azerbaijan	IDU	Azerbaijan	FJ435572	FJ435514	NA	Unpublished
1b2k_CY_CYHCV037_2005	NA	NA	Cyprus	ST/IDU	Georgia	EU684614	EU684686	NA	[39]
1b2k_CY_CYHCV093_2007	NA	NA	Cyprus	ST/IDU	Georgia	EU684649	EU684728	NA	[39]
1b2k_AM_P077_2006	35	M	Amsterdam	IDU	Russia	JF949902	JF949908	Confirmed	This study
1b2k_AM_P079_2006	42	M	Amsterdam	NA	Georgia	JF949897	JF949903	Confirmed	This study
1b2k_AM_P108_2007	35	M	Amsterdam	IDU	Georgia	JF949898	JF949904	Confirmed	This study
1b2k_AM_P135_2005	35	F	Amsterdam	NA	Georgia	JF949899	JF949905	Confirmed	This study
1b2k_AM_P159_2007	39	M	Amsterdam	NA	Georgia	JF949900	JF949906	Confirmed	This study
1b2k_AM_P179_2000	21	M	Amsterdam	IDU	Georgia	JF949901	JF949907	Confirmed	This study
1b2k_FR_M21_2007	30	M	France	IDU	Georgia	FJ821465	FJ821465	Confirmed	[29]
1b2k_IE_HC9A99966_2006	NA	NA	Ireland	NA	Russia	AB327058	AB327018	Confirmed	[28]
1b2k_RU_747_1999	NA	NA	Russia	IDU	Russia	AF388411	AY070214	Confirmed	[14,23]
1b2k_RU_796_1999	NA	NA	Russia	NA	Russia	AF388412	AY070215	Confirmed	[14,23]
1b2k_RU_ALT30_2000	34	F	Russia	NA	Russia	AB327055	AB327015	Confirmed	[27]
1b2k_RU_HIA1002_2003	NA	NA	Russia	NA	Russia	DQ001221	AB327011	Confirmed	[27]
1b2k_RU_KNG318_2002	30	M	Russia	IDU	Russia	AY764172	AB327010	Confirmed	[27]
1b2k_RU_KNG327_2002	25	M	Russia	NA	Russia	AY764176	AB327012	Confirmed	[27]
1b2k_RU_N687_1999	NA	NA	Russia	NA	Russia	AY587845	AY587845	Confirmed	[27]
1b2k_RU_PSA108_2005	37	M	Russia	NA	Russia	AB327053	AB327013	Confirmed	[27]
1b2k_RU_PSA62_2005	50	M	Russia	BT	Russia	AB327054	AB327014	Confirmed	[27]
1b2k_UZ_AZ15	22	M	Uzbekistan	BT/IDU	Uzbekistan	AB327056	AB327016	Confirmed	[26]
1b2k_UZ_UZIDU19_2006	NA	NA	Uzbekistan	IDU	Uzbekistan	AB327120	AB327122	Confirmed	[26]

Table 1. Epidemiological and sequence information of the CRF01_1b2k isolates used in this study. *M, male; F, female; NA, not available; IDU, intravenous drug usage; BT, blood transfusion; ST, sexual transmission.*

Collation of HCV sequence alignments

To investigate the evolutionary origin and spread of HCV CRF01_1b2k, a data set comprising all subtype 2k/1b (n = 27), 2k (n = 15), and 1b (n = 71) isolates for which both core/E1 and NS5B sequences were available was collated from GenBank and the HCV Sequence Database [25] (see Table S1 at http://jvi.asm.org/content/86/4/2212/suppl/DC1). This collection included the 6 newly sequenced isolates obtained from HCV patients in Amsterdam (see above). Alignments for both genome regions were constructed manually, and each alignment contained exactly the same set of taxa. The date and sampling location of each sequence were obtained from the literature or via personal communication (Table 1).

Estimation of genome region-specific rates of evolution

The evolutionary rates of the core/E1 and NS5B regions used in this study could not be estimated directly from our data, because the sample size and range of sample dates were not large or wide enough. In line with previous studies of HCV epidemic history (e.g., see reference [32], we employed an independent data set with significant temporal information to provide the substitution rates of the subgenomic regions of interest. Specifically, we used the data and analysis strategy of a recent study that reported rates of evolution for all HCV genome regions for subtypes 1a and 1b [42]. That study utilized an alignment-partition approach, which was implemented in the BEAST program, to estimate region-specific rates [34]. We applied a codon-structured nucleotide substitution model [43], an uncorrelated relaxed lognormal molecular clock [44], and a Bayesian skyline coalescent model [45] to both subtype 1a and 1b whole-genome alignments, from which we obtained rate estimates for the precise subgenomic regions (core/E1 and NS5B) used in our analysis of CRF01_1b2k. The Markov chain Monte Carlo chains (MCMCs) were run for 200 million generations and sampled regularly to yield a posterior tree distribution based upon 10,000 estimates. For further analysis details, see reference [42].

Phylogenetic analysis

Preliminary phylogenetic analyses were undertaken to confirm that CRF01_1b2k originated from a single recombination event. Neighbour-joining (NJ) trees of the core/E1 and NS5B data sets were estimated using the PAUP* program [46] with an HKY85 nucleotide substitution model and a gamma distributed among site rate heterogeneity (data not shown).

Next, in order to directly test the hypothesis of a single recombinant origin, we performed Bayesian MCMC analysis of the core/E1 and NS5B data sets in two ways: (i) we constrained the CRF01_1b2k isolates to be a monophyletic clade, and (ii) no phylogenetic constraints were imposed. The hypothesis of a single origin was then tested by performing a Bayes factor (BF) comparison of the marginal likelihoods [47,48] obtained from these two analyses. This revealed an insignificant difference between the two competing hypotheses; thus, a single recombination event was assumed in following analyses.

Molecular clock analysis

In order to estimate the date of the recombination event that formed CRF01_1b2k, we created separate data sets for the core/E1 and NS5B regions that contained the CRF isolates, plus all available closely related parental subtype reference sequences (belonging to subtype 2k for the core/E1 region and to subtype 1b for the NS5B region). A hierarchical phylogenetic model [49] was used to combine both data sets and thereby provide a joint estimate of the time to the most recent common ancestor (TMRCA) of the CRF clade, while accounting for uncertainty in both genome regions.

As the CRF clade in the different genome regions is known to represent a common evolutionary history, jointly estimating the age of this clade will maximize the explanatory power of the data [49] and is thus more powerful than analysing the regions independently, as has been done previously (e.g., see reference [37]. However, due to the recombination of the different subtypes, we cannot simply assume that a single tree represents the entire genome. Instead, we allow independent trees for each genomic region but maintain the TMRCA of the CRF clade in each region to lie within a small time of each other, while estimating the mean of these as the parameter of interest.

Specifically, separate phylogenies, molecular clock models, and substitution models were estimated for the core/E1 and NS5B regions. The genome region-specific rates (estimated as described above) were used as prior distributions for the evolutionary rates for the core/E1 and NS5B regions. For the NS5B region, the rates estimated from subtype 1b were applied, while for the core/E1 region, the average of the 1a and 1b rates was used (because a subtype 2k-specific rate was not available).

For each pair of sampled phylogenies (core/E1 and NS5B) in the posterior distribution of the MCMC, three node dates were obtained (as labelled in Figure 2): A, the joint TMRCA of the CRF clade; B, the date of the parental node of the CRF clade in the core/E1 subtype 2k phylogeny; and C, the date of the parental node of the CRF clade in the NS5B subtype 1b phylogeny. The former date, together with the more recent of the last two dates, therefore defines a time range during which the recombination event must have occurred (see Figure 2). The posterior distribution of this time range was then compiled by repeating the above-described procedure for each pair of phylogenies in the MCMC output. The BEAST analysis model settings were the same as those outlined in the section on genome region-specific rates above.

CRF01_1b2k transmission history

Further Bayesian MCMC phylogenetic analyses were performed solely on the CRF01_1b2k isolates in order to estimate the epidemic history and basic reproductive number, R0, of the strain since its emergence. BEAST model settings were the same as those outlined above, except that different coalescent models were employed to reconstruct the transmission history of the CRF. Both the GMRF skyride [50] and exponential-growth coalescent models were used.

Results

Estimation of genome region-specific rates of evolution

The rates of evolution of the core/E1 and NS5B genomic regions used in this study were estimated from subtype 1a and 1b whole-genome data sets (see Materials and Methods) and are given in Table 2. The rates for the NS5B region corresponded between subtypes, with similar 95% highest posterior density (HPD) intervals, while the rate estimates for the core/E1 region did show some variation among subtypes (Table 2).

Phylogenetic analysis

To establish the evolutionary origins of the HCV 2k/1b strain, we analysed the core/E1 (644 nt) and NS5B (741 nt) regions of 27 CRF 1b/2k, 15 subtype 2k, and 71 subtype 1b isolates. The 2k/1b isolates were sampled between 1999 and 2007 and were from the following locations: Ireland, Uzbekistan, Azerbaijan, Cyprus, Amsterdam, France, and Russia (Table 1). Since the BF test supported the hypothesis that the

2k/1b isolates were monophyletic, a single origin of the CRF was inferred (BFs for the comparisons of monophyly and nonmonophyly models were 0.31 for the core/E1 data set and −0.68 for the NS5B data set). Furthermore, the monophyletic origin of the CRF clade was supported in both genome regions when no phylogenetic constraints were imposed. However, monophyly of CRF 1b/2k isolates was not supported by a high posterior probability (0.67) in the NS5B maximum clade credibility (MCC) tree, most likely reflecting the uncertainty associated with the star-like phylogeny and relatively short sequence length. However, the recombinant nature of many of the isolates in this study was confirmed by direct observation of the breakpoint in the NS2 gene region (confirmed isolates are represented by filled circles in Figure 1).

	No. of substitutions/site/year (10e-3)		
Genomic region	**HCV subtype 1a**	**HCV subtype 1b**	**Average rate**
Core/E1	1.75 (1.41, 2.10)	1.36 (1.01, 1.76)	1.56 (1.21, 1.93)
NS5B	0.89 (0.71, 1.07)	0.91 (0.68, 1.14)	0.90 (0.70, 1.11)

Table 2. Evolutionary rate estimates of the genomic regions used in this study. *The estimates were obtained from HCV subtype 1a and 1b whole genomes using a genomic partition model (see Materials and Methods). The numbers inside parentheses represents the 95% highest posterior density credibility interval.*

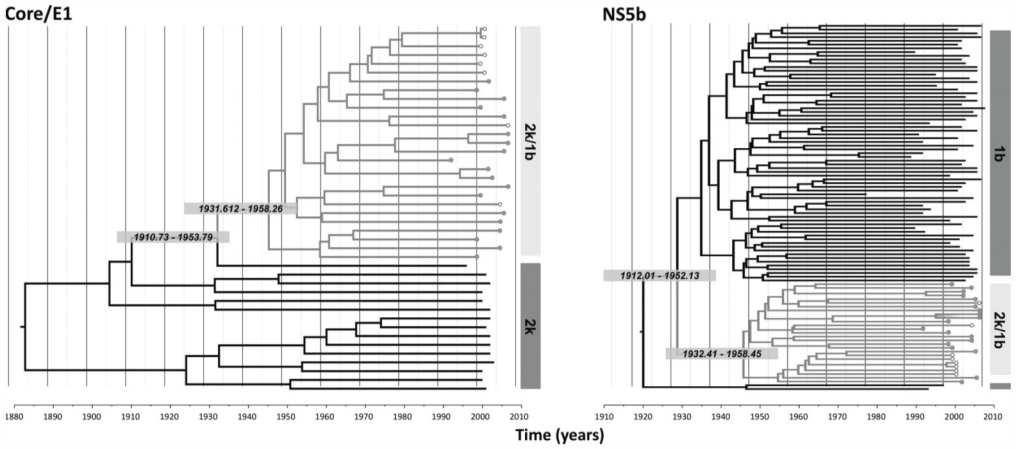

Figure 1. Molecular clock phylogenies of CRF01_1b2k and its parental subtypes, estimated from the core/E1 alignment and the NS5B alignment. *The horizontal bars in each hylogeny contain the dating estimates for two nodes: the common ancestor of the CRF clade (1931 or 1932 to 1958) and the common ancestor of the CRF and the most closely related parental strain (1910 to 1912 to 1952 or 1953). Filled circles, 2k/1b isolates that were confirmed as being recombinant by sequencing of the breakpoint in the NS2 region; open circles, those isolates for which NS2 sequences were not available. The trees shown are the maximum clade credibility trees from the Bayesian MCMC analysis*.

Molecular clock analysis

Figure 1 and Table 3 provide the estimated TMRCAs of the CRF clade obtained using the joint and independent molecular clock analyses. The estimates obtained separately from the core/E1 and NS5B data sets were in close agreement and exhibit overlapping 95% HPD intervals. These estimates also agree with the joint estimate of TMRCA of the CRF (node A), which was 1946 (1932 to 1959; Table 3). We also estimated the date of the most recent common ancestor of the CRF clade and its most closely related parental isolate (Figure 2). These estimates were again similar for both genome regions, at about

1933 (Table 3). Lastly, by comparing the HPDs of the node A date with the dates of the more recent of the two parental nodes (either B or C), we were able to generate bounds for the time of the recombination event that generated the CRF, which was between 1923 and 1956.

	TMRCA (year)[b]		
Clade (node[a])	Core/E1	NS5B	Joint (hierarchical) estimate
CRF (A)	1945.2 (1931.6, 1958.3)	1945.8 (1932.4, 1958.5)	1946.0 (1932.5, 1959.0)
CRF + 2k (B)	1932.0 (1909.7, 1952.8)	NA	NA
CRF + 1b (C)	NA	1933.1 (1912.0, 1952.1)	NA

Table 3. Estimates of TMRCA of the CRF clade obtained from separate genome regions and from joint (hierarchical) phylogenetic analysis*. [a] The node labels highlighted in Figure 2, [b] The numbers inside parentheses represent the 95% highest posterior density credibility interval. NA, not available.*

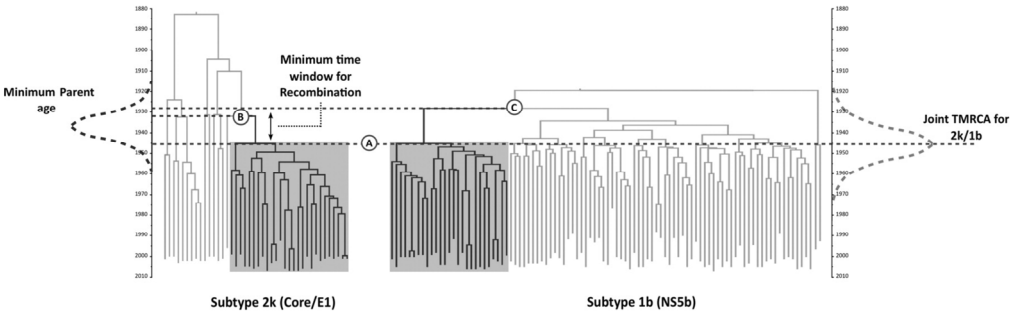

Figure 2. An illustration to explain the structure of the joint phylogenetic method that we employed to estimate the date of the recombination event that generated CRF01_1b2k*. The core/E1 tree is shown on the left and the NS5b tree on the right; in both, the shaded box highlights the CRF01_1b2k clade. The left axis depicts the time of the ancestral nodes (node B in the core/E1 tree and node C in the NS5B tree). The right axis shows the TMRCA of CRF01_1b2k (node A). Both CRF node A (estimated jointly from the core/E1 and NS5B regions) and the youngest parental node (either B or C) were used to calculate the minimum time window for the recombination event to have occurred.*

CRF01_1b2k transmission history

Figure 3 shows the estimated epidemic history of CRF01_1b2k, as estimated using the Bayesian skyride plot method, which depicts the effective population size of the CRF epidemic over time. The plot indicates approximately constant exponential growth since the emergence of the CRF lineage (Figure 3) until the mid-1990s, after which the effective population size declines or stabilizes (either is plausible, given the size of the credible region of the estimate). This decrease/stabilization coincides with the advent of screening for HCV in blood donors, which greatly reduced the risk of HCV infection via blood transfusion [51–53].

To ascertain the CRF's exponential growth rate (r), the CRF data set was also analysed using an exponential-growth coalescent model. The estimated growth rate was 0.116 year-1 (95% HPD interval, 0.079 to 0.159). This estimate was subsequently used to calculate R0 values for the CRF01_1b2k strain under a plausible range of average durations of infectiousness (D), using the equationR0 = rD + 1 [10]. Both the estimated growth rate (r = 0.1 year-1) and the estimated R0 values (R0, ~2 to 4) are compatible with a number of equivalent estimates for other HCV subtypes, including those from IDU risk groups [10,13,31,32].

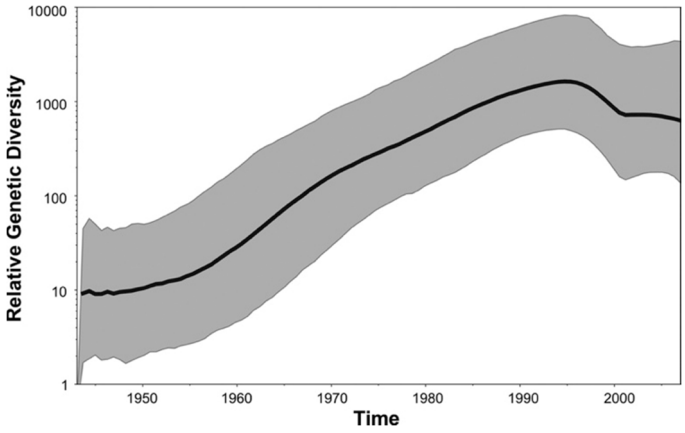

Figure 3. Estimated Bayesian skyride plot of the CRF01_1b2k clade. *The vertical axis represents the product of viral generation time and the effective number of infections (Ne). The solid line shows the best estimate, and the shaded area shows the 95% credible region of this estimate.*

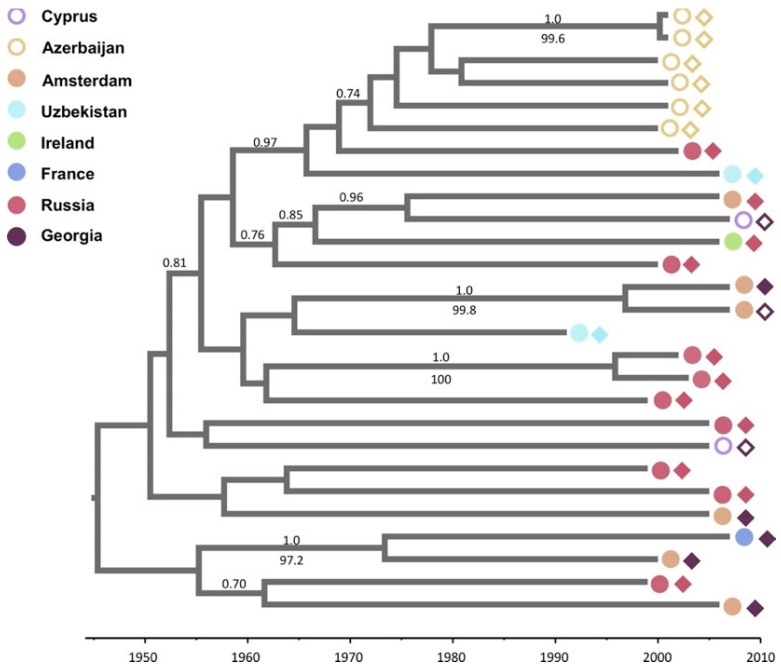

Figure 4. Molecular clock phylogeny of the CRF01_1b2k clade, estimated using a relaxed uncorrelated lognormal clock model and the SDR06 nucleotide substitution model (see Materials and Methods). *Sequences are colour labelled according to the country of origin (diamonds) and the country of sampling (circles). All isolates were found to have an epidemiological link to Russia or the former Soviet Union (Table 1). Filled circles/diamonds, 2k/1b isolates that were confirmed as being recombinant by sequencing of the breakpoint in the NS2 region; open circles/diamonds, those isolates for which NS2 sequences were not available. The numbers above the branches show the posterior probability of nodes in the MCC tree; numbers below the branches represent bootstrap support values using maximum likelihood.*

The MCC tree of the CRF01_1b2k clade (Figure 4), when combined with all available epidemiological information (Table 1), indicates a clear pattern of phylogenetic clustering according to the geographic location and risk factor of each patient. For example, where these details were available, 14 out of 15 isolates were associated with IDU, while only 1 was isolated from a patient with a history of blood transfusion (Table 1). These observations further support the view that CRF01_1b2k transmission is strongly linked with the IDU transmission route. Although it is not possible to reconstruct the location of the common ancestor of the CRF lineage with any certainty (because our sample size is small and the basal branches of the phylogeny are poorly supported), all of the isolates included in this study are from or have an epidemiological link to the former Soviet Union, and the oldest strain was sampled in Russia in 1999. A well-supported cluster of strains from Azerbaijan originated in about 1970, and therefore, the CRF has likely circulated there since that time. Hence, it appears that CRF01_1b2k disseminated throughout the Soviet Union before its dissolution in the late 1980s and for some time prior to the discovery of the CRF in St. Petersburg in 1999.

Discussion

As the only known HCV recombinant in widespread circulation, the existence and emergence of CRF01_1b2k present an interesting question in HCV epidemiology and evolution. Investigating its evolutionary origins and transmission history helps to understand the circumstances that led to its unique properties. In contrast to HIV, which has 49 known CRFs and a much greater number of unique recombinant forms [54], recombination typically contributes little to the generation and maintenance of HCV genetic diversity. Given that HCV has a higher global prevalence than HIV and, thus, all else being equal, there is a high likelihood of dual infections with divergent HCV strains, it is unlikely that epidemiological factors are restricting the opportunities for HCV to generate CRFs. Mixed infections with divergent HCV strains have been reported for many different populations and are noted to be prevalent among high-risk groups, particularly IDUs and some haemophiliacs [55–59].

Since the opportunities for HCV recombination are not limited, it is more likely that fundamental molecular and evolutionary differences between HIV and HCV explain why HIV has many CRFs and HCV has few. These could include differences in the rate of template switching or differences in genomic or immunological constraints, such that HCV recombinants have, on average, lower fitness than HIV recombinants and therefore are rarely transmitted [60]. Although both viruses are associated with chronic infections, unlike HIV, HCV can be spontaneously cleared by the host. This may explain in part the differences in the number of recombinants between HIV and HCV, where partial protective immunity against the latter reduces that chance of *in vivo* recombination of HCV strains [61–63]. However, the high rate of mixed infections observed suggests that this is likely at best to play a minor role in HCV recombination. The low frequency of HCV recombinants is more likely to reflect mechanistic constraints on viral replication. There is evidence that template switching in HCV is especially rare and that the replication complex is typically encoded on the same genomic strand that it will replicate and transcribe [64]. It is also interesting that when replication complexes are exchanged between different genotypes, the replication efficiency is substantially reduced [65]. The pseudodiploidy of the HIV genome certainly increases the likelihood of recombination occurring due to the ability of the virus to package two RNA templates [66], while the secondary RNA structure in the HCV genome may limit the production of viable hybrid HCVs [67,68].

Our study of previously reported and newly obtained HCV isolates provides the first estimates of the date of the recombination event that generated CRF01_1b2k. We estimated the time of origin of CRF01_1b2k to be between 1923 and 1956, which is not much later than the origin and global spread of the parental subtype 1b [30]. This date is significantly earlier than we expected: we expected that the CRF's creation might be linked to the dramatic increase in IDU behaviour following the breakup of the former Soviet Union. This result is robust to the manner of isolate sampling: if, because of non-random sampling, our isolates are more closely related to each other than under random sampling, then the TMRCA of CRF01_1b2k would be biased toward more recent dates. Furthermore, despite its small size, our data set provides a relatively short time window during which the recombinant must have arisen (Figure 2). The involvement of subtype 1b in the recombinant is not surprising, as it is one of the most prevalent subtypes worldwide. However, to fully appreciate the origin of CRF01_1b2k, we need to consider the evolutionary history of both parental subtypes and of the recombinant lineage itself. Genotype 2 harbours considerable genetic diversity, especially in West Africa, which is where the genotype is thought to have originated [69]. Although the small number of subtype 2k isolates sampled to date likely underestimates the true extent of subtype 2k distribution, such viruses have been isolated from Martinique and Madagascar, implicating a role for the historical trans-Atlantic slave trade in the dissemination of the virus from West Africa [69].

The current distribution of subtype 2k is associated with francophone regions and former Soviet Union countries. In contrast, CRF01_1b2k is more spatially limited, with all isolates being directly or indirectly linked to the former Soviet Union. As the nonrecombinant subtype 2k isolates that are most closely related to CRF01_1b2k are from Moldova and Azerbaijan (see Figure S1 at http://jvi.asm.org/content/86/4/2212/suppl/DC1), it seems most likely that CRF01_1b2k was generated in the former Soviet Union. An equivalent analysis of subtype 1b viruses provides no reliable phylogeographic linkage, due to the low phylogenetic resolution of the NS5B data set.

Our estimated date of CRF origin coincides with an interesting period of history in the former Soviet Union, which was an early leader in transfusion technology. Under the leadership of Alexander Bogdanov in the 1920s, a nationwide network of blood transfusion centres and research institutes, as well as the Central Institute of Hematology in Moscow, Russia, in 1926, was established throughout the Soviet republics [70]. This expanded into a network of ~1,500 blood donating centres across the republics [71]. The Soviets also adopted blood storage and preservation techniques at an early stage. They established more than 60 primary and 500 subsidiary blood storage centres by the mid-1930s, which shipped blood across the entire Soviet Union [70]. During the Second World War, these networks were swiftly readapted to support the front line; in Moscow alone, about 2,000 blood donations were given per day [70,71]. The impressive scale of the blood service in the former Soviet Union is likely to have favoured HCV transmission by increasing the efficiency and geographic range of the virus's dissemination. Whether specific medical practices at this time increased the probability of mixed viral infections remains unknown. It is interesting to note that Bogdanov himself was fascinated by the ideological interpretation of blood sharing and frequently practiced what he called “physiological collectivism”: the exchange of blood with others through mutual transfusions [70].

Although unscreened blood transfusions can provide a credible hypothesis for the origin of CRF01_1b2k in the Soviet Union some time from 1923 to 1956, we must also attempt to explain how subtype 2k or the CRF itself arrived in the Soviet Union from West Africa or the Caribbean. Migration from Africa to the former Soviet Union did occur during the late 1950s and 1970s as a result of alliances forged by the Soviet government with newly independent African states such as Ghana and Angola [72]. However,

these connections are too late to have contributed to the emergence of CRF01_1b2k, according to our dating estimates. Although we cannot reject the hypothesis that the CRF was formed in West Africa and subsequently moved to the Soviet Union, our results are more consistent with the recombination event occurring in the latter. This uncertainty is likely to be reduced with further samples, especially subtype 2k viruses, from African and former Soviet Union locations.

The epidemic history CRF01_1b2k (Figure 3) since its emergence is similar to that estimated for other epidemic subtypes of HCV (e.g., see reference [30]. The growth in the CRF01_1b2k effective population sizes coincides with a substantial increase in blood transfusion, including during the Second World War, and with the subsequent rise in intravenous drug usage. CRF01_1b2k transmission seems to have slowed or stabilized since the early 1990s, coinciding with the onset of the anti-HCV screening of donors. In the absence of any data to the contrary, the transmission of this recombinant and its spread from the former Soviet Union reflect the peculiar epidemiological properties of the risk groups that it has been associated with rather than any intrinsic properties of the virus.

We demonstrate the practicality and benefits of using a hierarchical phylogenetic model to jointly estimate parameters of interest when analysing multipartite sequence data that result from genetic exchange. This method yields more accurate parameter estimates than previous methods (e.g., see references [38] and [37] by incorporating the phylogenetic information and uncertainty in different genomic regions. We recommend that this improved statistical framework be used in future investigations of recombination in fast-evolving RNA viruses.

This study has made significant steps in understanding the epidemic history and spread of the unique circulating HCV recombinant 2k/1b. Most significantly, we show that this strain originated many decades before the post-Soviet rise in injection behaviour with which it is currently associated. On the basis of the date of its origin and its molecular epidemiology, there are reasonable grounds to suppose that the Soviet Union's revolutionary blood service was instrumental in the CRF's early generation and continental-scale spread. This infrastructure may have facilitated the pan-Eurasian spread of other parenterally transmitted blood-borne infections, and this is an interesting question for future research.

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Part II

Acute Hepatitis C Virus Infections in
HIV-Infected Men who have Sex with Men

Chapter 5

Alarming Incidence of Hepatitis C Virus Reinfection after Treatment of Sexually Acquired Acute Hepatitis C Virus Infection in HIV-Infected MSM

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Abstract

Background: Recent data indicate that seroprevalence of sexually transmitted hepatitis C virus (HCV) infection among MSM is stabilizing in Amsterdam. However, little is known about the incidence of HCV reinfection in MSM who have cleared their HCV infection. We, therefore, studied the incidence of reinfection in HIV-infected MSM who were HCV RNA-negative following HCV treatment of acute primary infection.

Methods: Our study population comprised HIV-infected MSM at two large HIV out- patient clinics in Amsterdam, who were previously diagnosed with a sexually transmitted acute HCV infection and tested HCV RNA-negative at the end of treatment. We defined HCV reinfection as detectable HCV RNA in individuals with an undetectable HCV RNA at the end of treatment accompanied by a switch in HCV genotype or clade. Person–time methods were used to calculate the incidence of reinfection.

Results: Fifty-six persons who became HCV RNA-negative during primary acute HCV treatment were included. Five of the 56 cases relapsed and were not analysed. Eleven persons were re-infected. The incidence of HCV reinfection in this group was 15.2 per 100person-years (95% confidence interval 8.0–26.5). The cumulative incidence was 33% within 2 years.

Discussion: An alarmingly high incidence of HCV reinfection was found in this group. This high reinfection rate indicates that current prevention measures should be discussed, frequent HCV RNA testing should be continued after successful treatment and, in case of possible relapse, clade typing should be performed to exclude re-infection.

Introduction

In the last decade, the sudden increase in incidence of acute hepatitis C virus infection (HCV) among HIV-infected MSM in Europe, Australia and the United States has led to a substantial number of studies on this new public health problem. It has become clear that transmission takes place in specific clusters of HIV- infected MSM engaging in high-risk sexual behaviour [1–3]. Subsequently, targeted prevention messages have been developed, focusing on sexual risk behaviour, recreational drug use and regular testing for HCV. Furthermore, treatment of HCV co-infection in this population has proven to be very successful in the acute phase, and recommendations on treatment have been published [4,5].

The response to this epidemic has clearly been extensive, and a recent study in Amsterdam has suggested that the prevalence of new primary HCV infections may no longer be increasing (A.T. Urbanus et al., presented at AIDS Conference 2010, abstract WEPDC 104). The question remains, however, whether prevention messaging and early testing and treatment also prevents HCV reinfection in MSM co-infected with HCV and HIV who have cleared their infection.

HCV reinfection occurs frequently among IDUs who continue high-risk behaviour [6]. Incidence rates of re- infection vary depending on population, definition of re-infection and methods and frequency of testing [6–10]. Reports on HCV reinfection by sexual transmission among MSM have been published only rarely [11,12] (H.-J. Stellbrink et al., presented at CROI 2011, poster 645; presented at International Congress on Drug Therapy in HIV Infection 2010, poster 200; and J. Sasadeusz et al., presented at EASL 2011, poster presentation), and no specific incidence rates have been presented yet.

Therefore, the objective of the current study was to examine the incidence of HCV reinfection among HIV- infected MSM attending two HIV outpatient clinics in Amsterdam, who were HCV RNA-negative at the end of treatment for their initial acute HCV infection.

Methods

Study population

We included 56 HIV-infected MSM at the HIV outpatient clinics of two major hospitals in Amsterdam, who had been previously diagnosed with and treated for an acute HCV infection between 2003 and 2011; none had detectable HCV RNA at the end of their HCV treatment. All patients had been treated with weekly injections of peg-interferon and daily doses of ribavirin, the majority for a duration of 24 weeks [13]. In the majority of the cases, no HCV parental transmission routes were identified by clinical history and sexual transmission was the most likely mode of transmission.

Data collection

Sociodemographic, clinical and virological data, such as age, use of HAART, CD4 cell counts, HIV RNA levels, levels of alanine aminotransferase (ALT) and genotype of primary HCV infection were collected from medical files. A subset of the MSM at risk of reinfection (n = 21) was included in a prospective study of acute infection with HCV in MSM (MSM Observational Study of Acute Infection with hepatitis C, MOSAIC study). For these patients, additional data on risk behaviour are presented. Data collection exists of an extensive self-administered questionnaire regarding classic risk factors for HCV transmission, such as IDU and sexual risk behaviour, collected at baseline and follow-up visits.

Virological testing

All plasma samples available after the end of treatment were tested for HCV RNA with the Siemens VERSANT transcription-mediated amplification (TMA) assay which has a detection limit of 5 IU/ml. Genotyping of the first TMA-positive sample after the end of treatment was performed by amplifying and sequencing a 389 base pair fragment of NS5B, as described by Murphy et al.[14]. If the genotype was similar to that in the treated primary infection, a 573 base pair fragment of E2 including the hyper variable 1 region (HVR1) was amplified and sequenced directly to identify clade shifts and differentiate between relapse or reinfection.

Definition of reinfection and relapse

Reinfection was defined as having detectable HCV RNA following an undetectable level at the end of treatment, with demonstration of the presence of a different genotype compared with primary infection or, if genotype was similar, a different clade compared with primary infection, as indicated by phylogenetic analysis of the E2/HVR1 region. If in the phylogenetic pretreatment and posttreatment sequences from the same viral subtype (e.g. 1a) large genetic distances were present, as indicated by distinct clustering, this was defined as a clade switch and, therefore, as a reinfection with the same viral subtype. *Relapse* was defined as a positive HCV TMA after a negative HCV TMA at the end of treatment and no genotype or clade switch compared with the primary infection.

Phylogenetic analysis

Sequences were aligned using Clustal X version 2 [15]. Phylogenetic trees were inferred using maximum-likelihood methods, using a Generalized Time Reversible Model with a gamma distribution of mutations (GTR + G) as implemented in MEGA software package version 5 [16]. Bootstrap values were determined from 500 bootstrap resamplings of the original.

Statistical analysis

The incidence rate of reinfection was estimated by dividing the number of reinfections by the total duration of follow-up. The individuals who had relapses were excluded from this calculation. The cumulative incidence was estimated by Kaplan–Meier methods.

In case of reinfection, follow-up time was calculated as the time between the end of treatment and the date of re- infection; the latter was estimated by taking the midpoint between the last negative HCV RNA test and first positive HCV RNA test. If no reinfection occurred, the censor date for follow-up was the date of the last HCV RNA test.

We compared sociodemographic, clinical, virological and behavioural characteristics, including peak ALT levels during follow-up between patients with and without reinfection. Risk behaviour was compared between MSM with and without reinfection for whom risk questionnaires were available. Differences between the two groups were tested with the x2-test or Fisher's exact test for categorical variables and Student's t-test or Mann–Whitney U-test for continuous variables. Analyses were performed using SPSS (version 17.0; SPSS Inc., Chicago, Illinois, USA). The incidence rate and its confidence interval (CI) were calculated with OpenEpi [17]and are given per 100 person-years.

Results

In total, follow-up was obtained for 56 HIV-infected MSM treated for acute HCV infection who were HCV RNA-negative at the end of treatment. Patients were treated between 2003 and 2011. Patient and virological characteristics are shown in Table 1.

Characteristics	All (N= 51), % (n)	Not re-infected (N = 40), % (n)	Re-infected (N = 11), % (n)
Baseline			
Median age in years (IQR)	44.3 (39.1–48.8)	44.6 (40.7–48.8)	42.3 (36.6–49.1)
Genotype primary infection			
Gt 1	69 (35)	73 (29)	55 (6)
Gt 2	2 (1)	3 (1)	–
Gt 3	4 (2)	5 (2)	–
Gt 4	22 (11)	15 (6)	45 (5)
Unknown	4 (2)	5 (2)	–
Median CD4 cell count at end of treatment (cells/ml)	305 (240–403)	300 (240–308)	335 (243–388)
Follow-up			
Total follow-up time in years	72.2	63.0	9.2
Median follow-up time in years (IQR)	1.3 (0.5–1.6)	1.4 (0.5–2.3)	0.7 (0.3–1.6)
Median time between tests in months (IQR)	3.0 (1.9–4.3)	3.0 (2.0–4.5)	2.7 (1.7–4.1)
cART use	75 (38)	73 (29)	82 (9)
Median maximum ALT during follow-up (U/l)	37 (26–63)	34 (25–56)	67 (28–136)
Median CD4 cell count at last HCV RNA-negative visit (not re-infected)/first HCV RNA-positive sample (re-infected) (cells/ml)	450(400–620)	450 (400–583)	440 (345–675)
Genotype switch at reinfection			
Gt 1 → Gt 4			18 (2)
Gt 4 → Gt 1			36 (4)
Gt 1 → Gt 2			18 (2)
Gt 1 → Gt 1, different clade			18 (2)
Gt 4 → Gt 4, different clade			9 (1)

Table 1. Baseline and follow-up characteristics of hepatitis C virus–HIV-co-infected patients at risk of hepatitis C virus reinfection. *ALT, alanine aminotransferase; cART, combination antiretroviral therapy; HCV, hepatitis C virus; IQR, interquartile range; Gt, genotype.*

Five of the 56 experienced relapse, as evidenced by sequencing of the E2/HVR1 region, and were excluded from the incidence calculations.

According to our definition, 11 of the remaining 51 persons became re-infected. The total follow-up time for the 51 persons was 72.2 years [median 1.3 years, interquartile range (IQR) 0.5 – 1.6]. The incidence of HCV reinfection was 15.2 per 100 person-years (95% CI 8.0–26.5). Among the 11 individuals with a reinfection, the median time until reinfection was 8.4 months (IQR 3.6–19.2). The majority of re-infected patients switched from genotype 4 to 1.

Three persons became re-infected with the same genotype (clade switch). Figure 1 shows the phylogenetic tree of pretreatment and posttreatment E2-HVR1 sequences of these patients together with pretreatment and posttreatment sequences of relapse patients. Pretreatment and posttreatment sequences from patients O1, O2, O3, P06 and P44 clearly cluster together and they were, therefore, classified as 'true' relapsers, corresponding with the clinical observation of RNA rebound at the first time point available after treatment withdrawal. In contrast, pretreatment and posttreatment sequences from patients P01, P31 and P48 do not cluster and they were, therefore, considered reinfections. Although patients P01 and P31 became HCV RNA-positive again within 6 months after the end of treatment, the first sample taken at 4 weeks was negative, supporting our phylogenetic evidence of re-infection.

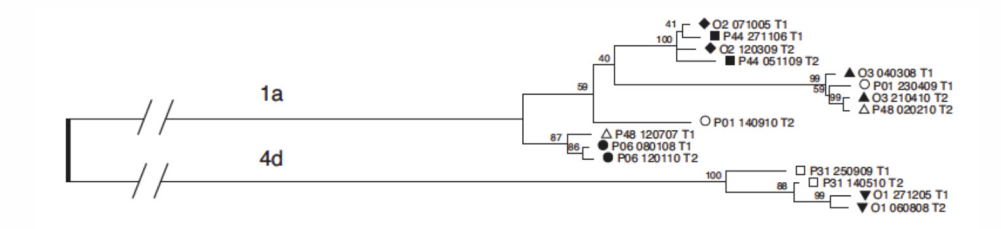

Figure 1. Phylogenetic tree of relapsers and patients with a reinfections with the same genotype. *Phylogenetic tree of sequences before and after treatment from relapsers and patients with a reinfection with the same genotype. Relapsers are presented by filled symbols and reinfections are presented by open symbols. Each patient is presented by a unique symbol. The numbers in the labels indicate sampling dates. Note: reinfections with a different genotype are not presented in this tree.*

The cumulative incidence of reinfection is demonstrated in Figure 2; after 2 years, the cumulative incidence of re- infection was 33% (95% CI 16–50).

In order to examine whether ALT levels are useful for indicating a new infection, we compared peak ALT levels during follow-up in patients with and without re- infection. The peak ALT levels were in general low (with a maximum of 160 U/l), although the median ALT peak during follow-up was higher in individuals with a re- infection than in those without a reinfection (P = 0.01). Interestingly, in four cases with a reinfection, no increased ALT levels were observed, whereas in individuals without evidence of reinfection, ALT levels were elevated frequently (Figure 3).

CD4 cell counts did not differ between patients with and without reinfection (Table 1). In addition, analysis of HIV load data from the eight of nine patients with a re- infection, who were on combination antiretroviral therapy (cART), showed that all patients had undetectable HIV loads around the time of HCV reinfection. From one re-infected patient on cART, no HIV load data were available.

Analysis of the 21 MSM with behavioural data revealed that re-infected MSM (n = 7) significantly more often reported non-injecting recreational drug use at inclusion than MSM without reinfection (n = 14) (P = 0.048). In this small study population, no statistically significant differences in sexual risk behaviour were found.

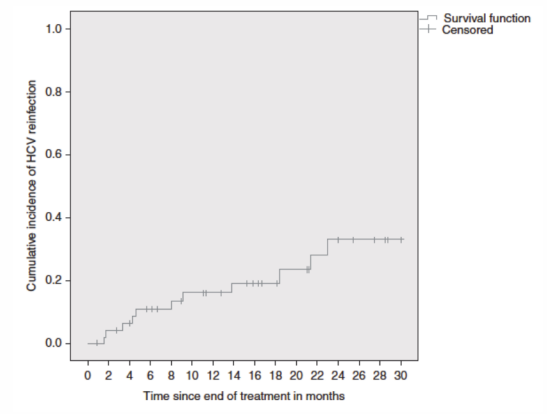

Figure 2. Cumulative incidence of hepatitis C virus (HCV) reinfection after successful treatment of primary HCV infection.

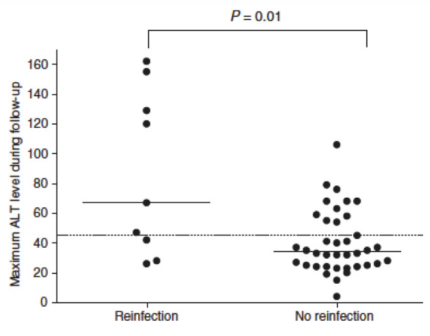

Figure 3. Peak alanine aminotransferase levels during follow-up. *Peak alanine aminotransferase (ALT) levels in units per litre during the observation period (end of treatment until last negative or first positive time point). The dotted line represents the maximum normal ALT value.*

Of the 11 re-infected patients, four were treated for their reinfection; of those four, two achieved a sustained virologic response (SVR), one had a relapse, and one is still in follow-up for SVR time.

Discussion

With this study, we demonstrate an alarmingly high incidence rate of sexually transmitted HCV reinfection among HIV-infected MSM previously successfully treated for primary HCV infection.

Most importantly, these findings stress the importance of repeated risk counselling for HCV transmission which should be provided not only before and during treatment but also after its completion. MSM re-infected with HCV showed higher rates of non-injecting recreational drug use. Sexual risk behaviour, including recreational drug use during sex, was highly prevalent (data not shown). Unfortunately, because of our relatively small study population with behavioural data, we were not able to examine risk behaviour more precisely and longitudinally.

The high incidence rate in this study implies that ALT levels, which can be elevated during an acute HCV infection, should be measured regularly in this population. Because these levels are not always elevated during acute infection or might not coincide with the test moment, as shown in Figure 3, subsequent HCV RNA testing, especially in cases of high-risk behaviour, should be performed regularly, as antibodies remain in general present after successful treatment.

Along with risk behaviour, the role of biological susceptibility to HCV reinfection, although still unclear, is an important consideration. Importantly, discussion is ongoing whether previous infection with HCV can generate partial protective immunity to reinfection or increase the chance of clearance of re-infection.

Several studies that compared incidence rates of primary infection and reinfection among IDUs have presented results that argue both for [9,18,19] and against [6,7,10] this phenomenon. Differences between these studies are probably due to variations in intervals of testing, age of the participants, frequency of ongoing drug use and adjustment for behaviour in the analyses. The higher incidence of reinfection in our study compared with the incidence of primary infection in Amsterdam [2,20] and elsewhere a [3] indicates that, as expected, there is no complete protection. Yet, the finding that most re- infections in this study occurred with a different genotype compared with the primary infection suggests that genotype-specific immunity may develop in some individuals.

Additionally, underlying a persons' ability to develop protective immunity, genetic profiles may play a role in susceptibility to HCV reinfection. The association of genetic variations near the interleukin-28B (IL28B) region with spontaneous HCV clearance and treatment- induced clearance has been well described for HCV- mono-infected patients [21–28]. Similar results have been found regarding HCV treatment response in persons co-infected with HIV, although in this population the association is less clear for those treated during the acute infection [29–34]. The effect of IL28B polymorphisms on HCV reinfection has not been described. In accord with the effects in primary infection, one would expect the responder genotype to be at least more likely to allow clearance of reinfection than the nonresponder genotype. Whether initial partial protective immunity is also more established in individuals with a beneficial IL28B genotype remains undetermined.

Apart from HCV-specific immune responses, HIV co- infection may play an important role. The exact role of HIV co-infection in primary sexually transmitted HCV infection is not well known. CD4 cell depletion in the gut may diminish the immune response against HCV sexual transmission [35]. Nevertheless, CD4 cell counts did not differ between patients with and without reinfection, indicating that the level of immune suppression caused by HIV co-infection does not influence the risk of reinfection following successful HCV treatment.

The results of this study may lead to a change in the current definitions of HCV relapse and reinfection. When no further genotyping or sequencing is performed, a recurrent HCV viraemia within 6 months after a negative test at the end of the treatment is currently considered a relapse [36]. Our study demonstrates that early recurrence of HCV could well be a reinfection with another genotype or strain. This distinction has important clinical ramifications and should, therefore, be recognized by clinicians. The definition of relapse or re- infection, especially in population with a high incidence of infection, should, therefore, always be based on virological characteristics and not on a specific interval between the end of treatment and recurrence of HCV RNA in the serum.

Finally, from a clinical and cost-effective perspective, the results of this study will encourage discussion about the validity of repetitive HCV treatment in patients with numerous subsequent reinfections owing to continued risk behaviour.

Apart from small numbers, this study has other limitations. We have not studied the possible existence of HCV-mixed infections during primary infection. Therefore, we cannot entirely exclude the possibility that reinfections were previously existing infections that became detectable after a dominant strain had been cleared [37]. However, the fact that the median interval from the first HCV RNA-negative test to the first HCV RNA-positive test after treatment was 8 months, with several negative results in between, strongly suggests that all reinfections were recently transmitted infections.

Furthermore, as this was not a prospective study, time between tests was not similar for all patients, and a re- infection followed by a quick, spontaneous clearance might have been missed. Nevertheless, as the median time between tests was 3 months, we do not expect this to have significantly influenced the incidence rate.

In conclusion, a high incidence rate of HCV reinfection among HIV-infected MSM in Amsterdam was demonstrated in this study, emphasizing the need for more extensive risk behaviour counselling and secondary prevention by regular and frequent HCV testing in this population. Future research should focus on the reasons for continuing high-risk sexual behaviour in order to improve targeted prevention. In addition, research should try to elucidate the virological and host factors associated with reinfection and its outcome in HIV-infected individuals.

Acknowledgements

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Chapter 6

Characterization of Multiple Hepatitis C Virus (HCV) Infections following Acute Infection in HIV-Infected Men who Have Sex with Men: is Immunity to HCV Genotype Specific?

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Manuscript submitted

Abstract

Background: High rates of hepatitis C virus (HCV) reinfections among HIV-infected men who have sex with men (MSM) following clearance of a primary infection, suggest a lack of protective immunity. Here, we investigated incidence and genotype of HCV super- and reinfections in a cohort of acute HCV infections in HIV-infected MSM.

Methods: Genotyping was performed longitudinally to identify new infections in 85 HIV infected MSM following acute infection. If the primary genotype was still present at the most recent viremic time point, longitudinal E2/HVR1 sequence analysis was performed to distinguish a new infection with the same genotype (clade-switch) from intra-host evolution. Incidence rate and cumulative incidence of secondary infections were estimated and the effect of primary genotype (1a versus non-1) on the risk of acquiring a second infection with the same genotype was determined using Cox proportional hazards analysis.

Results: Among 85 patients with a median follow-up of 4.8 years, incidence rate of secondary infections was 5.39 cases/100 person-years (95% CI 3.34-8.26). Cumulative incidence of genotype-switches was markedly higher than the cumulative incidence of clade-switches (26.7% vs. 4.8% at 5 years respectively). In patients with HCV-1a as primary infection, the risk for acquiring another HCV-1a infection was reduced compared to those with a primary non-HCV-1a subsequently acquiring HCV-1a (HR = 0.25, 95% CI 0.07 – 0.93).

Conclusion: Risk of acquiring a secondary infection with the primary genotype was strikingly reduced compared with acquiring a secondary infection with another genotype.

Introduction

Sexual transmission of hepatitis C virus (HCV) is considered to be rare [1], but over the past decade there is increasing evidence of sexual transmission of HCV among HIV-infected men who have sex with men (MSM) [2,3]. Given the reduced spontaneous clearance [4] and more rapid progression to liver fibrosis and cirrhosis [5], the increased incidence of HCV among this population is of great concern. Nevertheless, the infection can be cured, especially during the acute phase, with reported success rates above 60% [6,7]. With new antivirals becoming available, success rates are expected to increase further, although this comes with a substantial cost [8].

Unfortunately, after viral eradication, patients may become reinfected again as observed among people who inject drugs [9–12]. Whether clearance of a primary infection, either treatment-induced or spontaneously, results in some degree of protection against (persistence of) reinfection is still undecided given the inconsistent findings in studies that addressed this issue thus far [12–16].
To date, the number of studies on HCV reinfection among HIV-infected MSM has been very limited. We previously reported a very high reinfection rate of 15.2 per 100 person-years among patients successfully treated for acute HCV infection [17]. This finding was confirmed and extended in a recent study from the UK, that investigated the rate of reinfection among both treatment induced and spontaneous clearers of acute HCV infection [18].
The true incidence of new infections among HIV-infected MSM is likely to be underestimated for several reasons. First, depending on interval of testing, secondary infections that were rapidly cleared may have been missed. Second, patients with persistent infection were not included in both studies, whereas persistent viremia may have been caused by infection with a new virus, with or without (observed) clearance of the primary virus. Third, without genetic analysis of the 'relapsing' virus, recurrence of viremia within 24 weeks following clearance of viremia may be misclassified as relapse [9,19].

Increasing our knowledge on the incidence of multiple infections and assessing the genetic relatedness of primary and successive viral strains will provide more insight into correlates of immunity against HCV and is crucial for vaccine development and for targeted preventive strategies. We therefore studied the occurrence of multiple HCV infections in persistently infected patients and in patients who cleared the infection with or without treatment. The presence of new infections with a different genotype (genotype switch), or with a different strain from the same genotype (clade switch) was assessed by systematically sequencing the virus present during the entire viremic period.

Methods

Study population

The study population consisted of 85 HIV-infected MSM attending the HIV-treatment clinic of the Academic Medical Center (AMC) in Amsterdam, the Netherlands, who acquired HCV infection sexually between 1994 and 2011. Acute HCV infection was defined by a positive HCV RNA test preceded by a negative HCV RNA test in patients without evidence of past HCV infection. Patients were either prospectively identified during acute infection or retrospectively by determining HCV RNA in anti-HCV positive patients in stored sera from earlier time points. The majority of patients are participants of the

MSM Observational Study of Acute Infection with hepatitis C (MOSAIC), a prospective cohort study on acute hepatitis C infection in HIV-infected MSM [6]. Informed consent was obtained from MOSAIC participants and all patient material and data was processed according to hospital guidelines.

Virological assessments

HCV RNA presence was assessed by either TMA (VERSANT® HCV RNA Qualitative Assay, Siemens) or bDNA (VERSANT® HCV RNA 3.0 Assay, Siemens). For genotyping, a 389-base pair (bp) fragment spanning positions 8616-8275 relative to the H77-strain (AF009606) of the NS5B region was amplified and sequenced as described by Murphy et al. [20]. For the detection of new infections with the same genotype (i.e. clade-typing), a 590-bp fragment from the envelope, spanning positions 1295-1885 relative to the H77-strain, which includes the HVR1 (designated 'E2/HVR1'), was amplified and directly sequenced.

Analysis of multiple infections

For each patient, the first and most recent RNA positive samples were selected for NS5B-genotyping. If a genotype switch was observed between the first and last RNA positive time points, samples taken between these two time points were genotyped to determine the interval of genotype switch.

If, at the most recent time point, the original genotype was still present, sequence analysis of E2/HVR1 was performed longitudinally on the stored sera, to detect secondary infections with the same genotype (i.e. clade switch).

Phylogenetic analysis was used to determine whether the evolving primary virus was still present or replaced by a new viral strain from the same genotype. Sequences were analysed using Codoncode version 3.7.1, aligned using Clustal X version 2.0.11, and edited using GeneDoc version 2.7 software. For visual inspection of sequences, maximum likelihood trees were constructed for each genotype under a Hasegawa-Kishino-Yano evolutionary model with invariant sites plus a gamma distribution of among-site rate heterogeneity (HKY+I+G) as implemented in MEGA version 5 [21]. This substitution model exhibited the best-fit evolutionary substitution-model for this fragment, using the akaike information criterion (AIC) in modeltest version 3.7 [22] as implemented in Paup*4.0 [23]. Trees were unrooted and bootstrap values were determined from 1000 bootstrap resamplings of the original data.

To define an objective criterion for distinguishing intra-host evolution from a clade-switch, nucleotide substitution rates were estimated for the E2/HVR1 region for all patient-specific internal branches using a Bayesian Markov Chain Monte Carlo (MCMC) approach as implemented in the program BEAST, version 1.7.4[24]. For each genotype, the model was run separately by applying an uncorrelated relaxed lognormal molecular clock [25] and a coalescent exponential population size with a random starting tree [26,27]. The MCMC chains were run for 100.000.000 states to obtain a good convergence and effective sample size (ESS) >200. The model was run whilst enforcing monophyly for each patient, thereby forcing the model to impose an unrealistically high nucleotide substitution rate for branches with sequences that derived from a heterologous virus. Substitution rates for all branches were extracted from the maximum clade credibility tree as created by TreeAnnotator version 1.7.4 and constructed using FigTree version 1.4.0. Mean substitution rates from all internal branches from patient-specific monophyletic clades were calculated and a rate exceeding the mean plus three times the standard deviation (SD) was considered as evidence for the occurrence of a clade-switch between the two time points.

Case definitions

Cleared infections were defined as absent HCV RNA for at least 60 days in untreated patients or when a sustained viral response (SVR) was achieved 24 weeks after treatment discontinuation. If a new virus was present within the 24 week period following end of treatment, the response was regarded as SVR.
A *reinfection* was defined as recurrence of HCV RNA with a viral strain other than the primary one after a cleared infection. A *superinfection* was defined as the detection of a new viral strain other than primary virus during follow-up without documented HCV RNA negative time points in between. Throughout the manuscript, the term *new* infection refers to either reinfection or superinfection.

Statistical analysis

The incidence rate of new infections following primary infections and its confidence interval (CI) were calculated using person time methods and are given per 100 person-years. The mid-P test was used to compare incidence rates. The date of primary HCV infection was estimated as the midpoint between the date of last RNA negative sample and the date of the first RNA positive sample. Follow-up time was calculated from estimated time of infection until the date when either a genotype or clade switch occurred, or the last date of HCV genotyping if no viral switch had occurred. Cumulative incidence curves were estimated within a competing-risks framework to determine the incidence of the first genotype switch compared to the first clade switch during follow-up. In addition, using Cox proportional hazards analysis, the effect of genotype (1a versus non-1a) at primary infection on the risk of a secondary infection with genotype 1a infection was determined. During treatment, patients were considered not to be at risk for a new infection. A p-value <0.05 was considered to be statistically significant. The R language and environment for statistical computing, version 2.8 and SPSS statistical software (version 19.0, SPSS Inc, Chicago, IL) were used for the data analysis.

Results

At the time of HCV infection, the median age was 41.6 (IQR 36.2-46.8) years and the median CD4+ cell count was 500 (IQR 393-638) cells/µL. HIV load was available for 80 out of 85 patients: in 39 (48.8%) no HIV RNA was detectable at the time of HCV infection. Median interval between HCV RNA testing was 2.2 (IQR 0.9-4.8) months. Fifty-six out of 85 patients (65.9%) were treated with peg-interferon and ribavirin during the acute stage of infection, and 46/56 (82.1%) achieved SVR. Five out of 29 patients not on treatment (17.2%) cleared the primary infection spontaneously.

Genotype switches

NS5B genotyping resulted in the identification of 18 genotype switches with a switch from HCV-4d to HCV-1a being the most common (n=9). Other observed genotype switches were 1a to 4d (n=3), 1a to 2b (n=2), 1a to 3a (n=1), 1b to 4d (n=1), 1a to 1b (n=1) and 3a to 1a (n=1). Genotype switches occurred in both treated and untreated patients following both cleared and persistent infections. Sixteen of these 18 genotype switches were secondary infections, and two were tertiary infections in two patients.

Clade switches

Given the high homogeneity of NS5B sequences [9], for the remaining 69 patients without a genotype switch, phylogenetic analysis of consecutive E2/HVR1 sequences was performed to determine whether changes in E2/HVR1 over time were compatible with either intra-host evolution or a clade switch.

A total of 380 sequences were analysed. Visual inspection of ML-E2/HVR1 trees indicated that in general, sequences clustered per patient, with some intermingling of sequences from different patients in the beginning of the infection. However, in six HCV-1a infected patients, variants were detected during follow-up that formed distinct clusters, suggesting replacement of the primary strain with another HCV-1a strain (Figure 1). Intermingling in the beginning of infection was also observed in the HCV-4d tree. Here, sequences derived from one patient occasionally formed separate clusters during follow-up (Figure 2, e.g. patient 004 and 013), making distinction between intrahost evolution and occurrence of a clade switch not entirely straightforward.

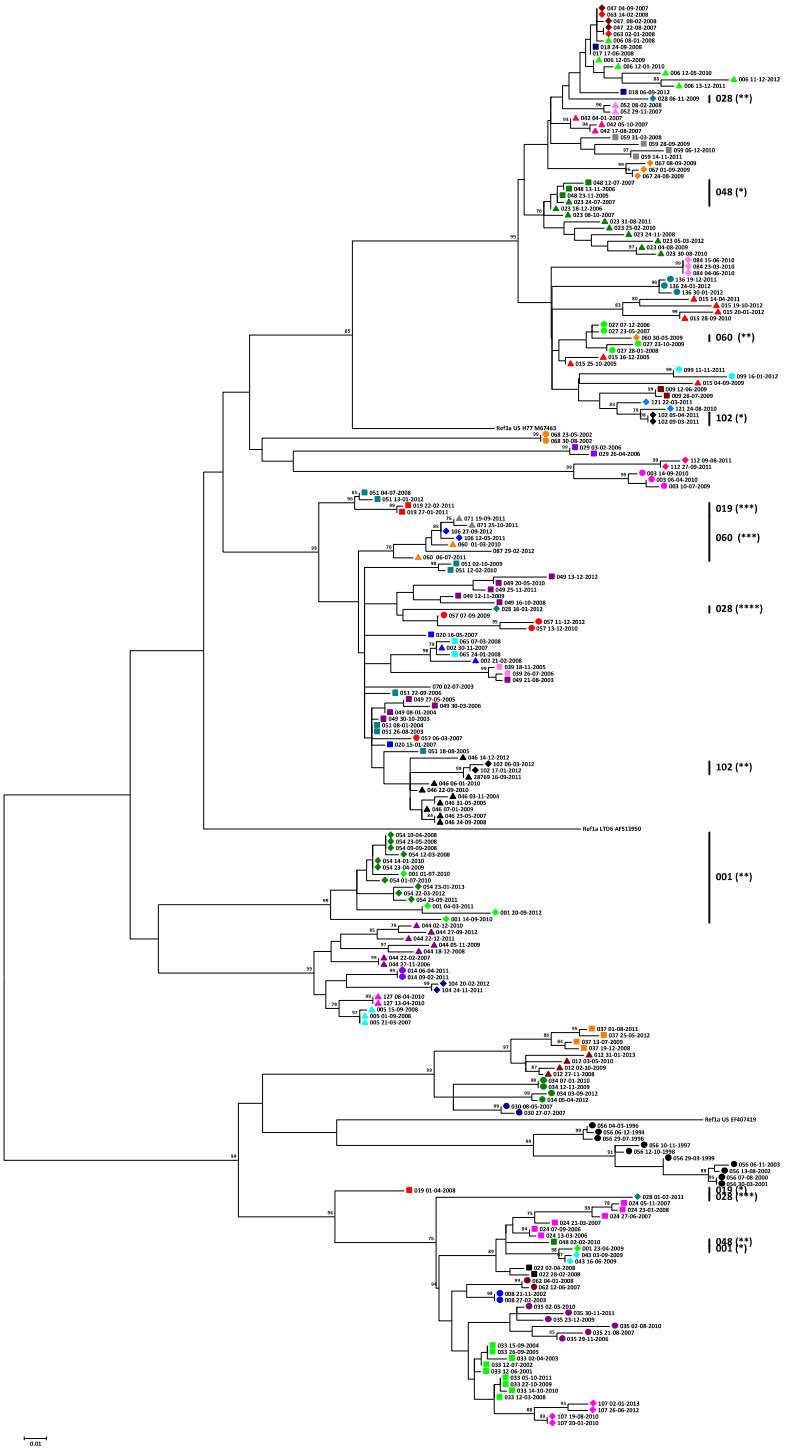
Incidence of new infections: association with clearance and primary genotype

In total, 24 new infections, both super- and reinfections, were observed in 19 patients. In four out of 19 patients multiple viral switches were observed. Most new infections were reinfections, following either spontaneous or treatment induced clearance of the primary infection, with a total of 19 reinfections among 64 previous cleared infections and five new infections following 44 persistent infections. Interestingly, in the five spontaneously cleared infections, three reinfections occurred. Figure 4 summarizes all new infections in a flow chart in relation to treatment and its outcome. Figure 5 illustrates individual flow charts of reinfected patients.

Given 19 secondary infections among 85 patients with a median follow-up time (excluding the treatment period) of 2.87 years (IQR 1.51-5.87), overall incidence rate of a secondary infection was 5.39 cases per 100 person-years (95% confidence interval (CI): 3.34-8.26). The incidence rate of reinfection among those with a resolved primary infection (n=51) was 14.5 per 100 person-years (95%CI 8.41-23.34). In contrast, incidence rate of superinfections was significantly lower with a rate of 1.6 cases per 100 person-years, (IQR 0.5-3.9, $p < 0.01$.)

Cause-specific cumulative incidence curves are shown in Figure 6a. At five years following primary infection, the cumulative incidence of a clade switch was 4.8% (95% CI 0.0-10.1), whereas the cumulative incidence of a genotype switch was 26.7% (95% CI 13.3-38.1). In Cox proportional hazards analysis, patients with an HCV-1a during primary infection had a decreased risk for acquiring an HCV-1a again as secondary infection (HR = 0.25, 95% CI 0.07 – 0.93) compared to patients with a non-HCV-1a at primary infection (Figure 6b, p=0.03, log-rank test).

Figure 1. Maximum likelihood phylogeny of HCV genotype 1a. *ML-tree of all E2/HVR1 HCV-1a sequences. Bootstrap values ≥75 indicative of well-supported clades are shown. Symbols are patient specific. Tip labels denote patient code followed by sampling date. Multiple HCV-1a infections in the same patients are indicated on the right of the phylogenetic tree. Number of asterisks behind patient code indicates the number of infection.*


028 (**)
048 (*)
060 (**)
102 (*)
019 (***)
060 (***)
028 (****)
102 (**)
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Ref1a LT06 AF511950
Ref1a US EF407419
0.01

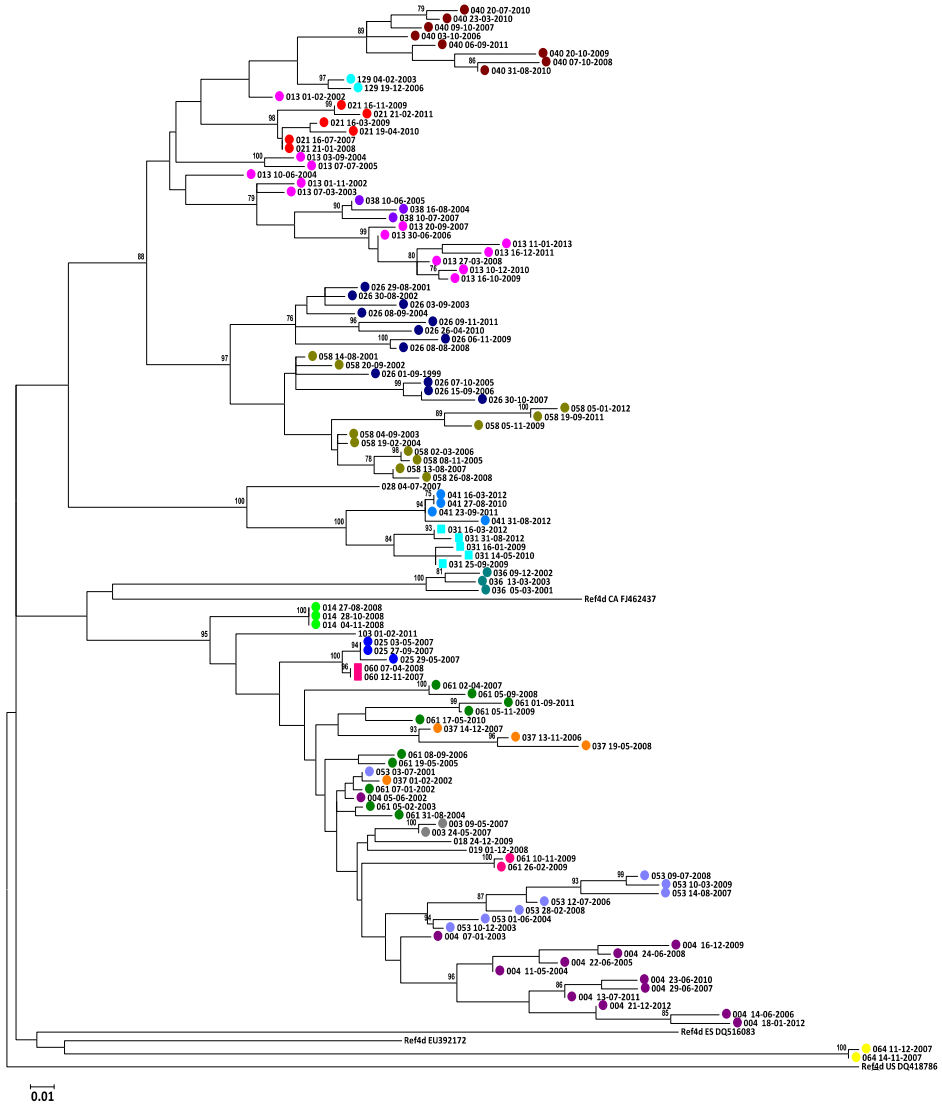

Figure 2: Maximum likelihood phylogeny of HCV genotype 4d. *ML-tree of all E2/HVR1 HCV-4d sequences. Bootstrap values ≥75 indicative of well-supported clades are shown. Symbols are patient specific.*

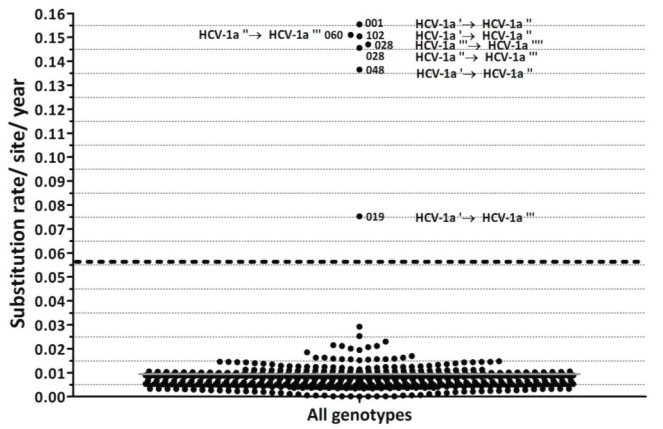

Figure 3: HCV E2/HVR1 substitution rates per site per year among HIV-infected MSM. *Dotplot of monophyly enforced substitution rates for all branches from all patients (see method section). The horizontal dotted line represents the cut off (5.62e-2 substitutions/site/year (mean + 3*SD). ID019 was infected with HCV-4d between his first and third HCV-1a infection. The apostrophe sign (') indicates the infection order.*

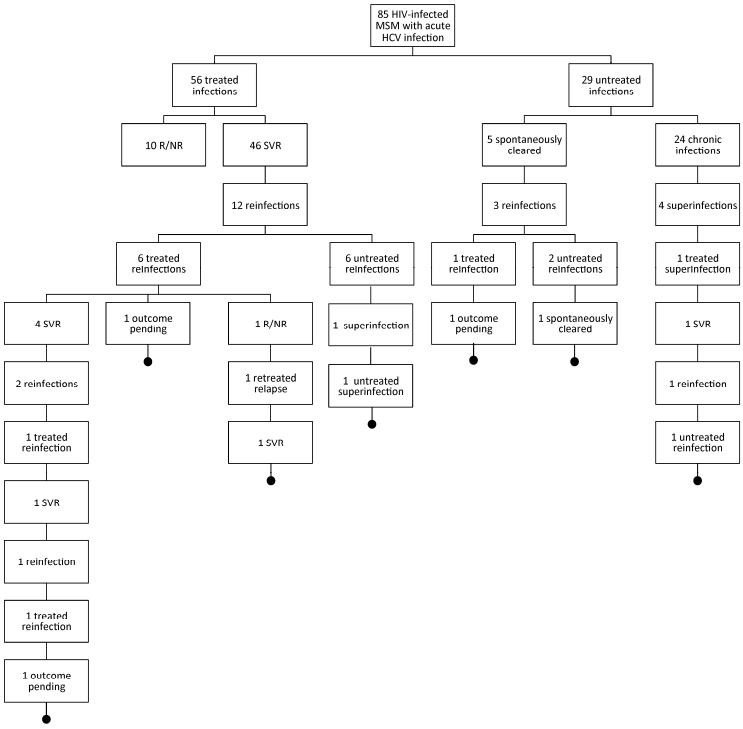

Figure 4. New infections following primary HCV infection. *Flow chart summarizing all observed genotype- and clade-switches during follow-up. R= relapse, NR= non response, SVR= sustained viral response. Bullets indicate the end of follow-up.*

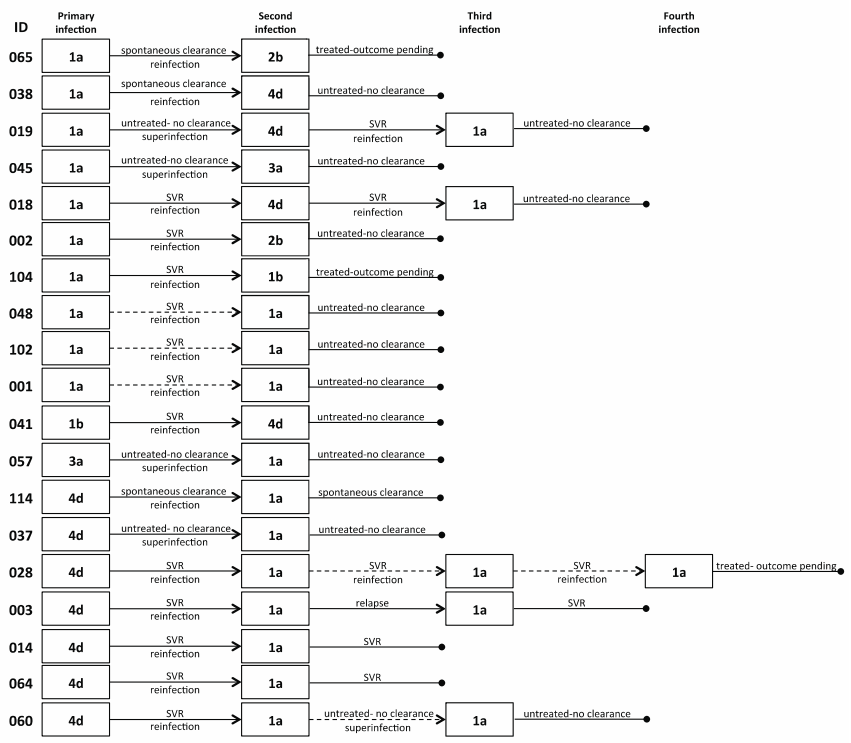

Figure 5.Individual flowgrams of reinfected patients. *Bullets indicate the end of follow-up*

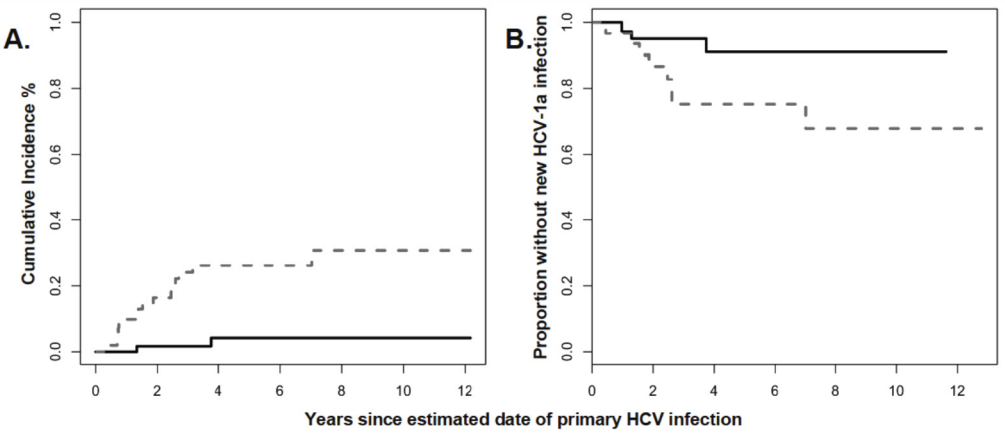

Figure 6. Cumulative incidences for genotype- and clade-switches and Kaplan-Meier estimates for new HCV infections following primary HCV infection. *A) Cumulative incidences, estimated within a competing-risks framework, for genotype- and clade-switches following primary HCV infection among 85 HIV-positive MSM. Lines represent new infections with the same genotype as the original HCV infection (clade-switch, solid line) and new HCV infections with a genotype different from the genotype at primary HCV infection (genotype-switch, dashed line). B) For all patients, Kaplan-Meier estimates for new HCV-1a infections (n=11) following a primary infection with HCV-1a (solid line) (n=51) or a non-HCV-1a primary infection (dashed line) (n=34), were calculated. A Cox proportional hazards model was used to compare primary infection with HCV-1a to primary infection with non-HCV-1a.*

Discussion

Given the high similarity of circulating viruses in an emerging epidemic, identification of new infections with the same genotype as the original one can be challenging, in particular in patients with persistent viremia or a relapse following treatment. We therefore sequenced a genetically highly diverse fragment of the second envelope gene (E2) that includes the hyper variable region 1 (HVR1). By sequencing consecutive samples from each patient, the intra-host nucleotide substitution rate of this region was determined and a threshold for genetic divergence between two sequences in a certain timeframe was defined that distinguishes new infections from intra-host evolution. This allowed us to precisely estimate the incidence of new infections with the original or a different genotype in both persistent and cleared acute HCV infections.

The overall incidence rate of secondary infections was 5.39 cases per 100 person-years. However, among patients with cleared infections, incidence of secondary infections was as high as 14.5 per 100 person-years. A novel and important finding is that, for genotype 1a, the most common genotype, the risk of acquiring a genotype 1a infection again was markedly reduced. This suggests that, even in HIV-infected individuals, partial, genotype-specific immunity is generated at the time of the primary infection.

The high incidence of reinfection following spontaneous or treatment-induced clearance of the primary infection observed in this study is in line with the high reinfection rate of 15.2 cases/100 person-years observed in an earlier smaller study on the incidence of reinfection following treatment induced clearance in HIV-infected MSM with acute HCV from two HIV-clinics [17]. The incidence rate is somewhat higher than observed in a recent study from the UK [18], which showed a reinfection rate of 8.0/100 person-years among HIV-infected MSM who cleared their primary HCV infection. The higher estimate in our study might be explained by our extensive sequence analysis, which enabled us to identify clade switches in patients who 'relapsed' within 24 weeks after the end of treatment. This results in the identification of additional reinfections whereas such patients were considered not to have cleared their primary infection and were thus excluded in the UK study. In addition, the smaller test interval in our study might have resulted in the identification of reinfections that would have been missed otherwise [28].

Martin et al. reported a non-significant trend towards a lower incidence of reinfection among spontaneous clearers versus treatment-induced clearance. Our study population comprised only five spontaneous clearers among untreated patients, and three of them became reinfected suggesting that spontaneous clearance of a previous infectious does not reduce the risk of reinfection.

In this study, the replacement of the primary virus by other viruses in persistently infected patients was also investigated. We cannot prove that such replacements are indeed new infections since these could also have been caused by dynamic changes in dominance when different viral strains are present at the same time. However, we believe that the newly identified viruses are true superinfections, given the overall high incidence of new infections [29]. Another possibility is that such superinfections are in fact reinfections, where aviremic time points were missed. The likelihood of this scenario is small, given the small RNA testing interval in this study. In any case, the incidence rate of such superinfections was relatively low with 1.6 cases per 100 person-years. There are several explanations for a lower incidence of superinfection in persistently infected patients as compared to the incidence of reinfection in patients

with cleared infection. First, patients who are aware of their chronic HCV infection may be less likely to engage in high risk sexual behaviour. Second, in the HCV cell-culture system it has been shown that superinfection is excluded at a post-entry step [29,30]. Third, cross-neutralizing antibodies are present in most patients during the chronic phase of infection [31]. These circulating antibodies may immediately neutralize any new virus, before they are able to establish a new infection.

Perhaps the most remarkable finding of this study is the strikingly reduced incidence of new infections with the original genotype as compared to a new infection with a different genotype. Whether the incidence of such new infections is truly reduced, or whether they are cleared more rapidly and therefore may be missed as a consequence of our testing interval of 2.2 months remains unknown. Indeed, reinfections are characterized by lower viral load and a shorter duration of viremia, suggesting the existence of acquired immunity [15,32]. However, our study demonstrates that there is a strong genotype-specific component to this acquired immunity, resulting in partial protection against the genotype present at the primary infection. Whether the genotype-specific immunity is driven by B- or T-cells or even NK-cells [33] needs to be further explored. For vaccine development this suggests that a strategy directed at generating genotype-specific responses may be more successful than pursuing the holy grail of a pan-genotype vaccine for a virus as variable as HCV. However, other studies need to confirm our findings as the ongoing epidemic in Amsterdam may convey immunity only against locally circulating variants of the same genotype with saturation of infection with these variants among the population at risk.

In conclusion, this study confirms the high rate of HCV reinfections following primary infection among HIV-positive MSM, demonstrating the need for public health interventions in this high-risk group. In addition, this study highlights that observational cohort studies with frequent sampling of individuals with acute HCV infection are important to better understand the correlates of immunity against HCV [34]. Such studies ultimately will contribute to the development of a protective vaccine as the most powerful public health intervention.

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Chapter 7

Coalescent Analysis of Serial Hepatitis C Virus Sequences in HIV/HCV Coinfection: Is Time to the Most Recent Common Ancestor a Reliable Proxy for Infection Date?

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Manuscript submitted

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Abstract

Background: Knowledge of the duration of hepatitis C virus (HCV) infection may have important clinical implications, as it provides a time frame for the stage of liver disease. However, because of its asymptomatic nature, acute HCV infections often go unnoticed and the HCV infection date is therefore usually unknown. Here, we investigated if the date of the most recent common ancestor (MRCA) estimated using a coalescent model could be used as a proxy for the HCV infection date.

Methods: The study population consisted of 63 HIV-infected men who have sex with men (MSM) with incident HCV infection attending the HIV clinic of the Academic Medical Center in Amsterdam. Patients were included if they had (i) HCV infection of known duration: i.e. a positive HCV RNA test preceded by a negative HCV RNA and anti-HCV test (ii) a maximum interval between the first positive and the last HCV RNA negative test of six months, (iii) presence of at least two HCV RNA positive samples. Infection date was estimated using the midpoint between the last negative and the first positive HCV RNA time point. The MRCA date was inferred using the coalescent framework with a Bayesian Markov Chain Monte Carlo (MCMC) algorithm as implemented in BEAST version 1.7.4. Genotype was determined longitudinally by sequencing a fragment of the NS5B polymerase and intra-host evolutionary analysis was assessed using serially sampled E2/HVR1 sequences. Effect of unsampled diversity in early years of infection was assessed by censoring sequences sampled during the early years of infection.

Results: Patients were infected with HCV-1a (n=41), HCV-4d (n=16), HCV-1b (n=5) and HCV-2b (n=4). Of 44 treated patients, 81% (n=36) achieved SVR. A total of 259 sequences were analysed with a median of 3 sequences per patient. Main finding of study was that for the majority of patients (58) infection date and MRCA date correlated well with a median difference of 113 days. The absolute difference between infection date and MRCA date was positively associated with treatment and total duration of follow up. The large interval between infection date and MRCA date, in the remaining 5 patients could be explained by reinfection with the same genotype, resulting in coalescent events more in the past. Censoring sequences from the early years of infection resulted in shifts in the MRCA date estimates proportionally to the censored time.

Conclusion: The Bayesian coalescent-based framework is a robust method for analysing serial sampled sequences and inferring dates of a MRCA. However, provision of reliable estimates of infection dates for seroprevalent cases in the absence of prior knowledge about actual estimated infection dates will remain challenging given the dependence on sequences sampled early during infection. A practical clinical application of this Bayesian coalescent-based framework could be the identification of co-infection or superinfection as a MRCA date substantially older than the known infection date may be caused by infection with multiple strains.

Introduction

Infection with hepatitis C virus (HCV) is a major public health concern with an estimated 130 – 170 million people infected worldwide [1]. Chronic HCV infection causes liver damage with progressive fibrosis, which may result into liver cirrhosis and hepatocellular carcinoma after several decades [2]. HCV is single stranded RNA virus which is transmitted via blood contact. Sexual transmission is considered rare [3]. However, outbreaks of sexually transmitted HCV among men who have sex with men (MSM) with HIV coinfection have been reported in several developed countries since 2000 [4].

Acute HCV infection frequently goes unnoticed as symptoms are mostly mild or absent. Therefore, the date of HCV infection is usually unknown. However, the duration of HCV infection may have important implications for clinical decision-making and epidemiological surveillance. Knowledge of the HCV infection date enables the determination of the rate of disease progression, e.g., advancement to liver cirrhosis. Furthermore, reliable estimates of incidence, a key aspect of epidemiological surveillance, can only be obtained when date of HCV infection is known since this enables to distinguish new cases from prevalent ones.

For fast evolving RNA viruses such as HCV, it is possible to infer the divergence time since the most recent common ancestor (MRCA) directly using viral sequences [5,6]. If genetic distance increases proportional over time, a molecular clock can be assumed and evolutionary rates of genes can be inferred. The time for two lineages to coalesce into a single ancestor or to the MRCA can be estimated by employing the coalescence model which describes the ancestral relationship between two lineages retrospectively [7]. Most HCV infections are established by one or a few single founder viruses, because a genetic bottleneck occurs during transmission [8,9]. Therefore the MRCA date from serial sampled intra-host lineages is likely to coincide with the infection date, as has been shown for HIV [10]. However, accuracy of such estimates is influenced by sampling frequency and the time which has elapsed since transmission.
Aim of this study was to investigate if the estimated MRCA date obtained by molecular clock analysis coincides with the actual HCV infection date among an HCV/HIV co-infected cohort of MSM with incident HCV infection using a Bayesian statistical framework.

Methods and materials

Patient population and data collection

Patients were selected from a cohort of HCV/HIV co-infected MSM attending the HIV clinic of the Academic Medical Center (AMC) in Amsterdam, The Netherlands. For this study, patients were selected who met the following criteria: (i) documented HCV infection as evidenced by a positive HCV RNA test preceded by a negative HCV RNA test in patients who had been anti-HCV antibody negative before first detection of HCV RNA, (ii) a maximum interval of 6 months between the first positive and the last HCV RNA negative test, (iii) the availability of at least two HCV RNA positive samples. The infection date was estimated by calculating the midpoint between the dates of the last negative and the first positive HCV RNA tests. For each patient, details on treatment and treatment outcome were collected from clinical records. A minimum of one HCV RNA positive sample for each follow up year with viremia was selected

for sequence analysis. If available, additional early samples collected during the first year of infection were selected.

Virological assessments

HCV RNA presence was assessed by either TMA (VERSANT® HCV RNA Qualitative Assay, Siemens) or bDNA (VERSANT® HCV RNA 3.0 Assay, Siemens). For genotyping, a 389-base pair (bp) fragment spanning positions 8616-8275 of the NS5B region relative to the H77-strain (AF009606) was amplified and sequenced as described by Murphy et al. [11]. For the intra-host evolutionary analysis a 590-bp fragment from the envelope (E2/HVR1), spanning positions 1295-1885 relative to the H77-strain, which includes the hyper variable region 1 (HVR1) was amplified and directly sequenced.

PCR and sequencing analysis

HCV RNA was extracted from 200 µl EDTA plasma using the Boom method [12]. cDNA was synthesized with random hexamers as previously described [13]. A fragment of E2/HVR1 was amplified and population sequenced using genotype specific primers. Amplification of the E2 fragment was performed in a 25 µL volume using genotype-specific primers and FastStart Taq DNA Polymerase with additional ready-to-use PCR Grade PCR Mix kit (ROCHE Diagnostics GmbH). Final concentrations of 0.1 µM/µL E2 genotype-specific primer, 0.5 mM/L deoxynucleoside triphosphate, 0.1 µg/µL bovine serum albumin and 2.5 mM MgCl2 was used.

The E2 amplification was performed using a touchdown-PCR with the following cycling conditions: 2 min at 50 °C and 10 min at 95°C followed by 10 cycles each consisting of 30 sec at 95°C, 1 min at 66°C (genotype 1B) or 66°C (genotype 1a, 3a, 4d), and 1.5 min at 72°C with a ΔT= -0.6˚C/ cycle. This was followed by 45 cycles each consisting of 30 sec at 95°C, 1 min at 60°C, and 1.5 min at 72°C. The amplicons were gel purified and sequenced in both directions with same genotype specific primers using Big dye terminator (Applied Biosystems, Inc., Norwalk CT).

Phylogenetic analyses

The E2/HVR1 sequences were compiled into datasets according to genotype: 1a, 4d, 1b and 2b. The datasets were created and manually edited using BioEdit version 7.1 [14] ClustalX 2.1 [15] was used to align the sequences [15]. Jmodeltest 2.1 was used to select the best fitting nucleotide substitution model [16] and maximum likelihood (ML) trees were generated using MEGA v5 with the general time-reversible model with rate heterogeneity among sites (GTR + I) and 1000 bootstrap replicates [17].

Bayesian estimation of MRCA date

The date of the most common recent ancestor (MRCA) from the E2/HVR1 sequences was inferred using the coalescent framework with a Bayesian Markov Chain Monte Carlo (MCMC) algorithm as implemented in BEAST version 1.7.4 [18]. Each MCMC run was performed with at least 1 × 10e8 states and sampling of 10e4 trees. Convergence was assessed by the effective sample size (ESS) > 200 and visual inspection of the trace file using Tracer version 1.5 [19]. The Hasegawa, Kishino and Yano nucleotide substitution model with heterogeneity among sites (HKY + G) was used for all MCMC runs [20,21]. Sequences derived from the same patient were grouped into a taxon set without enforcing monophyly. Codons were partitioned into the first and the second base as one group and the third base as separate group. The best-fitting clock and demographic model was selected by calculating the Bayes Factors (BF), as implemented in Tracer version 1.5 [19,22]. In total six models were compared for each

dataset, the models were a combination of the strict or the uncorrelated lognormal relaxed molecular clock models and the constant size, exponential growth or Bayesian skyline plot (BSP) coalescent models [23]. Maximum clade credibility (MCC) trees were generated using Tree Annotator with 10% burn-in and visualized using FigTree 1.2 [24]

MRCA date sensitivity analysis: effect of unsampled diversity during early infection

To investigate the impact on the MRCA date estimate of unsampled genetic diversity present in the beginning of the infection, sequences of the early year(s) of infection from the dataset were censored in a stepwise manner. Four datasets were created for genotype 1a and 4d sequences separately, censoring sequences of the first, the first two, the first three and the first five years of infection. If censoring sequences sampled during the early years of infection would result into less than two retained sequences for a patient, sequences were kept in the analysis for calibration purposes. For each dataset the MRCA dates were inferred with the uncorrelated lognormal relaxed molecular clock and the exponential growth coalescent model. The settings for the MCMC runs were the same as described before.

Statistical analysis

The relationship between the infection date and MRCA date estimate was assessed using linear regression analysis. Correlation between ΔT - i.e. the differences between the infection date and the MRCA date in days - and several covariates (genotype, treatment history, number of sequences per patient, patient follow-up time and median sampling interval) was determined by calculating the Pearson correlation coefficient. A multivariate linear regression model was constructed to determine which of the covariates independently contributed to ΔT. Covariates that were correlated with ΔT were entered in the model in a stepwise manner. As the majority of the sequences from the patients that cleared the virus were obtained prior to treatment, these patients were categorized as untreated patients. The software used for this analysis was SPSS version 22.0.

Results

Patient characteristics

Table 1 summarizes the characteristics of the patient data. In total 63 patients were included in this study. The majority of patients were infected with genotype 1a (n = 41). Genotype 4d was the second most prevalent genotype (n = 16) and the remaining infections were genotype 1b (n = 5) and 2b infections (n = 4). Sequencing of the NS5B gene demonstrated that during follow up three patients became reinfected with another genotype (patients 3, 014 and 041). Most patients (n = 44) were treated with peginterferon and ribavirin, resulting in viral clearance in 36 patients. The median follow-up time until the last (available) viremic time point was 23 months (interquartile range (IQR) 5 - 60 months). The median sampling interval between available E2/HVR1 sequences was 9 months (IQR 4 -14 months). In total 259 E2/HVR1 sequences were analysed with a median of three per patient.

Number of patients	63
Genotype	
1a	41
4d	16
1b	5
2a	4
Treatment history	
Untreated	19
SVR*	36
Failure	10
unknown	1
Median follow-up time in months (IQR)	23 (5 – 60)
Median interval between last negative and first positive HCV RNA test	
Median sampling interval in months (IQR	9 (4 – 14)
Total number sequences	259
Median number of sequences per patient (IQR)	3 (2 - 5)

Table 1. patient characteristic. **SVR – sustained viral response.*

Molecular clock and coalescent model testing

The relaxed clock provided a better fit than the strict clock model in all datasets as indicated by the Bayes factors (Data not shown). The coalescent model providing the best fit differed per dataset, but without strong evidence in favour of one particular model. For genotypes 1b and 2b the Bayes factor provided weak evidence of a better fit of the BSP model over the exponential growth and the constant size model (2 ln BF<3). For genotype 4d the exponential growth model had a slightly better fit (2 ln BF < 3) and for genotype 1a the Bayes factor of the constant size model provided positive evidence of the best fit with (2 ln BF < 10). However, for most MRCA date estimates no significant differences were observed across the models and because the 95% highest posterior densities (HPDs) were slightly smaller for the exponential growth model, we continued the analyses with this coalescent model.

Phylogenetic analyses

MCC trees were constructed for all genotypes. The MCC tree for genotype 1a was constructed from 158 E2/HVR1 sequences sampled between 2001 and 2013. In general, each patient formed a monophyletic cluster. However, in four patients (patient 001, 019, 048 and 102), different lineages were present with large genetic distances between these lineages (Figure 1), demonstrating the presence of more than one distinct genotype 1a variants. All four patients were treated and were aviremic at the end of treatment. In one of these four patients (001) HCV RNA became detectable again 3 months after the end of treatment. In the absence of phylogenetic analyses, the early rebound after the end of treatment would have been classified as treatment failure. However, the clinical data combined with the tree topology strongly suggest that reinfection with genotype 1a virus occurred during the observation period. The root of genotype 1a MCC tree was dated in 1965 (95% high posterior density (HPD) from 1936 to 1982). At least six separate introductions of genotype 1a occurred between 1998 and 2007 as indicated by root dates of clusters.

For the MCC tree of genotype 4d, 77 E2/HVR1 sequences sampled between 1999 and 2011 were analysed (Figure 2). Again, sequences from each patient formed a monophyletic group. However, early lineages from four patients with very similar viruses (patients 004, 037, 053, 061) intermingled in the first year of infection (2001 and 2002), whereas lineages from later years formed separate clusters. The

root of the genotype 4d lineages is dated in 1969 (95% HPD from 1946 to 1986). Four separate introductions, which resulted in ongoing transmissions, were observed.
Only 5 and 4 patients were infected with genotypes 1b and 2b, respectively. For genotype 1b the earliest sample was from 2002 and the latest from 2011 and 16 sequences were analysed in the MCC tree (data not shown). The root of this tree was dated in 2002 (95% HPD 2001 - 2003). Only eight genotype 2b sequences were available, sampled between 2010 and 2012 and the MCC tree root was dated in 2007 (95% HPD 2002 - 2010).

MRCA date and infection date

The median MRCA date estimates and the corresponding 95% HPD interval were obtained for all 63 patients from the BEAST analysis and compared to the HCV infection date. Figure 3 shows the median MRCA date with the 95% HPD interval and the estimated HCV infection date with the interval between the last HCV RNA negative and first HCV RNA positive time points. The 95% HPD interval completely overlapped with the interval between the last HCV RNA negative and first HCV RNA positive time points in 17 out of the 63 patients.
The estimated MRCA date of patients 001, 019, 048 and 102 was considerably earlier than the estimated infection date, with roots ranging from 23 to 44 years before the infection date. This strongly suggests that in these patients sequential infections with the same subtype occurred during the observation period. For patient 034 the MRCA was dated 6 years prior to the infection date. This patient was successfully treated, but HCV RNA became detectable again 20 months later. Interestingly, his second lineage was genetically quite closely related to the first strain, which may suggest that a reinfection may have occurred from the same source, although a late relapse may also explain this observation. The above 5 patients with (possible) reinfections were excluded from further analysis.
For the remaining 58 patients, good agreements between the estimated MRCA and the infection dates were observed in general: the median absolute difference (ΔT) was 113 days (IQR 53 – 330 days). For the great majority of patients (84%) the estimated MRCA was dated earlier than the infection date. Closer examination of the 14 patients with a ΔT of more than one year indicated that 5 out of the 14 patients (36%) failed treatment, whereas treatment failure was observed in only 5 out of 44 patients (11%) with a ΔT of less than one year.
The relationship between the MRCA date and the infection date was further quantitatively analysed using linear regression, with the inferred regression line depicted in Figure 4. The slope of 0.83 and the high R^2 value of 0.92 confirmed a good agreement between the MRCA and infection date.
Next, we analysed which factors were correlated with ΔT. Table 2 shows the Pearson correlation coefficients for the factors analysed. A significant positive correlation was found between ΔT and duration of follow up, number of sequences per patient and the median sampling interval. In addition, ΔT was also correlated with treatment: in patients who were treated (and failed to clear the virus) ΔT was larger than in untreated patients. In a linear regression analysis, treatment and duration of follow up remained both independently associated with ΔT.

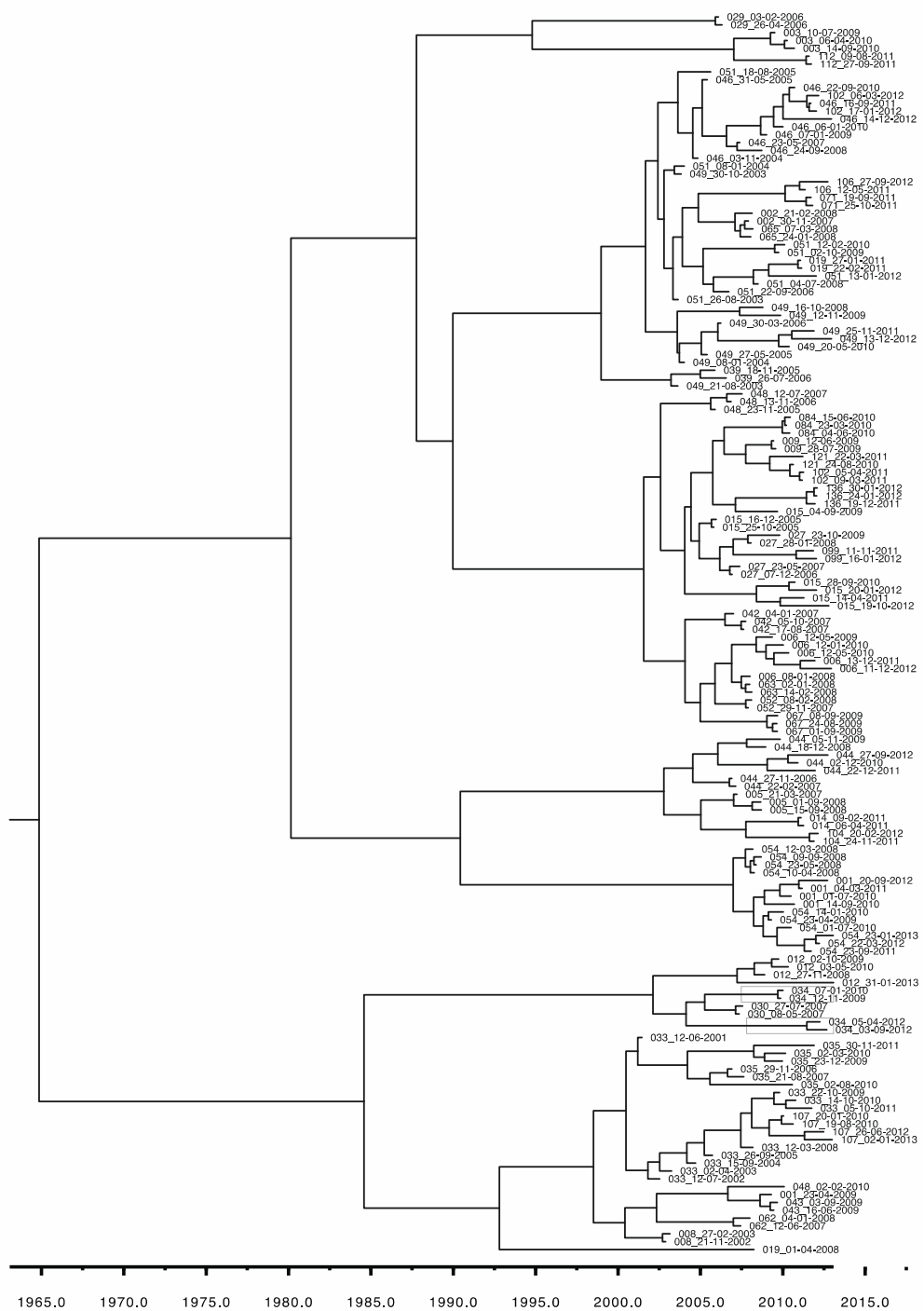

Figure 1. MCC tree genotype 1a. *The branches are proportional to time and the time scale is in years. The patient's ID number and the sampling date are denoted in the tip labels.*

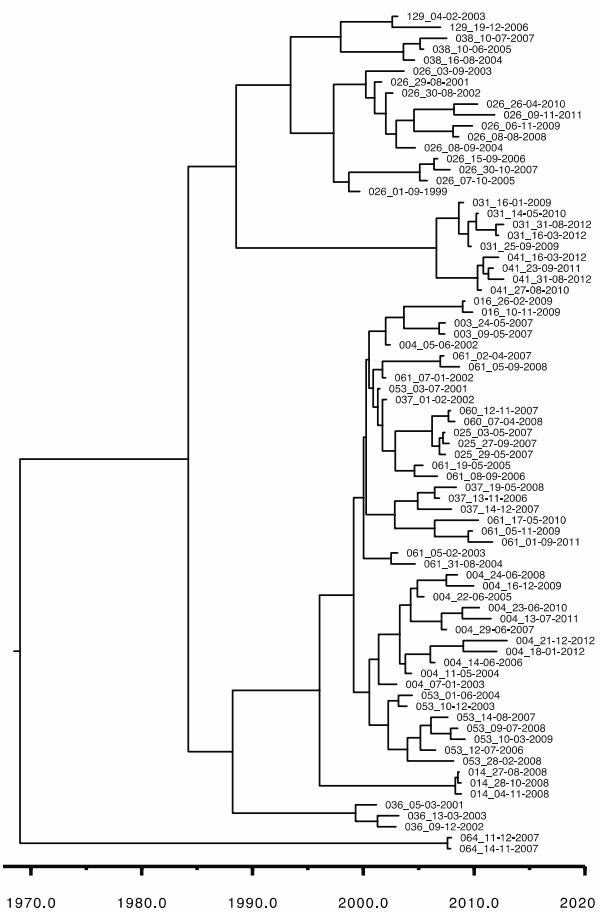

Figure 2. MCC tree genotype 4d. *The branches are proportional to time and the time scale is in years. The patient's ID number and the sampling date are denoted in the tip labels.*

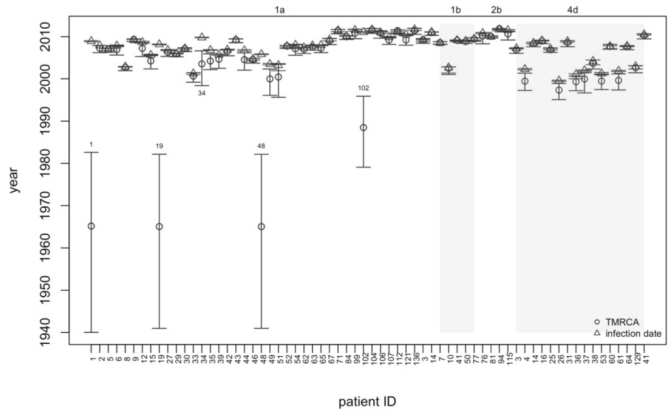

Figure 3. Infection and MRCA date. *The median TMRCA (circle) with the 95% HPD (whiskers) and the infection (triangle) date with the last RNA negative and first RNA positive time point (whiskers).*

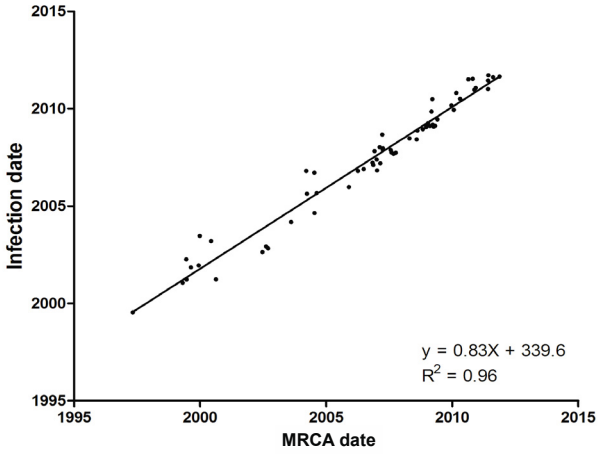

Figure 4. Regression analysis of the MRCA and the infection date. *The five patients with (possible) reinfections were excluded. Axis scale is in year.*

A .Pearson correlation coefficient		
covariates	Pearson p	p-value
Genotype (1 vs 4)	0.093	NS
Treatment* (no/yes)	0.543	<0.01
Number of sequences	0.546	<0.01
Duration of follow up (months)	0.555	<0.01
Median sampling interval (months)	0.265	0.04

B. Multivariate linear regression			
	ß	SE	p-value
Duration of follow up (months)	0.011	0.003	<0.01
Treatment (no/yes)	1.188	0.325	<0.01

Table 2. Covariates of ΔT in days (infection date – MRCA date).

MRCA date analysis censoring sequences of early infection

Figure 5 shows the regression analysis of the four datasets for which the sequences of the first years of infection were censored. For 24 patients the sequences sampled during the first year of infection could be censored and for 17 patients the sequences sampled during the first two years. The sequences of the first three years and the first five years of infection were censored for 15 and 11 patients, respectively. Results of the regression analysis describing the relation between the infection date and MRCA date is depicted in Figure 5.

The regression analysis shows a clear trend towards underestimation of MRCA dates in the absence of sequences of the first years of infection. MRCA dates shift to more recent estimated infection dates in proportion to the number of years from which sequences were excluded from the analysis. The median absolute ΔT was 333 days, 390 days, 2.5 years and 4.2 years for the datasets from which sequences of the first, the first two, the first three of the first five years were excluded respectively.

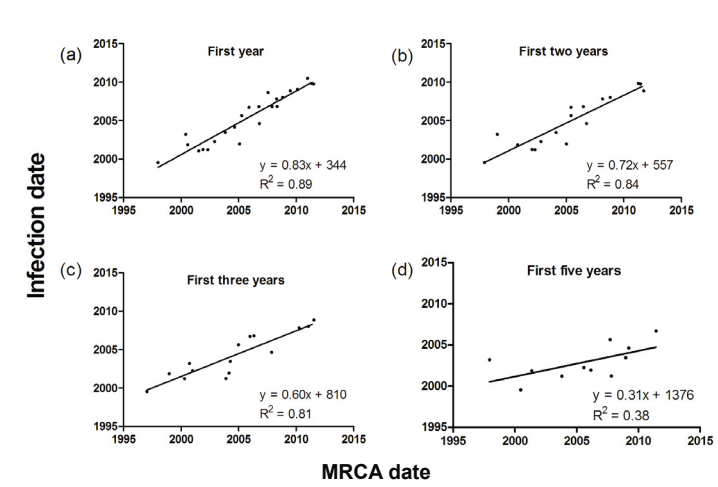

Figure 5. Linear regression analysis of MRCA and infection date with censoring of samples from early infection. *The axis scale is in year. a) sequences of within the first year of infection are omitted n = 24, b) sequences within the first two years n = 17. c) sequences within the first three years, n = 15 and d) sequences of the first five years, n = 11.*

Discussion

In this study we investigated whether and to what extent the MRCA date estimated from longitudinal HCV E2/HVR1 population sequences using a Bayesian coalescent-based method coincides with the actual estimated HCV infection date [25]. To answer this question we used a well-characterized cohort of HIV infected MSM with incident HCV infection, for whom the date of HCV infection could be reliably estimated by retrospective testing of stored sera collected for HIV RNA testing. A total of 256 serially sampled sequences from 63 HCV/HIV co-infected patients were analysed. Our main finding is that for 58/63 patients the MRCA dates and the infection dates correlated well and that the absolute differences between these dates were small. This study also confirms earlier observations that HCV started spreading among HIV infected MSM in the Netherlands around the year 2000, as the roots of the different clusters were placed around that year [4,26].

In four patients the MRCA was dated decades before the patients acquired HCV. This clearly indicates that in these patients a new lineage emerged, presumably as a consequence of reinfection, as all patients were treated and were HCV RNA negative at the end of treatment. Reinfection occurs frequently in this population as we and others have reported earlier [27–29]. Diagnosis of reinfection is usually straightforward when HCV RNA becomes detectable again in a patient who cleared the virus either spontaneously or upon treatment. However, if viremia rebounds early (within 6 months) following treatment-induced clearance, as was the case for patient 84 and 102, this by definition is categorized as a relapse, i.e. a treatment failure. In such cases sequence analysis of a region with sufficient phylogenetic resolution is necessary to discriminate between a relapse and a reinfection. However, even then distinguishing reinfection from a relapse is not always straightforward as illustrated by our observation in patient 34. The MRCA in this patient was dated six years before the actual estimated infection date, and the genetic distance between the virus that emerged 20 months after the end of treatment and the earlier variants was relatively small. This may indicate that the patient became reinfected from the same source with a similar virus. However, another possibility is that a late relapse

occurred with a minor variant that was not eradicated during treatment and slowly 'surfaced' following the treatment-induced bottleneck. Such late relapses have been described before and indicate that HCV may persist and even replicate without detectable viremia [30–32].

Excluding these 5 possible reinfection cases, the median difference between the actual infection date and the MRCA date of 113 days was remarkably small in the remaining 58 cases. Recently, Poon et al. performed a similar study using coalescent analysis on HIV datasets of individuals with well-documented dates of infection [10]. In this study, the HIV clonal sequence data of Shankarappa et al. consisting of 7 – 16 serial sampled clonal sequences over a period of 6 - 11 years following transmission was used as a proof-of-concept that serially sampled sequences can be used to estimate the date of HIV infection in individual patients [33]. MRCA dates based on this clonal data set resulted in an absolute difference between the infection date and MRCA date of 3 months, very similar to the 113 days observed in our study. This suggests that the use of multiple clonal sequences per time point does not necessarily result in a more accurate MRCA date estimate than the use of population sequences.

Poon et al. also used a next-generation sequencing derived dataset: for 19 patients, 100 randomly sampled pyrosequencing reads sampled at two time points were used to estimate the date of the MRCA. Again, MRCA dates correlated well with infection dates as indicated by a very small median absolute difference of 44 days. This smaller absolute difference between MRCA date and infection date when compared to the Shankarappa data and to our study might be explained by differences in duration of follow up. We observed that the interval between MRCA date and infection date increased with longer follow up durations. Indeed, excluding patients with a follow up of more than 500 days, which was the maximum follow up in the study of Poon et al, resulted in a median absolute difference between infection date and MRCA date of only 73 days (data not shown).

In our study the MRCA date tended to systematically predate the estimated infection dates, which was especially prominent for patients with a longer follow up. As our first sampling time point was within six months of the infection date, this indicates that within this time frame selective sweeps had not taken place yet, as this would have led to more recent MRCA dates. This is supported by our analysis where we censored the early time points and which resulted in progressively more recent MRCA dates. The reason why MRCA dates tended to be more predated in patients with longer duration of follow up remains unclear. It seems unlikely that, specifically in these patients, transmitted genetic diversity is larger which would explain coalescent events to occur more in the past. An alternative hypothesis may be that intra-host evolutionary rates are not equal during the course of time, resulting in an underestimation of the evolution rate in patients with a longer follow up.

We have demonstrated the robustness of the Bayesian coalescent-based framework for analysing serial sampled sequences in patients with known dates of infection. However, provision of reliable estimates of infection dates for seroprevalent cases in the absence of prior knowledge about actual estimated infection dates will remain challenging given the dependence on appropriate sampling especially during the early years after infection. A practical clinical application of this Bayesian coalescent-based frame work could be the identification of co-infection or superinfection as a MRCA substantially older than the known infection date may be caused by infection with multiple strains.

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Chapter 8

Hepatitis C Virus (HCV) Antibody Dynamics following Acute HCV infection and Reinfection among HIV-infected Men who have Sex with Men

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Abstract

Background: A decline of hepatitis C virus (HCV) antibody titres (anti-HCV), ultimately resulting in seroreversion, has been reported following clearance of viremia in both acute and chronic HCV infection. However, frequency of seroreversion remains unknown in human immunodeficiency virus (HIV)/HCV-confected patients. We describe anti-HCV dynamics among HIV-infected men who have sex with men (MSM) following acute HCV infection and reinfection.

Methods: Primary acute HCV infection was assumed when a subject was anti-HCV negative prior to the first positive HCV RNA test. Anti-HCV was measured at least annually in 63 HIV-infected MSM, with a median follow- up of 4.0 years (interquartile range [IQR], 2.5–5.7 years). Time from HCV infection to seroconversion, and from seroconversion to seroreversion, was estimated using the Kaplan–Meier method. Longitudinal anti-HCV patterns were studied using a random-effects model to adjust for repeated measures.

Results: Median time from HCV infection to seroconversion was 74 days (IQR, 47–125 days). Subjects who cleared HCV RNA (n = 36) showed a significant decrease in anti-HCV levels ($P < 0.001$). Among 31 subjects with sustained virologic response (SVR), anti-HCV became undetectable during follow-up in 8; cumulative incidence of seroreversion within 3 years after seroconversion was 37% (95% confidence interval, 18%–66%). Eighteen subjects became reinfected during follow-up; this coincided with a subsequent increase in anti-HCV reactivity.

Conclusions: A decline of anti-HCV reactivity was associated with HCV RNA clearance. Seroreversion was very common following SVR. Upon reinfection, anti-HCV levels increased again. Monitoring anti-HCV levels might therefore be an effective alternative for diagnosis of HCV reinfection.

Introduction

Hepatitis C virus (HCV) is a major cause of liver disease. Globally an estimated 2%-3% of people are infected [1, 2]. Following acute infection, the majority of HCV-infected individuals will develop chronic HCV infection and are at risk for long-term sequelae, including liver cirrhosis and hepatocellular carcinoma [3]. Major risk factors for contracting HCV infection are injecting drug use (IDU), blood transfusions from unscreened donors, and unsafe medical procedures [2]. The risk of sexual transmission of HCV in monogamous heterosexual couples is considered negligible [4]. Since the mid-1990s, an epidemic of HCV infection has emerged among HIV-infected men who have sex with men (MSM) in high-income countries [5-8]. These men denied IDU, and phylogenetic analyses of circulating HCV strains have revealed the presence of multiple MSM-specific clusters, thereby demonstrating that sex may be an alternative transmission route [9-11].

The window period between HCV infection and detectable anti-HCV has been estimated to range from 34 to 70 days in studies among HIV-negative blood product recipients [12-15] and HIV-negative IDU [16, 17]. Recently, a delayed anti-HCV response was reported among HIV-infected MSM with acute HCV, suggesting an important role for coinfection with HIV [18]. The reported median time to seroconversion in that study was 91 days, and 158 days in a subset of eight men sampled more frequently.

A decline in anti-HCV reactivity, ultimately followed by seroreversion, has been reported following spontaneous or treatment-induced clearance, mostly in the absence of HIV infection and only after long-term follow-up [19-27]. The available literature seems to agree that seroreversion may occur in cases of profound immunodepression [28]. Only few reports have described seroreversion among HIV-coinfected patients [27, 29-31]. Therefore, the frequency of HCV seroreversion among HIV-coinfected patients remains unknown. The objectives of the current study were to examine dynamics of anti-HCV reactivity following acute HCV infection among HIV-infected MSM. The high incidence of HCV reinfection in this population [32, 33] also allowed us to study anti-HCV dynamics following reinfection.

Methods

Participants

Participants eligible for this study included HIV-infected MSM, aged ≥18 years, diagnosed with acute HCV infection at the Academic Medical Center HIV outpatient clinic in Amsterdam. The majority of study subjects (42/63; 66.7%) participated in MOSAIC (MSM Observational Study for Acute Infection with hepatitis C); a multi-centre open prospective cohort study in the Netherlands that was initiated in 2009 [32]. For the present study, strict inclusion criteria were applied with respect to the maximum interval between HCV-RNA-negative and -positive visits (i.e., max. 6 months). Primary HCV infection was assumed when a subject was anti-HCV-negative prior to the first positive HCV-RNA test. Sociodemographic, clinical, and virological data, including age, use of combination antiretroviral therapy (cART), HIV viral load, CD4 cell count, concentrations of alanine aminotransferase (ALAT), HCV viral load, and HCV treatment data were retrieved from medical files.

Laboratory methods

To determine the interval between the last negative and the first positive HCV-RNA test, blood samples collected at earlier visits were tested retrospectively. HCV-RNA tests were performed using either transcription-mediated amplification (TMA; Versant, Siemens, limit of detection, LoD: 5-10 IU/ml) or COBAS Ampliprep/COBAS TaqMan (CAP/CTM, Roche Diagnostics, LoD: 15 IU/ml). Anti-HCV reactivity was tested at least every six months in the first year following infection (and reinfection), followed by annual testing. Anti-HCV testing was performed using a commercial microparticle enzyme immunoassay (MEIA) AxSYM HCV 3.0 (Abbott Laboratories). A positive anti-HCV test was defined as having a sample-to-cut-off (S/CO) value of ≥1.00. When a subsequent negative anti-HCV test was recorded (i.e., S/CO <1.00), this was considered seroreversion. Date of seroreversion was estimated as the midpoint between the first negative anti-HCV test after seroconversion, and the preceding sample. HCV genotype was determined by sequencing a 340 base pair fragment of the NS5B region [34].

Definition of HCV reinfection

Reinfection was defined as the presence of a different genotype compared with primary infection. To investigate the possibility of reinfection with the same genotype in patients without a genotype switch, consecutive E2/HVR1 sequences were analysed as previously described [32]. Relapse was defined as a positive HCV-RNA after a negative HCV-RNA at the end of treatment with the same viral strain.

Statistical analysis

The midpoint between the last negative and the first positive RNA test, and the midpoint between the last anti-HCV-negative and the first anti-HCV-positive test was estimated to be the dates of infection and seroconversion, respectively. When the last negative HCV-RNA coincided with the last negative anti-HCV test, and the first positive HCV-RNA coincided with the first positive anti-HCV test, the estimated dates of infection and seroconversion were estimated at the 1/3 and 2/3 time point between the last negative HCV and first positive HCV test, respectively.

First, we estimated the cumulative incidence and median time (1) from acute HCV infection to seroconversion and (2) from seroconversion to seroreversion, through Kaplan-Meier survival estimates. Univariable Cox proportional hazards analysis was used to evaluate associations of age, HCV genotype, CD4 cell count before infection, and nadir CD4 cell count before infection, on time to seroconversion. Associations of these variables were also evaluated on time to seroreversion. In addition, peak level of anti-HCV was examined in analysis of the latter. Second, differences in peak levels of ALAT concentration and anti-HCV reactivity between primary HCV infection and reinfection were compared using the nonparametric Wilcoxon's matched-pairs signed-rank test. Third, anti-HCV signal patterns were estimated from the time of primary infection until end of follow-up. Sequential anti-HCV measurements were corrected for within-subject correlation using a random effects model with random intercept. A random slope was added to the model 6 months after estimated infection; restricted cubic splines allowed for smoothly varying trends. In the analyses of primary HCV infection, measurements during treatment were included, but were censored at HCV reinfection. Statistical software STATA Intercooled 13.1 (STATA Corp, College Station, TX, USA) and R 3.0.1 [35] were used for analysis.

Results

General characteristics

Sixty-three HIV-infected MSM were diagnosed with acute HCV infection and included in this study (Table 1). Median age at the estimated date of infection was 42 years (IQR: 35-47), and the majority had Dutch nationality (87.3%). Genotype of primary HCV infection was most frequently genotype 1a (39/63; 61.9%) or 4d (15/63; 23.8%); other genotypes were 1b (n=5), 2b (n=3), and 3a (n=1). Anti-HCV reactivity was measured during a median observation time of 4.0 years following acute infection (IQR: 2.6-5.8), with a median test interval of 0.5 years (IQR: 0.2-1.0). Median time between the estimated date of infection and initiation of treatment was 6.7 months (IQR: 3.7-8.7), or 4.4 months (IQR: 1.8-7.5) after the first RNA-positive visit. Sampling intervals around HCV infection were wider in the group that did not clear HCV, compared to those who did (123 vs. 89 days; P=.015). During follow-up 18/63 subjects became reinfected; 16 were reinfected once, one was reinfected twice, and one was reinfected three times. Two out of 21 reinfections (9.5%) were cleared spontaneously. Remarkably, these two patients also spontaneously cleared their primary HCV infection. Treatment outcomes during follow-up of all primary infections and reinfections are shown in Figure 1.

Characteristics	All (n=63)	Persistent viremia (n=27)	Viral clearance (n=36)
Age at primary HCV infection	42 (35-47)	41 (36-45)	42 (35-49)
Dutch nationality, n/N (%)	55/63 (87)	22/27 (81)	33/36 (92)
Genotype of primary infection, n/N (%)			
1a		39 (62)	17 (63)
1b		5 (8)	3 (11)
2b		3 (5)	1 (4)
3a		1 (2)	0 (0)
4d		15 (24)	6 (22)
CD4 cell count before primary infection (cells/µL)	495 (350-660)	475 (320-680)	505 (380-660)
Nadir CD4 cell count before primary infection (cells/µL)	260 (130-410)	275 (190-445)	230 (80-370)
On cART at first HCV-positive visit, n/N (%)	41 (65)	15 (56)	26 (72)
HIV RNA load at first HCV-positive visit (copies/ml)	<50 (40-17590)	99 (<50-33,680)	<50 (<40-9,916)
Anti-HCV reactivity at first anti-HCV-positive visit (S/CO)	60.5 (12.9-85.1)	61.5 (21.0-85.2)	50.6 (12.5-84.2)
Days between last negative and first positive HCV-RNA test	107 (80-133)	123 (98-140)	89 (71-119)

Table 1. Characteristics of 63 HIV-Infected Men Who Have Sex With Men by Hepatitis C Virus Status After Primary Infection. *Reported values are median (interquartile range), unless indicated otherwise. Abbreviations: anti-HCV, hepatitis C virus antibody; cART, combination antiretroviral therapy; HCV, hepatitis C virus; HIV, human immunodeficiency virus; S/CO, sample-to-cut off value. Twenty-seven had persistent viremia; 36 cleared HCV (5 spontaneously cleared infection, and 31 cleared infection after treatment).*

Seroconversion window

All subjects (63/63; 100%) seroconverted during the observation period. Median time from infection to seroconversion was 74 days (IQR: 47-125; Figure 2). The cumulative incidence of seroconversion was 59% (95% CI: 47-71) at 3 months, 73% (95% CI: 62-83) at 4 months, and 98% (95% CI: 93-100) at 12 months. In univariable Cox regression, time to seroconversion was not significantly associated with age (hazard ratio, HR, per 10 year increment: 1.15; 95% CI: 0.82-1.59), genotype (1 versus non-1, HR: 1.06, 95% CI: 0.62-1.83), CD4 cell count (HR per 100 cells/µL increment: 1.06, 95% CI: 0.92-1.22), nor nadir CD4 cell count before infection (HR per 100 cells/µL increment: 1.01, 95% CI: 0.86-1.18).

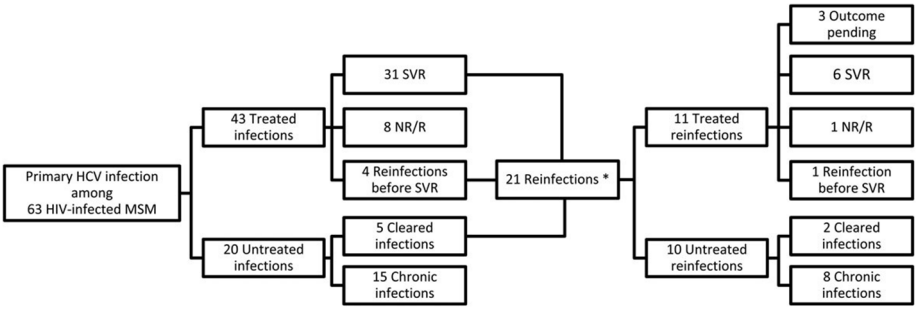

Figure 1. Flowchart showing treatment status and outcome during follow-up of 63 HIV-infected men who have sex with men with acute hepatitis C virus infection. **Twelve subjects became reinfected after reaching sustained virologic response (SVR), 4 before reaching SVR, and 2 following spontaneous clearance. Two subjects became reinfected again after treatment of their (first) reinfection episode. One reached SVR; the other became reinfected again before reaching SVR (this subject is also shown in Figure 5). Abbreviations: HCV, hepatitis C virus; HIV, human immunodeficiency virus; MSM, men who have sex with men; NR, nonresponse; R, relapse.*

Dynamics of anti-HCV reactivity following primary HCV infection

Upon seroconversion, anti-HCV reactivity increased to peak levels well above the detection limit with a median S/CO ratio of 89.4 (IQR: 58.6-115.3). Two distinct patterns of anti-HCV dynamics emerged 6 months post-infection; Figure 3 shows the modelled estimates for anti-HCV reactivity. Of 27 subjects that were persistently viremic (i.e., untreated subjects, non-responders, and relapsers), all but one showed a stable serological profile; 80% of all anti-HCV measurements in this group were above S/CO ratio 50. After HCV clearance, either spontaneously (N=5) or following treatment (N=31), anti-HCV reactivity decreased significantly (P<0.001). The median peak and subsequent nadir S/CO ratios were 89.4 (IQR: 58.6-115.3) and 5.4 (IQR: 1.3-50.8), respectively.

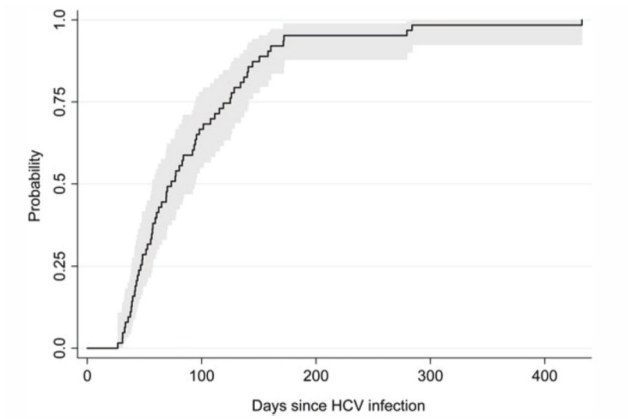

Figure 2. Kaplan–Meier estimate of the probability of seroconversion following acute hepatitis C virus (HCV) infection*. All 63 HIV-infected men who have sex with men seroconverted within the observation period. Shaded gray area: 95% confidence interval. Abbreviation: HIV, human immunodeficiency virus.*

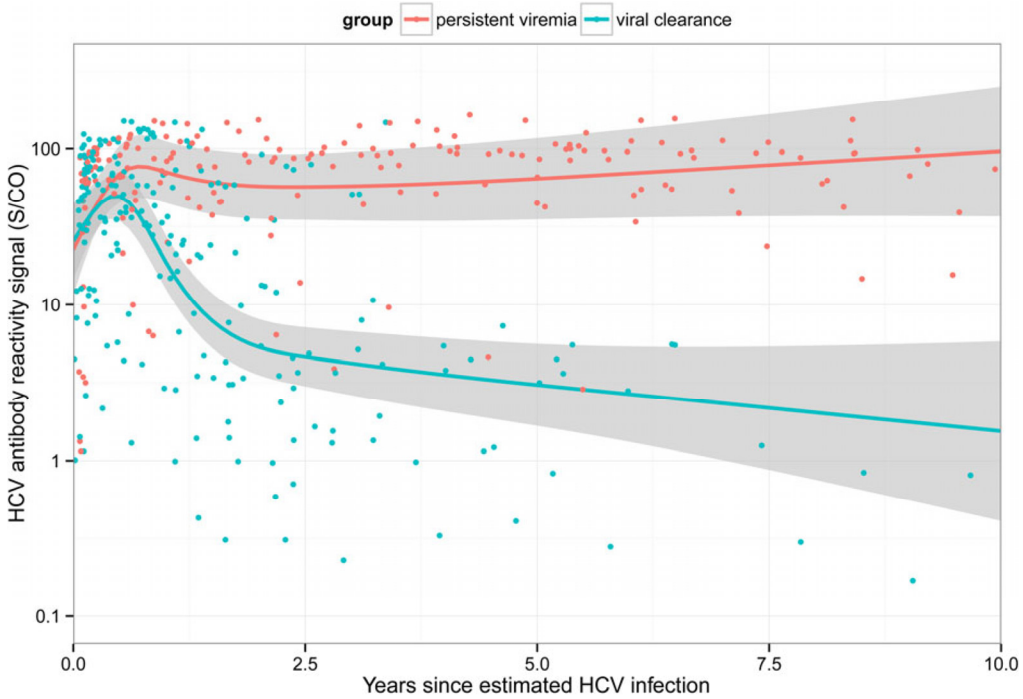

Figure 3. Longitudinal hepatitis C virus (HCV) antibody measurements following acute HCV infection, among 63 HIV-infected men who have sex with men. *Distinct patterns were apparent 6 months post infection for 36 subjects with viral clearance (n = 5 cleared spontaneously; n = 31 after treatment) and 27 subjects with persistent viremia (n = 15 untreated; n = 8 relapse; n = 4 nonresponse). Measurements during treatment were included in the random-effects model. Subjects were censored at HCV reinfection. Dots, observed values; solid lines, modelled estimate; shaded gray area, 95% confidence interval. Abbreviations: HIV, human immunodeficiency virus; S/CO, sample-to-cut off value.*

Incidence of seroreversion

Full seroreversion was observed in 8/31 subjects with SVR following primary HCV infection. Among those who spontaneously cleared HCV, partial seroreversion (i.e., a decrease, but not complete loss of anti-HCV signal) was observed. The cumulative incidence of seroreversion was 37% (95% CI: 18-66) within three years after seroconversion (Figure 4), or 51% (95% CI: 27-81) within three years after reaching SVR. The CD4 cell count at the visit before seroreversion was 490 cells/µL (IQR: 440-775) and the nadir CD4 cell count at that visit was 305 cells/µL (IQR: 140-380). In univariable Cox regression, seroreversion was significantly associated with lower peak anti-HCV levels during primary infection (HR, per 10 S/CO lower: 1.6, 95% CI: 1.1-2.3; *P*=.014). None of the other studied risk factors were significantly associated with seroreversion; age (HR, per 10 year increment: 0.88, 95% CI: 0.38-1.07), genotype 1 versus non-1 (HR: 2.62, 95% CI: 0.59-11.8), CD4 cell count before HCV infection (HR, per 100 increment: 1.25; 95% CI: 0.79-1.97), and nadir CD4 cell count before HCV infection (HR, per 100 increment: 1.18; 95% CI: 0.81-1.70).

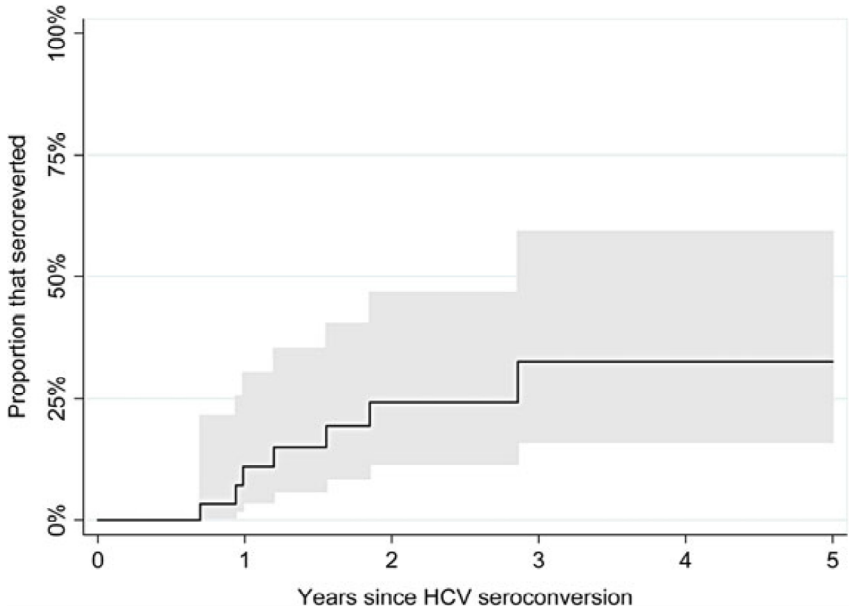

Figure 4. Cumulative incidence of hepatitis C virus (HCV) seroreversion (i.e., loss of detectable HCV antibodies after HCV seroconversion), among 31 HIV-infected men who have sex with men who cleared their primary HCV infection following treatment. *Shaded gray area, 95% confidence interval. Abbreviation: HIV, human immunodeficiency virus.*

Anti-HCV and ALAT during HCV reinfection versus primary acute infection

During follow-up, 21 reinfections were observed among 18 subjects. Reinfection occurred either following SVR (13 reinfections), before SVR was reached (three reinfections), or following spontaneous clearance (four reinfections), or without intermittent negativity (one reinfection; possible superinfection). Table 2 shows that peak anti-HCV reactivity levels (S/CO) were significantly higher during reinfection (median: 119.6, IQR: 103.4-146.6) compared to primary infection (median: 72.9, IQR: 57.1-105.5; *P*=0.014). Anti-HCV reactivity of the subject who had three reinfections is shown in Figure 5 to illustrate what may be observed during a course of multiple infections.

At the first RNA-positive date during primary infection, ALAT concentrations were elevated (i.e., more than two times the upper limit of normal; ≥80 U/L) in 13/18 (72.2%) of cases, with a median of 119 U/L (IQR: 56-470). ALAT levels were less pronounced upon reinfection with a median of 66 U/L (IQR: 26-222). Moreover, ALAT concentrations were elevated in only 8/18 (44.4%) cases at the first RNA-positive date of reinfection (Table 2).

	Anti-HCV	ALAT
Reactive/elevated at first HCV-RNA+ visit during primary infection	6/15 (40)	13/18 (72)
Reactive/elevated at first HCV-RNA+ visit during reinfection	13/14 (93)	8/18 (44)
Peak following primary HCV infection (median, IQR)	72.9 (57.1-105.5) S/CO	470 (336-840) U/L
Peak following HCV reinfection (median, IQR)	119.6 (103.4-146.6) S/CO	223 (164-482) U/L

Table 2. Maximum Observed Values for Hepatitis C Virus Antibody and Alanine Aminotransferase Concentrations During Primary Infection and Subsequent Reinfection for 18 HIV- Infected Men Who Have Sex With Men Who Were Reinfected During Follow-up. *Abbreviations: ALT, alanine aminotransferase; anti-HCV, hepatitis C virus antibody; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IQR, interquartile range.*

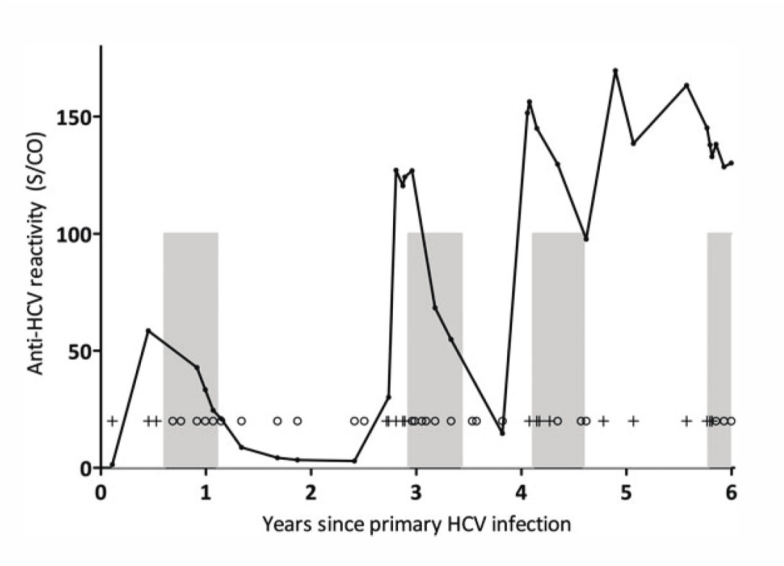

Figure 5. Hepatitis C virus (HCV) antibody (anti-HCV) sample-to-cut-off levels and qualitative RNA measurements in 1 HIV-infected man who has sex with men. *After resolving a primary HCV-4d infection after treatment, this subject became reinfected 3 times. All reinfections were of genotype 1a, and occurred at approximately 3, 4, and 5 years after primary infection. Notice that peak anti-HCV levels increase with every infection and that partial seroreversion is demonstrated after each RNA clearance. Connected dots, anti-HCV reactivity; +/o, RNA status (positive/negative); shaded gray area, HCV treatment period. Abbreviations: HIV, human immunodeficiency virus; S/CO, sample-to-cut-off.*

Discussion

In this study, dynamics of HCV-specific antibodies were studied among HIV-infected MSM with acute HCV infection. Our main findings were that (1) the seroconversion window in this population was comparable to the seroconversion window reported among HIV-uninfected subjects, (2) seroreversion was very common following successful antiviral treatment, and (3) after an initial decrease in anti-HCV levels following SVR, levels increased following reinfection to levels reached during primary infection (or higher).

The median time to seroconversion in our study was 74 days and comparable to the HCV seroconversion window reported for HIV-uninfected subjects [12-17]. However, in contrast to our study, in a group of HIV-infected MSM a delayed or even absent antibody response against HCV following acute infection was reported by Thomson et al. [18]. In our study, in a sensitivity analysis among subjects with narrower testing intervals around primary infection, estimates were comparable to the seroconversion window we obtained in the full dataset (data not shown). Also, in our study, all men seroconverted within the observation period, whereas in the Thomson study paper no anti-HCV antibodies were detected in 4/43 subjects at the end of follow-up [18]. As two of these subjects had spontaneously cleared HCV, seroreversion may partly explain these findings, as the authors do not give test intervals for these specific cases. In addition, differences in cART use (53% vs. 74% in our study) may partly explain the differences found with respect to seroconversion.

Among 17/63 subjects (27%) in our study, no anti-HCV antibodies could be detected four months after the estimated date of HCV infection. Screening for acute HCV is therefore preferably performed using nucleic acid testing instead of anti-HCV testing. Periodical testing of ALAT concentration levels may contribute to increased case finding, but ALAT levels can normalize within the serodiagnostic window. As a result, acute infections in patients with normal ALAT levels and an HCV-seronegative status may still be missed. ALAT levels may not always be elevated following HCV (re)infection, and when elevated, do not always indicate recent HCV infection [32]. Indeed, in our study 72.2% of reinfected subjects had elevated ALAT levels at the first RNA-positive date of primary infection, whereas only 44.4% of them had elevated ALAT at the first RNA-positive date of reinfection. This further emphasizes the need for HCV-RNA testing in patients at risk for reinfection.

Seroreversion (i.e., loss of antibodies) following HCV clearance was relatively common in our study, being 37% at three years after seroconversion. To our knowledge, we are the first to have systematically addressed the occurrence of seroreversion among HIV-infected patients with acute HCV infection. The observed incidence of seroreversion in our study is very high, especially when compared to the frequency of seroreversion reported after treatment of chronic HCV [19, 20, 22, 23, 25, 26, 36]. One explanation for the high seroreversion rate may be that in our study, treatment was initiated early in the course of infection, resulting in lower peak anti-HCV levels, as a loss of HCV-RNA coincided with decline in anti-HCV levels.

Seroreversion was observed only among those who cleared HCV following treatment; after spontaneous HCV clearance only partial seroreversion was observed, most likely because 4 out of 5 subjects that had spontaneously cleared their primary infection became reinfected during follow-up. We expect that seroreversion is also likely to occur after spontaneous clearance of HCV, as the observed slope of decline in anti-HCV was comparable to the slope in those who cleared following antiviral treatment. For the same reason, peak anti-HCV reactivity probably influenced the time to seroreversion.

The rapid decrease of anti-HCV reactivity, indicating loss of specific anti-HCV producing plasma cells in this population, may be partly due to the presence of HIV-coinfection, although most men were on cART and had relatively high CD4 cell counts. During HIV infection, the total B-cell number and number of memory B-cells may be significantly reduced [37]. While use of cART is associated with a normalization of the absolute number of B-cells, the memory B-cell subset is unlikely to be restored [37]. Also plasma cell disorders are reported more frequently among HIV-infected patients, but the exact mechanisms that drive this are still unclear [38]. An additional explanation for the rapid decline in anti-HCV reactivity following HCV clearance may be that humoral responses during antiviral therapy in patients with acute HCV infection differ from patients with chronic infection; the rapid decrease in viral antigen, required for stimulation of B-cells, and interferon therapy itself, may inhibit B-cell proliferation. Indeed, loss of anti-HCV reactivity has also been reported after treatment of acute HCV among HIV-negative individuals by Wiegand et al. [36].

Interestingly, following initial decrease in anti-HCV reactivity after HCV clearance, a subsequent increase in anti-HCV reactivity was observed in all reinfection cases. To our knowledge, this finding of 're-seroconversion' is unique and may supplement current screening strategies for HCV reinfection in this population. This may be especially helpful because ALAT levels are not always elevated during reinfection.

The level of anti-HCV reactivity might be a marker for the presence of neutralizing antibodies (nAbs) after infection, as has been proposed by Mizukoshi et al. [39]. In addition, nAbs generally are thought to develop only after initial control of viremia [40]. If this is indeed the case, nAbs titres may remain low

when treatment is initiated early after primary infection. To some extent, this could even explain the high rates of reinfection reported among HIV-infected MSM [32, 33].

Our study has a number of limitations. All subjects were identified at an HIV outpatient clinic; this may have led to selection bias because symptomatic patients may have a higher frequency of visit. Average testing intervals around infection were wider among subjects not treated for HCV compared to those who were, suggesting that patients who are willing to undergo HCV treatment are more compliant to clinical visits. We did not correct for the uncertainty of the estimated dates of infection, seroconversion, and seroreversion in our analyses. Instead, we applied a strict inclusion criterion of max. 6 months between the last negative and the first positive HCV-RNA test. Another limitation is that our study did not incorporate subjects with chronic HCV infection. However, literature suggest that decline of anti-HCV occurs rarely following treatment of chronic HCV infection [19, 20, 22, 23, 25, 26, 36]. Finally, the results of this study may only apply for the antibody assay used, an assay with sufficient linear range. Our results may be less applicable when assays with a more narrow linear range are used.

In conclusion, we have shown that the seroconversion window among HIV-infected individuals is comparable to HIV-uninfected individuals. Still, the median time to seroconversion was 74 days. Screening for acute HCV infection is thus still ideally performed using nucleic acid testing. Seroreversion was common following HCV clearance, and may cause misclassification of a reinfection as an initial infection in clinical practice. Finally, anti-HCV levels increased again following HCV reinfection to levels reached during primary infection. Although the antibody assay used is not a quantitative assay, a clear association existed between anti-HCV reactivity and viremia within subjects following acute HCV infection. Monitoring antibody dynamics following SVR could thus be a useful and inexpensive alternative and additional tool for evaluation and diagnosis of HCV reinfection in the HIV-infected MSM population.

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Chapter 9

Characteristics of Hepatitis C Virus (HCV) Neutralizing Antibody Responses during Acute HCV Infection: are Genotypes Neutralization Serotypes?

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Abstract

Background: The frequent occurrence of hepatitis C virus (HCV) reinfection following treatment-induced clearance in HIV-infected men who have sex with men (MSM) suggests that adaptive immune responses are insufficient to generate a response that protects against reinfection. However, the risk of acquiring a new infection with the same genotype that caused the primary infection appears to be reduced. To gain more insight in neutralizing antibody (nAb) responses generated during acute HCV infection in this HIV-infected population, we longitudinally investigated the breadth and potency of HCV specific nAb responses in HIV-infected MSM with treatment induced clearance of an acute genotype 1a infection.

Methods: Fifteen pegylated interferon (pegIFN)-treated HIV-infected MSM with documented primary HCV-1a infection were selected for this study. Patients were treated in acute phase of infection. Presence of nAbs against different genotypes was determined using a panel of 12 HCV pseudo-particles (HCVpp) which expressed envelopes of genotypes 1a (n = 6), 1b, 2a, 2b, 3a, 4a and 4d. Five HCVpp-1a were constructed using envelope sequences from strains locally circulating in the MSM population. The breadth and potency of nAb responses were quantitatively assessed by endpoint dilution at T1) RNA-positive time point T2) start of treatment, T3) last RNA positive sample and at T4) three and T5) six months after last RNA positive sample. Potency of nAb responses was used for a heatmap construction which clusters HCVpp with similar sensitivity to neutralization. In addition, we investigated whether reduced sensitivity to neutralization could be explained by the presence of mutations at positions implicated in escape from nAbs which could explain the observed antigenic clustering.

Results: One patient was excluded from further analysis because of aspecific neutralization. In 11 of the 14 remaining patients, nAbs against one or more HCVpps were present. Potency of response increased during viremia, with maximum titres ranging from 1:50 to 1:6400. Both breadth and potency of nAb response declined following clearance of HCV RNA. Breadth was strongly correlated with CD4+ cell count at the time of HCV acquisition. Interestingly, nAb responses were more potent against HCVpp genotype 1, the genotype all patients were infected with, compared to responses against genotypes other than 1. The heatmap analysis demonstrated the presence of two major antigenic clusters: one sensitive for neutralization, consisting of only genotype 1 HCVpp (A) and one cluster with reduced sensitivity to neutralization (B), consisting of both genotype 1 and non-1 HCVpp. Antigenic cluster B was divided further into a resistant cluster (B.1) and a cluster of HCVpp with an intermediate resistance profile (B.2). The HCVpp designated to antigenic cluster B.1, contained the D431G mutation, which has been associated with escape from monoclonal antibodies.

Conclusion: Our results suggest that nAb responses following acute HCV infection are skewed towards the infection genotype in patients with treatment-induced clearance. However, sensitivity to neutralization may vary and depend on the presence of escape mutations in the viral envelope. Breadth and potency of nAb response decline over time. These findings suggest the presence of partial humoral immunity following treatment-induced clearance of acute HCV in HIV-infected patients, which may explain the observed increased likelihood of reinfection with another genotype. The likelihood of reinfection with the same genotype may depend on the intrinsic sensitivity to neutralization of the transmitted virus.

Introduction

Since 2000, incidence of acute HCV infection has significantly increased among HIV-infected men who have sex with men (MSM) [1]. With no vaccine available and given the rapid disease progression in these patients if left untreated, the reported high incidence of HCV infection is of great concern [2]. A minor percentage of HIV-infected patients is able to clear HCV infection spontaneously [3,4]. Treatment of acute HCV in HIV-infected patients with pegylated-interferon (pegIFN) and ribavirin has reported success rates of 58%-76% which increase to 84% by the addition of telaprevir, a first generation protease inhibitor [5–7]. The continuing progress in the development of direct acting antivirals (DAA) has improved sustained viral response (SVR) rates for treatment of chronic HCV tremendously, and theoretically, cure of HCV infection is likely to occur in the majority of patients when treated during the acute phase [8]. However, there is a legitimate concern for resistance [9], and spread of resistant variants in populations with a high incidence is a not just theoretical risk [10]. Furthermore, treatment with the new cocktails of DAA is costly, and may not be affordable for a substantial proportion of infected patients [9]. Thus, the development of a vaccine is clearly an unmet need for populations with limited access to treatment or high incidence of new infections. To advance this field further, detailed dissection of HCV specific immune responses is necessary to gain more insight in the main correlates of protection against HCV.

Different components of both the innate and adaptive immune system are involved in clearance of HCV infection. The innate response has been shown to play an important role, as evidenced by the association between HCV clearance and polymorphisms in the gene encoding interferon-lambda 3 [20–22]. Furthermore, a critical role for T-cells is well established with several studies reporting that a sustained and vigorous T-cell response is a key factor in determining outcome of acute infection [19,23,24].
However, less is known about the role of antibodies in providing protection against chronicity of HCV infection. Using lentiviral pseudo-particles that express the HCV envelope it has been shown that neutralizing antibodies (nAbs) which are able to inhibit virus binding and entry are produced in both chronically infected patients and patients who cleared the infection spontaneously [25–27]. However, the time it takes for these nAbs to appear, may determine outcome of acute infection: patients with delayed nAb responses are more likely to develop chronic infection [27,28]. In addition, broader responses that are able to neutralize a broad range of HCV variants are also associated with clearance of infection [19]. Such broad nAbs are mostly targeting conformational epitopes in the envelope protein E2, with contact residues located mainly at two discontinuous regions spanning amino acid positions 424–443 (epitope II) and 529–535 (epitope III) [21–23]. These discontinuous regions overlap extensively with the CD81 binding domains, one of the main HCV entry receptors [22,24]. The region spanning position 529 – 535 is extremely conserved and escape mutations have not been described for this region. In contrast, epitope II is more variable and several escape mutations resulting in reduced neutralization sensitivity have been described [25].

Thus far, it has remained unclear whether treatment-induced or spontaneous clearance of acute HCV infection results in some degree of protection against reinfection and whether broad nAbs indeed play a role in neutralizing reinfection or can prevent a reinfection from becoming chronic. Observed reinfection rates after either spontaneous or treatment-induced viral clearance in high-risk populations, like HIV-

infected MSM and injecting drug users (IDU), suggest that clearance of a primary infection does not lead to protective immunity [3,11–17]. Nevertheless, several studies have shown that HCV RNA load is lower after reinfection compared to the primary infection [15,18,19], suggesting that some degree of non-sterilizing yet protective immunity exists that prevents chronicity.

In a cohort of HIV-infected MSM with sexually acquired acute HCV infections we recently reported a very high incidence of reinfection after treatment [11]. However, in a subsequent study we observed a reduced risk of reinfection with the genotype causing the primary infection, suggesting that immunity following clearance of primary infection might be genotype specific (chapter 6). An explanation for this observation could be that nAbs present following treatment-induced clearance of a primary HCV infection could have a greater affinity for the genotype of the primary infection, thereby preventing (chronicity of) a secondary infection. Most studies investigating nAb responses published to date have used very limited pseudo-particle panels consisting of just one prototype 1a variant [19]. Goal of the current study was to comprehensively study breadth and potency of nAb responses in HIV-infected MSM successfully treated for an acute HCV infection, using an antigenically heterogeneous panel of HCV pseudo-particles (HCVpp), expressing envelope glycoproteins from different genotypes of both locally and globally circulating variants.

Methods

Study population

Patients were selected from a cohort of HIV-infected MSM with acute HCV infection attending the HIV-treatment clinic of the Academic Medical Center in Amsterdam, The Netherlands. In this cohort, HCV is diagnosed by routinely measured alanine-aminotransferase (ALT) elevation and subsequent detection of HCV RNA. In addition, patients in this HIV risk-group are screened for anti-HCV antibodies routinely once a year. Additional HCV RNA testing was done on stored sera and the date of HCV acquisition was estimated by calculating the midpoint between the first RNA-positive sample and the last RNA-negative sample prior to HCV infection. Anti-HCV testing was performed using a commercial micro particle enzyme immunoassay (MEIA) AxSYM® HCV 3.0 (Abbott Laboratories).

Selection criteria for this study were 1) acute primary HCV genotype 1a infection, treated successfully with pegIFN and ribavirin, 2) a maximum interval of 6 months between the last negative and first positive HCV RNA test 3) the absence of documented reinfection at least 1 year following the end of treatment.

HCV pseudo-particle construction

Breadth, potency and dynamics of nAbs in serum were assessed using a panel of 12 HCVpp expressing a genotype 1, 2, 3 or 4 envelope. Six genotype 1a HCVpp (HCVpp-1a) were constructed: one for the prototype HCV H77 (AF009606) and five using envelope sequences from strains locally circulating among HIV-infected MSM (MSM1-MSM5). These sequences were representative of five distinct phylogenetic clusters, identified by sequencing of a 389 nucleotide fragment of the gene encoding the non-structural protein 5B (NS5B), which was used for genotyping [29]. The phylogenetic tree showing the different clusters, is depicted in Figure 1. HCVpp for genotypes 1b (HCVpp-1b), 2b (HCVpp-2b), 3a (HCVpp-3a), and 4d (HCVpp-4d) were constructed using envelope sequences from non-MSM chronically infected

patients. HCVpp for genotypes 2a (HCVpp-2a, AY734977) and 4a (HCVpp-2a, AY7349986) were made synthetically based on Genbank deposited sequences.

HCV E1E2 glycoprotein expression plasmids were constructed by purifying HCV-RNA from 200 µL serum or plasma using the Boom HCV RNA extraction method [30]. Complementary DNA (cDNA) was synthesized using random hexamer primers as described before [31]. A PCR product ranging from the sequence corresponding to the C-terminal part of the Core protein (which acts as signal sequence for E1) to the end of E2 was generated and cloned using the pcDNA™ 3.3-TOPO® TA Cloning Kit (Invitrogen). HCVpp were produced by co-transfection of expression plasmids for HCV E1E2 glycoproteins and murine leukemia virus (MLV) gag-pol, together with a transfer and luciferase reporter plasmid [25] in a ratio of 1:2:2. A control pseudo-particle was included using Vesicular Stomatis Virus (VSV) G glycoprotein and a negative (no envelope) pseudo-particle control was included that lacked the envelope glycoproteins proteins. Transfection was performed in human embryonic kidney (HEK) 293T cells using the X-tremeGENE (Roche applied sciences) transfection kit. After a 48-hour incubation at 37˚C with 5% CO_2, cell supernatants were harvested and filtered through a 0.45-µm nitrocellulose membrane.

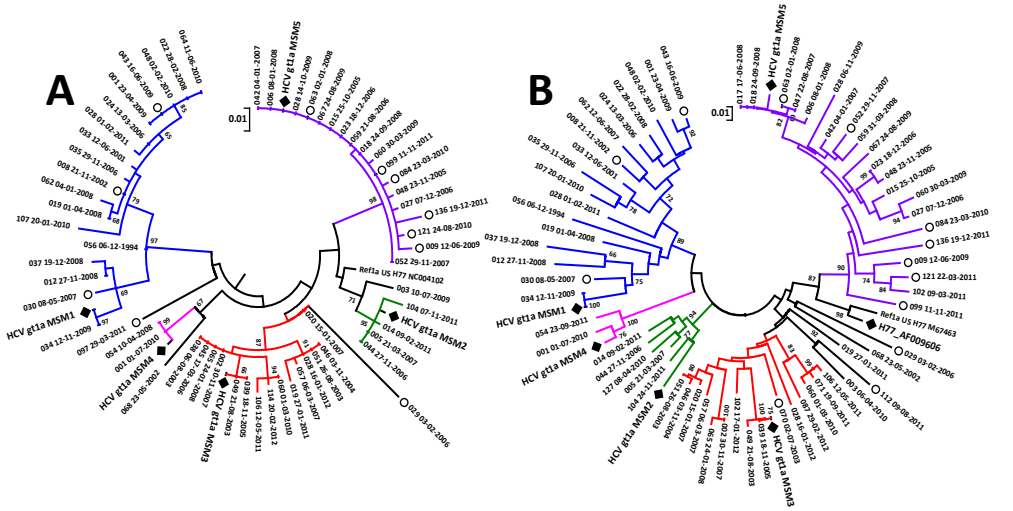

Figure 1. NS5B nucleotide (A) and E2/HVR1 amino acid (B) phylogenetic tree illustrating sequence clusters. *A) NS5B molecular phylogenetic tree and B) E2 phylogenetic tree. Trees were constructed as described in chapter 6 of this thesis. Clades are indicated with different colours. Blue=MSM1, green=MSM2, red=MSM3, pink= MSM4 and purple=MSM5. The strains used for constructing pseudo-particles are indicated with a bold diamond. Sequences from the first RNA positive sample from each patient are indicated with open bullets. Overall, MSM clusters were concordant between trees.*

Neutralization assay

Serum samples were selected at the first HCV RNA-positive time point (T1), start of treatment (T2), last RNA-positive time point (T3) and at three (T4) and six months (T5) after the last HCV RNA-positive sample. As a patient-specific negative control, serum from 0.5-1 year prior to the first HCV RNA positive sample was selected (T0).

HCVpp infection was performed by adding 200 µL of filtered medium containing HCVpp to each well of a 96-well micro-titre plate containing human hepatoma (Huh)-7 cells at 50% confluence, which was seeded the day before. Plates were spin-inoculated at 700 rpm for 48 minutes and were incubated at

37˚C with 5% CO_2 for 72h. Following removal of medium, infected cells were lysed with 50 µL luciferase substrate (Promega) and luciferase activity was measured using the FLUOstar OPTIMA (Isogen life science) plate reader.

Neutralization assays were performed by combining 100 µL HCVpp with 100 µL heat-inactivated (30 minutes at 56˚C) plasma or serum followed by incubating for 1 hour at 37˚C with 5% CO_2. Two hundred µL of virus-plasma mixture was pipetted to each well of a micro-titre plate in triplicate. Two–fold serial dilutions were prepared in DMEM with 10% Fetal Calf Serum. Dilutions ranged from 1:50 and continued until no neutralization was observed as mentioned above.

Neutralization was considered to be present if the mean relative light units (RLU) of triplicates in the presence of serum/plasma dilution (RLUtest) was below the mean RLU minus three times the standard deviation (SD) of triplicates from the T0 control serum from each patient (neutralization= RLUtest < T0 – (3*SD T0)).

Statistical analysis

Neutralizing responses were quantified by endpoint dilution. Breadth of response was defined as the number or percentage of neutralized HCVpp. Potency of response was determined by two-fold serial dilutions of serum. For analytic purposes, a potency score ranging from 0-8 was assigned to each dilution step, with 0 indicating lack of neutralization and 8 indicating neutralization observed at a titre of 1:6400. The highest dilution resulting in neutralization -irrespective of the number or type of HCVpp neutralized- was used for this potency score: for instance, a serum with a 1:200 neutralizing titre of any HCVpp was assigned a potency score of 3 and a serum with a 1:800 neutralizing titre was assigned a score of 5. Statistical comparisons of breadth and potency of nAbs between groups were done using the unpaired t-test if normally distributed or the Man-Whitney test in the case of non-normal distribution. P-values < 0.05 were considered significant. Potency scores were also used for heatmap construction to graphically group HCVpp with similar sensitivity to neutralization by our panel of sera [32]. For this, the heatmap tool available in the Los Alamos National Laboratory HIV database was used. Stability of clusters was tested with 1000 bootstraps.

Comparative analysis of envelope sequences

We investigated whether neutralization phenotype of the different genotype 1 HCVpp in our panel could be explained by the presence of known nAb escape mutations that have been reported in the literature. Sequence variation in the HCVpp-1a envelope sequences was assessed by comparing putative amino acid nAb escape mutations at positions, L413 [33], N415 [34], N417 [35], G418 [36], S419 [33], D431 [37,38], N434 [37], T435 [37], L438 [37], A439 [37], S501 [39], V506 [39], K610 [33] and E655 [34] with the H77 clone as a reference (AF009606).

Results

NAb dynamics during HCV viremia and subsequent viral clearance

Fifteen patients with successfully treated acute primary HCV genotype 1a infection were selected, with a maximum interval between the last negative and first positive HCV RNA test of 6 months. These patients had remained HCV RNA negative for at least 1 year following the end of treatment. Median age was 43.7 years (IQR, 38.0-53.4) and median CD4+ cell count was 470 cells (IQR, 390-640) at time of HCV acquisition. Eleven patients were on antiretroviral therapy at time of HCV acquisition. Patient and virological characteristics are summarized in Table1.

One patient was excluded from further analysis because of non-specific neutralization of the VSV-G control particle. In the remaining 14 patients, a median of six out of 12 HCVpp were neutralized by serum at the lowest dilution (1:50, Figure 2). In three patients no neutralization was observed at any time point. Two patients exhibited a broad response, neutralizing 11 out of 12 HCVpp at the lowest dilution. The median highest dilution at which at least one HCVpp was neutralized during follow-up was 1:400. In one patient a neutralizing titre of 1:6400 against a HCV1a-pp (MSM2) was present.

Characteristics	All n=15 , n (%)
Baseline	
Median age at time of HCV infection in years (IQR)	43.7 (38.0-53.4)
HIV treatment at time of HCV acquisition	11 (73.3)
HIV RNA undetectable at time of HCV acquisition	8 (53.3%)
(if detectable, n=7) median HIV load at time at acute HCV(IQR)	83729 (3329-1777378)
Median CD4+ cell count at last negative HCV RNA test prior to HCV infection cells/µL	470 (390-640)
Median interval between last negative and first positive HCV RNA test in months (IQR)	3.7 (2.8-5.6)
Median interval between last anti-HCV negative and first anti-HCV positive test in months (IQR)	4.9 (2.6-6.8)
Median interval from HCV acquisition to start of HCV treatment	4.4 (3.6-6.9)
Follow-up	
Median follow-up time in years (IQR)	3.2 (2.2-6.1)
Median duration of HCV viremia in months (IQR)	3.3 (1.8-7.2)
Median HCV RNA testing interval in months (IQR)	3.3 (0.9-5.4)

Table 1. Patient characteristics. *IQR, inter quartile range.*

During follow up, breadth of response, as indicated by the median number of HCVpp neutralized, varied between 2 and 4 (Figure 3a). No clear time dependent trend in breadth of response during the viremic period could be distinguished, as shown in Figure 4 which illustrates breadth of responses over time in individual patients. In some patients breadth was already high at the first RNA positive time point, whereas in others the breadth increased over time. However, in all patients, a decline in breadth was observed at T5, six months after the last positive HCV RNA time point, compared to the maximum number of HCVpp neutralized before (p=0.04, comparing the maximum number of HCVpp neutralized at T1 – T4 with T5).

Potency of response increased during viremia, with highest neutralization titres at T4, 3 months after the last RNA positive sample (Figure 3b). Loss of viremia coincided with loss of potency and titres were lowest at T5 (p=0.03, comparing maximum potency at T1-T4 with T5).

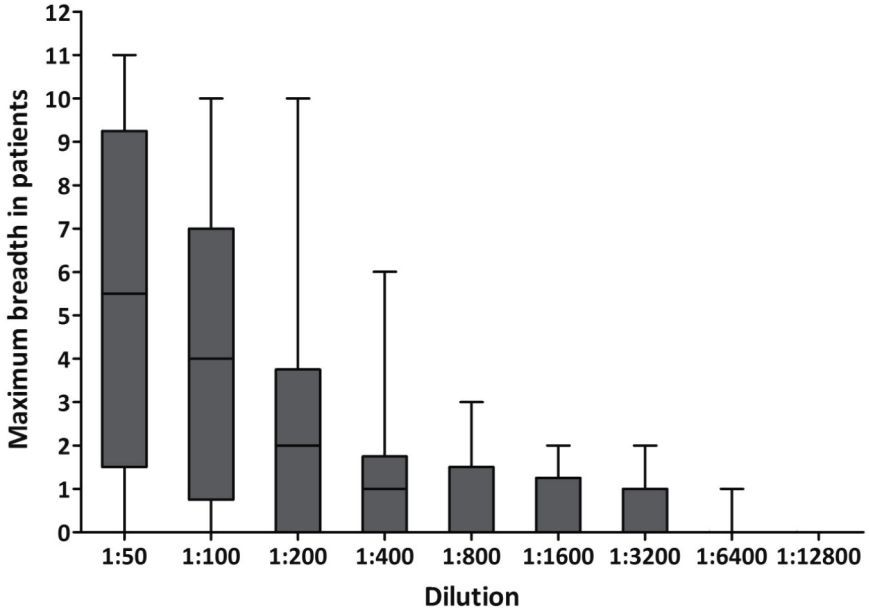

Figure 2. Breadth and potency at different dilutions. *For each patient the maximum number of HCVpp neutralized at different dilutions of serum was determined. Boxplots summarize the distribution of the number of HCVpp neutralized for all patients. Each boxplot delimits the interquartile range (IQR) of the maximum number of HCVpp neutralized. The thick line segment indicates the median, and the whiskers represent the range that is within 1.5times IQR above the upper quartile or below the lower quartile.*

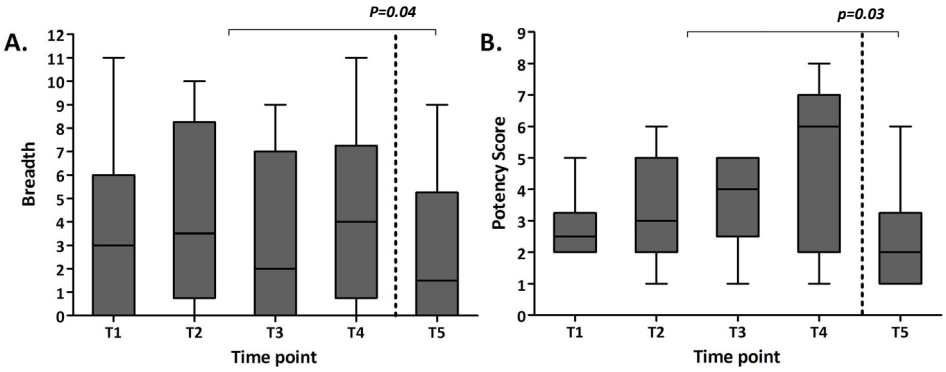

Figure 3. Breadth and potency of the nAb responses at different time points during follow-up. *A) For each patient the number of HCVpp neutralized per time point was determined. Boxplots summarize the distribution of the number of HCVpp neutralized at the 1:50 dilution. In all patients a decline in breadth of response was observed at 6 month after HCV RNA clearance compared to the maximum number of HCVpp neutralized at T1–T4 (p=0.04). B) For each patient the highest neutralization titre was determined per time point. Following the first RNA positive time point, potency of responses in general increased up to 3 months after viremia (T1-T4), followed by a drop 6 months after RNA clearance (T5), p=0.03. Boxplots summarize the highest titres expressed as potency scores for all patients. T1= first HCV RNA positive time point, T2= start of treatment, T3=last RNA positive time point, T4= 3 months after last RNA positive sample and T5= six months after the last HCV RNA positive sample.*

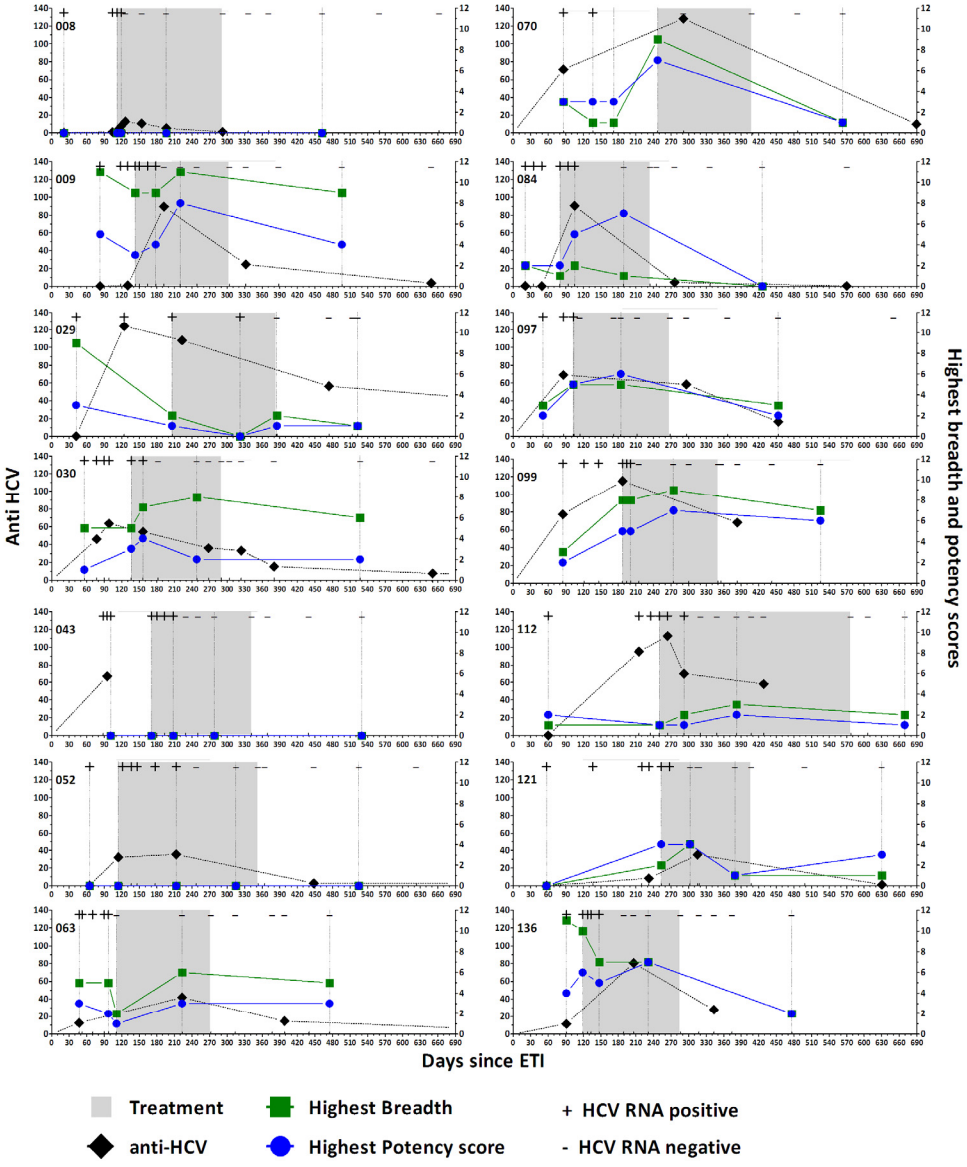

Figure 4. Individual patient data of commercial anti-HCV testing, breadth and potency of nAb response over time. *Anti-HCV reactivity as determined by the AxSYM® is depicted on the left y-axis, breadth and potency scores on the right y-axis. On the X-axis time since infection is plotted in days. Grey shades indicate time at which patients received treatment. Individual graphs demonstrate that within a patient, anti-HCV reactivity, breadth and potency of nAbs, if present follow the same trend over time, although temporal dynamics of increase and decline over time of nAbs may vary between patients.*

Genotype specific neutralizing responses

Since all patients were infected with genotype 1a, breadth and potency of neutralizing responses against HCVpp-1 were compared with responses against HCVpp-non-1. Figure 5a shows the breadth of neutralizing responses against HCVpp-1 vs. HCVpp-non-1. Neutralizing responses were broader against HCVpp-1 compared to non HCVpp-non-1, as at different dilutions the breadth against HCVpp-1 was always higher compared with the breadth directed against HCVpp-non-1. In addition, in only one out of 14 patients a neutralization titre exceeding 1:400 against a HCVpp-non-1 was present, whereas this was the case for half of the patients against a HCVpp-1 (p= 0.01, Chi-square test). In addition, potency against HCVpp-1 was significantly higher compared to HCVpp-non-1 (Figure 5b, p=0.001).

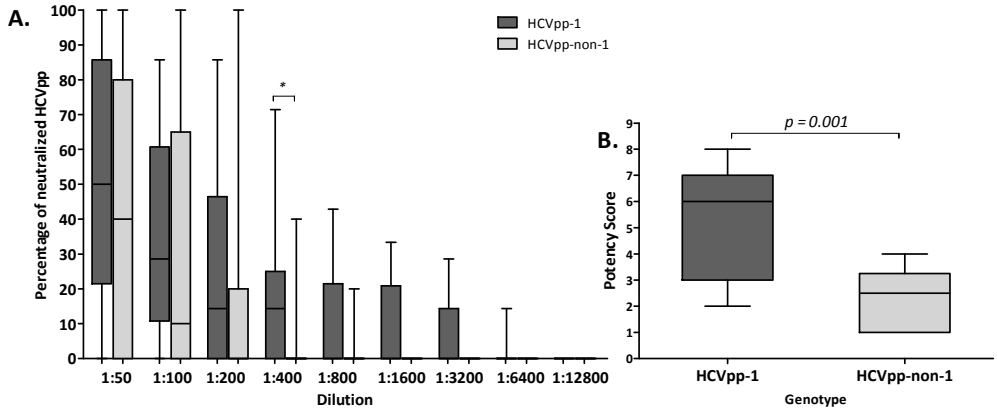

Figure 5. Breadth and potency of Nab response at different dilutions: the effect of genotype. *A) For each patient the highest number of HCVpp-1 and HCVpp-non-1 neutralized at different dilutions of serum -irrespective of time point- was determined. Boxplots summarize the distribution of the number of HCVpp-1 and HCVpp-non-1 for all patients. Patients infected with HCV genotype 1a, developed a broader and more potent response against HCVpp-1, compared to HCVpp-non-1. B) For each patient the highest titre against HCVpp-1 and HCVpp-non-1 irrespective of time point was determined. Boxplots summarize the distribution of titre expressed as potency score for HCVpp-1 and HCVpp-non-1. Patients infected with HCV genotype 1a developed more potent nAb responses against HCVpp-1 compared to HCVpp-non-1.*

Anti-HCV reactivity during follow-up

Overall, anti-HCV reactivity as measured by the AxSYM® platform, a commercial micro-particle enzyme immunoassay, followed the same trend as nAb responses, as depicted separately for each patient in figure 4. However, anti-HCV reactivity at T1, the first RNA positive time point, was detectable in only five out of the twelve patients with available anti-HCV data at that time point. Interestingly, in four of the seven patients without detectable anti-HCV reactivity at T1, nAb responses could be detected.

Anti-HCV reactivity peaked around the time treatment was started, in general 3 to 6 months after the estimated date of infection, but declined following loss of viremia, similar to the neutralizing responses. In one out of three patients without detectable neutralizing responses (patient 008), anti-HCV peak reactivity was low (max 12), and the patient seroreverted within a year of HCV acquisition.

Antigenic clustering of HCV pseudo-particles

Two-dimensional antigenic dendrograms were constructed by hierarchical clustering of the HCVpp based on their sensitivity to neutralization, which enables clustering of HCVpp displaying similar antigenic profiles. As illustrated in Figure 6, two main clusters are present with high bootstrap support: cluster A, consisting of antigenically related HCVpp-1 (HCVpp-H77, HCVpp-1b, MSM1 and MSM3) and cluster B, consisting of both HCVpp-1 and HCVpp-non-1. Interestingly, cluster B contained two subclusters; cluster B.1, containing both MSM5 and HCVpp-non-1, and cluster B.2, containing two antigenically related HCVpp-1 (MSM2 and MSM4). The dendrogram confirmed the overall increased potency of sera against HCVpp-1, as indicated by the dark red colours in the heatmap.

As the dendrogram is two-dimensional, patient sera could also be clustered based on their ability to neutralize different HCVpp. Different clusters clearly were present, e.g. sera that did not neutralize any of the HCVpp obviously clustered together. However, antigenic clustering did not coincide with the infection cluster MSM1-5, i.e. potency scores were not higher against the HCVpp derived from the same phylogenetic cluster the patient was infected with.

HCV-1a envelope sequence analysis

Next, we investigated whether reduced sensitivity to neutralization was associated with the presence of mutations at amino acid positions implicated in escape from nAbs, which could explain the observed antigenic clustering. Mutations were observed at six out of the 14 putative escape positions (Table 2). HCVpp H77, which did not possess any of the putative escape mutations, was the most sensitive HCVpp from our panel. In HCVpp MSM5, the most resistant variant in our panel, a D431G mutation was observed, which has been shown to result in escape from the well characterized monoclonal antibody CBH-2 [37,40]. N434D, another putative escape mutation, was present in HCVpp MSM2 which showed an intermediate resistance profile. HCVpp MSM3 harboured the same mutation but in this study was relative sensitive to neutralization [33,41]. S501N was present in HCVpp MSM1, a variant relatively sensitive to neutralization. Interestingly, at position 438, where a change to F has been implicated in resistance [37], changes to V or I were present only in the HCVpp variants with reduced neutralization sensitivity. In HCVpp MSM2 and MSM4, these mutations were present in combination with mutations at position 434, which may contribute to resistance as demonstrated for the combination of L438F and N434D [37].

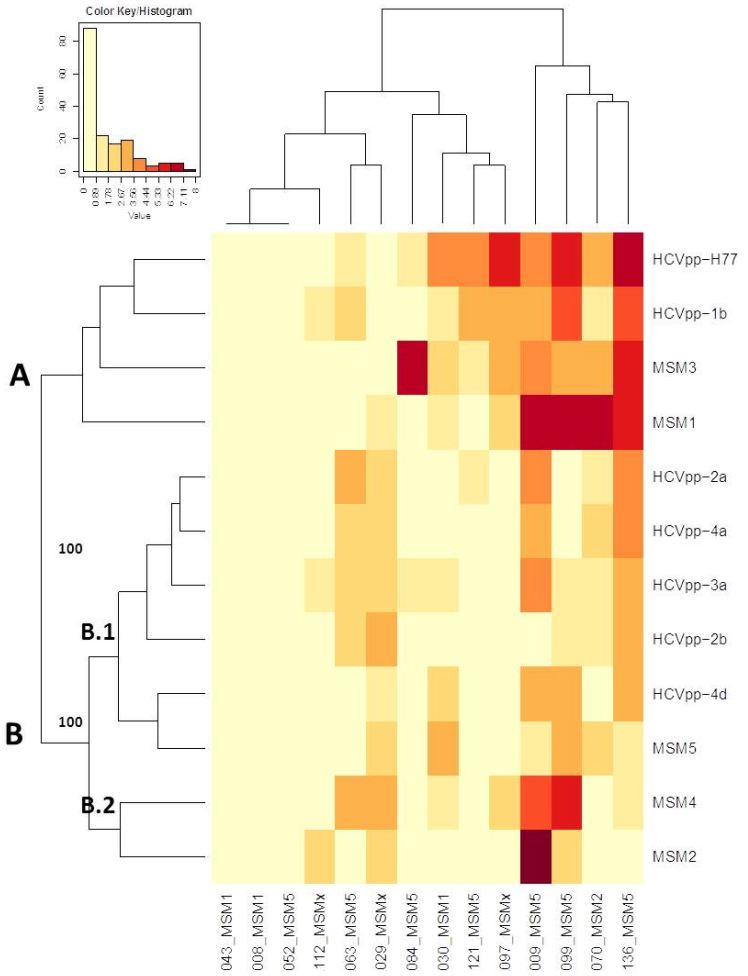

Figure 6. Heatmap and clustering analysis of genotypes HCVpp-1,-2,-3 and -4. *Using the highest potency score, irrespective of time point, three antigenic clusters were observed:, A, B.1 and B.2. Stability of clusters was tested with 1000 bootstraps and is indicated above branches. As indicated in the legend, the darker the colour, the higher the potency score. On the x-axis IDs are indicated followed by the corresponding MSM cluster-number from the HCV-1a variant that the patient was infected with. "MSMx" indicates that HCV-1a variant did not cluster with any designated cluster or that E2/HVR1 sequencing of this strain failed. HCVpp-H77 was the most neutralization sensitive variant.*

		Mutation positions relative to the H77clone													
HCVpp	**Antigenic cluster**	**L413I**	**N415Y**	**N417S/T**	**G418D**	**S419N**	**D431G**	**N434D**	**T435A**	**L438F**	**A439E**	**S501N**	**V506A**	**K610R**	**E655G**
H77	A	-	-	-	-	-	-	T	-	-	-	-	-	D	-
MSM1	A	-	-	-	-	-	A	E	-	-	-	**N**	-	D	D
MSM3	A	-	-	-	-	-	A	**D**	-	-	-	-	-	D	-
MSM2	B.2	-	-	-	-	-	A	**D**	-	**V**	-	-	-	N	-
MSM4	B.2	-	-	-	-	-	T	E	-	**I**	-	-	-	D	-
MSM5	B.1	-	-	-	-	-	**G**	-	-	**I**	-	G	-	N	-

Table 2. Analysis of escape mutations. *Each column indicates a site for which escape from nAbs has been documented. Amino acid positions are relative to the H77 clone. Bold-underlined amino acids are previously described escape mutations.*

Effect of CD4+ cell count prior to HCV infection on the breadth and potency of nAbs

The median CD4+ cell count prior to HCV acquisition in all patients (n=14) was 485 per mm^3 (IQR, 412.5-640). The median CD4+ cell count in patients without neutralizing responses (n=3) was lower than in patients with neutralizing responses (respectively, 450 (range 130-510) and 500 (IQR, 420-640; range 310-780)) but this difference was not statistically significant (p=0.350).

The CD4+ cell count prior to HCV infection was correlated with the breadth of neutralizing responses (Pearson R^2 0.52, p= 0.004), the correlation with potency of nAbs was not statistically significant (Pearson R^2 0.21, p=0.160). Correlations of CD4+ cell counts with breadth and potency of nAb responses are depicted in Figure 7.

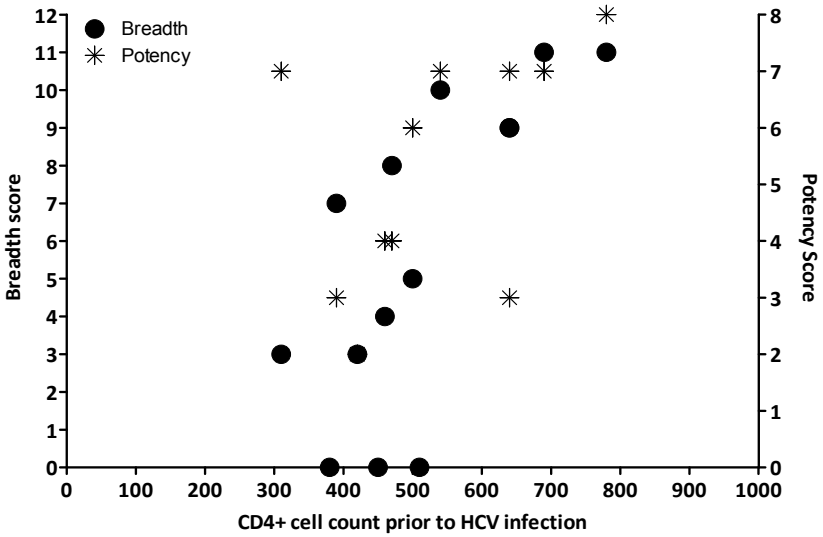

Figure 7. Correlation of CD4+ cell counts prior to HCV acquisition with breadth and potency of nAbs. *The x-axis indicates the CD4+ cell count at last RNA-negative time point prior to HCV acquisition. Maximum breadth during follow-up is depicted on the left y-axis. The right y-axis indicates the maximum potency of nAbs during follow-up.*

Discussion

This study demonstrates that nAb responses against HCV can be detected already early in infection in the majority of HIV infected patients treated for acute HCV infection. However, these antibodies are mainly directed towards the infecting HCV genotype. This observation provides an explanation for the observed reduced risk of reinfection with the genotype causing the primary infection, as reported in chapter 6 of this thesis.

Thus far, the precise role of antibodies in protection against HCV infection remains elusive. A few studies have shown that the presence of a broad nAb response early during acute infection is correlated with spontaneous clearance of HCV infection, which indicates an important role of nAbs in determining outcome of infection [18,19]. However, in a study in chimpanzees it was found that protection from reinfection challenges was independent from nAb responses [46]. Others have suggested that a vigorous CD4+ cell response, rather than nAb response, is the sole determinant of outcome of acute infection

[47]. Nevertheless, a proof-of-principle study using a humanized mice model, recently demonstrated that a cocktail of three broad nAbs protected mice against an infectious challenge and was even able to abrogate infection [48]. Part of the observed discrepancies in nAb studies might be explained by the limited panel of HCVpp used in most studies, and the tendency to generalize neutralization results of the H77 prototype strain. However, as we and others have observed, this prototype strains is rather sensitive to neutralization compared to other HCV-1a variants [49].

Our study is the first to study nAbs during acute HCV infection using a heterogeneous panel of both locally and globally circulating strains of four different genotypes. When comparing nAbs against HCVpp-1 and HCVpp-non1, a clear difference in neutralization sensitivity was observed using sera from acutely HCV-1a infected patients, which suggests the presence of neutralization serotypes. Interestingly, Tarr et al. also observed that binding patterns of polyclonal sera from chronically infected patients, to E1E2 cell lysates of different genotypes also corresponded with the infecting genotype [49]. However, the association between neutralization of a panel of different HCVpp and serum genotype was much less prominent in their study, as sera from genotype 1 infected patients were also able to neutralize genotype 2 and 3 HCVpp. A reason for this apparent discrepancy might be that the sera investigated in in the study of Tarr et al. were from chronically infected patients and that nAbs responses tend to become broader over time during chronic infection [19,50]. Additional evidence for the existence of neutralization serotypes stems from vaccine studies in chimpanzees, mice and humans, where nAb responses against genotype 2 and 3 appear to be reduced compared to responses against genotype 1a when using recombinant genotype 1a derived E1E2 glycoprotein as a vaccine [51–53]. If confirmed, the development of a protective B-cell vaccine generating a broad protective response will remain challenging.

The differential sensitivity to polyclonal sera, even within HCVpp of the same genotype as we observed for genotype 1 in this study, is not surprising given the huge sequence variability of the E1E2 glycoprotein. This underlines the need to use a broad panel of different viral envelopes when studying HCV neutralization. Indeed, two major antigenic clusters were identified based on the neutralization phenotype against the 12 different envelopes. Four of the HCVpp-1 were sensitive to neutralization, including the H77 prototype, whereas three other HCVpp-1 with envelopes of locally circulating strains displayed varying levels of resistance. The most resistant variant, HCVpp-MSM5, phenotypically clustered with non-1 genotypes. The antigenic profile of this variant might be explained by the presence of the D431G mutation in epitope II, which has been shown to convey resistance to CBH-2-like antibodies without compromising viral fitness [25]. This variant also had amino acid changes at positions L438, S501 and K610. All three positions have been associated with escape from nAbs, albeit in the context of different residues. While mutagenesis studies are required to predict the impact of these specific mutations on their sensitivity to neutralization, it is tempting to speculate that the D431G mutation in combination with one or more mutations at positions associated with resistance confers the neutralization resistant phenotype of HCVpp-MSM5 [25,38,43].

In our cohort of acutely infected patients we noted a substantial inter-patient variability in the breadth of response: in three out of the 14 patients no nAbs were detected against any of the HCVpp used in this study. The presence of HIV coinfection might be a plausible explanation for the lack of detectable neutralizing responses in these patients as reduced B-lymphocyte responsiveness to antigenic stimulation is a well-known feature of HIV infection [55,56]. In the three patients without detectable nAb responses, insufficient CD4+ helper cell functionality might explain the functional impairment of B-cells as breadth of response was positively correlated with the number of CD4+ cells. Patients with a higher

CD4+ cell count exhibited broad responses already in the early stage of infection or after a slight delay. In all patients both breadth and potency of response declined once treatment started, coinciding with the decline in viremia. This may suggest that treatment limits further broadening and increases of potency of neutralizing responses, which would imply that starting treatment (too) early during infection might actually be detrimental for the development of broad protective responses and negatively influence the risk of reinfection. Albeit speculative at present, this theoretical possibility, deserves consideration given the implications for future therapeutic strategies. Further comprehensive studies are needed that address whether and to what extent the nAb response induced by the primary infection influences the risk of reinfection with the original infecting and more distant genotypes and whether and to what extent this risk is affected by early treatment. Ultimately, detailed characterization of protective responses elicited by the primary infection represents an important step in rational design of a protective vaccine, the golden bullet towards elimination of HCV.

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Part III

Summary and General Discussion

Chapter 10

Summary

Hepatitis C virus (HCV) is a small (55-56 nm in size), enveloped, positive-sense single-stranded RNA virus that belongs to Hepacivirus genus in the Flaviviridae family. Transmission of HCV mainly occurs by blood-to-blood contact, but sexual transmission has been reported in HIV-infected men who have sex with men (MSM). Worldwide the predominant genotype is HCV genotype 1 followed by HCV genotypes 2 and 3. Because of the mild or asymptomatic nature, acute HCV infections are rarely observed. Mild symptoms are fatigue, nausea, flu-like symptoms, abdominal pain and occasionally jaundice. HCV is often detected in a late stage with high chance of developing chronic inflammation, progressive liver fibrosis, and eventually liver cirrhosis. The majority of patients (~80%) are not able to spontaneously clear their infection and become chronically infected. It has been shown that a strong and sustained CD4+ cell count, CD8+ T-cell response and the early presence of broadly neutralizing antibodies (nAbs) are associated with HCV clearance. Unfortunately, clearance of a primary infection does not lead to sterilizing immunity as in high-risk populations, like HIV-infected MSM and injecting dug users, reinfections with the same or other genotypes have been reported. However, duration and level of viremia following a secondary infection are reduced compared to the primary infection, suggesting that there is a degree of protective immunity present in patients. There is still no vaccine available preventing HCV infections, but current antivirals are able to cure hepatitis C infections for >90% of patients. These impressive results are achieved because of the use of directly-acting antiviral agents (DAAs). However, with current costs of DAAs, treatment cannot be afforded by all patients in need. Therefore, a prophylactic vaccine preventing new infections or a therapeutic vaccine lowering the HCV burden in patients remains needed.

This thesis describes the epidemiological, immunological and virological aspects of hepatitis C infections in both acute and chronically infected patients. Part I describes studies in chronic hepatitis C infected patients and Part II describes studies performed in acute HCV infections in HIV-infected MSM.

Genetic diversity of hepatitis C virus during chronic infection

The addition of NS3/4A protease inhibitors such as telaprevir, has significantly improved SVR-rates when given in combination with pegylated interferon (pegIFN) and ribavirin (RBV), compared with current standard of care in hepatitis C virus genotype 1 infected patients. In patients with a failed sustained response, the emergence of drug-resistant variants during treatment has been reported. It is unclear to what extent these variants are present prior to treatment, are selected or persist in untreated patients. In **chapter 2** we assessed using ultra-deep pyrosequencing, whether after 4 years follow-up, the frequency of resistant variants is increased compared to pre-treatment frequencies following 14 days of telaprevir treatment. In this study resistance associated mutations were detectable at low frequency at baseline. In general, prevalence of resistance mutations at follow-up was not increased compared to baseline. Only one patient had a small, but statistically significant, increase in the number of V36M and T54S variants 4 years after telaprevir-dosing. We concluded that in patients treated for 14 days with telaprevir monotherapy, long-term persistence of resistant variants is rare. In **chapter 3** we studied evolutionary dynamics of NS3 resistant variants, by analysing clonal NS3 protease sequences from thirty-two HCV genotype 1–infected patients following treatment with narlaprevir. Narlaprevir retreatment resulted in a 2.58 and 5.06 log10 IU/mL viral load decline in patients with and without mutations, respectively (P = <0.01). A slower viral load decline was observed in those patients with resistance-associated mutations detectable by direct population sequencing. These mutations became

undetectable within six months following treatment with the exception of the R155K mutation, which persisted in two patients up to 3.1 years after narlaprevir dosing.

There are seven HCV circulating genotypes, >100 subtypes and only a few recombinant variants. Since its initial identification in St. Petersburg, Russia, the recombinant hepatitis C virus (HCV) 2k/1b has been isolated from several countries throughout Eurasia. To our knowledge the 2k/1b strain is the only recombinant HCV to have spread widely, raising questions about the epidemiological background in which it first appeared. In **chapter 4** we investigated the epidemiological background in which the recombinant 2k/1b strain, the only recombinant HCV to have spread widely, first appeared. We estimate that 2k/1b originated sometime between 1923 and 1956, substantially before the first detection of the strain in 1999. The timescale and the geographic spread of 2k/1b suggest that it originated in the former Soviet Union at about the time that the world's first centralized national blood transfusion and storage service was being established. We also reconstructed the epidemic history of 2k/1b using coalescent theory-based methods, matching patterns previously reported for other epidemic HCV subtypes. This study demonstrates the practicality of estimating dates of recombination from flanking regions of the breakpoint and further illustrates that rare genetic-exchange events can be particularly informative about the underlying epidemiological processes.

Acute hepatitis C virus infections in HIV-infected men who have sex with men

Recent data indicate that sexually transmitted HCV infection among MSM was stabilizing in Amsterdam, but little was known about the incidence of HCV reinfection in MSM who had cleared their primary HCV infection. Therefore the incidence of HCV reinfection in HIV-infected MSM who were successfully treated for their acute primary HCV infection was studied in **chapter 5**. In this study we observed an alarmingly high incidence of HCV reinfections and concluded that current prevention measures should be discussed and that frequent HCV RNA testing should be continued after successful treatment. In the case of a suspected relapse, clade typing should be performed to make a distinction between a reinfection and a viral relapse. Next to other factors (like interval of testing) without genetic analysis of the “relapsing” virus as suggested in chapter 5, misclassified relapses might underestimate the true incidence of new HCV infections among HIV-infected MSM. Therefore, in **chapter 6**, the incidence of HCV super- and reinfections in a cohort of acute HCV in HIV-infected MSM was investigated by systematically sequencing the virus present during the entire viremic period. Using a Bayesian coalescent-based framework we observed new infections that would have mistakenly been diagnosed as viral relapse if genetic analysis would have been omitted. We found that for patients with HCV-1a as primary infection, the risk for acquiring another HCV-1a infection was reduced compared to those with a primary non-HCV-1a subsequently acquiring HCV-1a (HR = 0.25, 95% CI 0.07 – 0.93). From this study we concluded that acquiring a second infection with the primary genotype was strikingly reduced compared with acquiring a second infection with another genotype.

Because of its asymptomatic nature, acute HCV infections often go unnoticed and the HCV infection date is therefore usually unknown. In **chapter 7** we investigated if the date of the most recent common ancestor (MRCA) estimated using the Bayesian coalescent-based framework could be used as a proxy for the HCV infection date. Our main findings in this study were that 1) for 58 patients the median difference between the MRCA and the estimated infection date was 113 days and 2) linear regression analysis indicated a good correlation between these two dates. Censoring sequences from the early years of infection resulted in shifts in the MRCA date estimates proportionally to the censored time. From this we concluded that the Bayesian coalescent-based framework is a robust method for analysing

serial sampled sequences and inferring dates of a MRCA. However, provision of reliable estimates of infection dates for seroprevalent cases in the absence of prior knowledge about actual estimated infection dates will remain challenging given the dependence on sequences sampled early during infection. A practical clinical application of this Bayesian coalescent-based framework could be the identification of co-infection or superinfection as a MRCA date substantially older than the known infection date may be caused by infection with multiple strains.

A decline of hepatitis C virus (HCV) antibody titres (anti-HCV), ultimately resulting in seroreversion, has been reported following clearance of viremia in both acute and chronic HCV infection. However, the frequency of seroreversion was still unknown in HIV/HCV-coinfected patients. In **chapter 8** we describe anti-HCV dynamics among HIV-infected MSM following acute HCV infection and reinfection. In this study we found that individuals who cleared HCV RNA (n = 36) showed a significant decrease in anti-HCV levels ($P < 0.001$). Anti-HCV became undetectable during follow-up in 8 out of 31 successfully treated patients. Cumulative incidence of seroreversion within 3 years after seroconversion was 37%. Eighteen subjects became reinfected during follow-up; this coincided with a subsequent increase in anti-HCV reactivity. From these results we concluded that a decline of anti-HCV reactivity was associated with HCV RNA clearance and that seroreversion was very common following SVR. Upon reinfection, anti-HCV levels increased again. Monitoring anti-HCV levels might therefore be an effective alternative for diagnosis of HCV reinfection.

The frequency of observed reinfections in HCV treated HIV-infected MSM suggests that treatment-induced viral clearance does not lead to sterilizing immunity against HCV. Therefore in **chapter 9** we longitudinally investigated presence, breadth, potency and dynamics of HCV-specific neutralizing antibody (nAb) responses in HIV-infected MSM with treatment induced clearance of an acute genotype 1a infection. Breadth of response did not peak during follow-up but was strongly reduced at six months after viral clearance. During follow-up potency of nAbs increased during viremia and peaked at the last RNA positive measurement. Loss of viremia coincided with decrease of potency which was lowest at 6 months after viral clearance. Interestingly, nAbs were more potent against HCV genotype 1 pseudo-particles compared to non genotype 1. In addition, both the breadth and potency of nAb responses directed at HCV in HIV-infected individuals was correlated with the CD4+ T-cell count.

Chapter 11

General Discussion

Most emerging viral infectious diseases such as those caused by influenzavirus [1,2], hantavirus [3,4], nipah virus [5], norovirus [6] and ebola virus [7] all have in common that they are caused by RNA viruses [8]. These viral pathogens have a high degree of genetic diversity as a result of their error-prone RNA polymerases that lack proofreading activity [9]. Some genetic variants will be better adapted to their environment and consequently have a better chance of reproduction [10,11]. This theory is also known as "natural selection" or "survival of the fittest" and is generally regarded as the basis of the existence of all species, including humans and viruses [12]. The adaptive properties of RNA viruses should not be underestimated as new viral variants can emerge, for example from the animal kingdom, and cause epidemics or even pandemics in the human population [1–8].
Hepatitis C virus (HCV) is an RNA virus that was first discovered in 1989 as the causative agent of non-A non-B viral hepatitis [13]. As it also harbours an error-prone RNA polymerase without proof-reading activity [14,15], HCV displays high diversity which is illustrated by the classification into seven major genotypes and many different subtypes [16,17]. Several outbreaks of HCV have been reported in patients at risk such as HIV-infected men who have sex with men (MSM) and patients receiving blood-related products [18–23]. HCV has a major impact on the global health with an estimated 150-170 million individuals currently infected [24,25], a substantial proportion of whom will progress to development of liver cirrhosis or hepatocellular carcinoma [26–28]. There is no evidence for an animal reservoir, nor for the existence of insect vectors of HCV transmission. Humans thus serve as primary reservoirs who directly contribute to the further spread of HCV [29] through behavioural aspects such as injecting drug use or specific sexual practices [30,31] and globalization [32,33].

In this thesis several epidemiological, immunological and virological aspects of HCV in both acute and chronically infected individuals were addressed. Among these are the consequences and challenges for humans as result of the prevalence and viral evolution of HCV, which were mainly discussed in the first part of this thesis. In addition, complex mechanisms that contribute to viral diversity and adaptation, such as the analysis of resistance-associated mutations (chapters 2, 3), immune escape (chapter 9) and genetic recombination (chapter 3), were discussed.

When a novel infectious disease emerges, it is essential to investigate the cause and impact of the problem, to try to eliminate it, for example by specific treatment, and finally to try to prevent it. Thus far we successfully achieved the first two for HCV infections: We know the cause and impact of HCV-infections on human and public health, and our ability to cure individual patients has increased drastically [25,34–37]. However, with regards to prevention and elimination of HCV substantial challenges remain.

Our elimination attempts and its challenges

For the past 10 years, the standard of care for HCV-infected patients has been treatment with pegylated interferon (pegIFN) combined with ribavirin (RBV) [38,39]. The disturbingly low sustained viral response (SVR) rate (~50%), especially if infected with HCV genotypes 1 and 4, was of great concern [40,41]. The infection could not always be cured and significant IFN-related side-effects made it challenging for some patients to complete their treatment regimen [42–46].
Since 2011 the approved standard of care for chronic HCV genotype 1 infection has been pegIFN combined with RBV and a first generation protease inhibitor [47,48]. The concerted action of these drugs was thought to effectively cure the infection by directly inhibiting viral replication and enhancing

the hosts immune system [49,50]. The addition of a first generation protease inhibitor to the standard of care indeed further increased SVR rates, but was mainly effective against HCV genotype 1 [158–161].

A legitimate concern using first generation direct-acting antiviral agents (DAAs) – whether in combination with pegIFN/RBV or not - was the a priori presence or emergence of resistant variants. These variants could be selected during treatment and consequently become the dominant strain resulting in treatment failure [54,55]. In chapters 2 and 3, two studies are described that thoroughly investigate, using sensitive molecular techniques, the presence and persistence of resistance-associated variants in patients treated with first generation protease inhibitors [56,57]. In these studies we confirmed that the concern about resistance development is justified by showing a rapid selection of resistant variants leading to treatment failure in patients. However, we also demonstrated that the wild-type virus slowly regained its dominance within four months after discontinuation of treatment, and that the relative abundance of resistance-associated mutations after four years was comparable to levels prior to treatment. In one patient we found a higher abundance of the V36M resistance mutation after four years of follow-up. However, by using molecular clock analysis we demonstrated that this variant was generated de novo during follow-up rather than reflecting persistence of a resistant variant generated during treatment. Apparently, this resistant variant thus emerged without the selective pressure of treatment. This raises the question to what extent we should be concerned about the pre-existence of resistant variants in patients and how such pre-existence will influence treatment outcome. Most recent studies using highly sensitive sequencing and molecular clonal techniques, as were used in our studies, confirm the presence of pre-existing naturally occurring resistance-associated variants at low frequencies [58–61]. Observed variants often harbour mutations that confer low-level resistance, whereas high-level resistance is rarely observed [61]. This is not unexpected, as most low-level resistant variants have comparable viral fitness to the wild-type virus [54,55]. Studies investigating the pre-treatment viral population in patients have demonstrated that the presence of resistant variants can negatively influence outcome of treatment with protease inhibitors [62–64]. This implies that analysis of the presence of resistant variants prior to treatment can help to predict treatment outcome in patients using first generation protease inhibitors.

Interestingly, in chapter 3, the resistant variant R155K was observed in two patients and was still present up to 3 years after treatment discontinuation. This persistence suggests that viral fitness was unaffected by this mutation, possibly due to the presence of compensatory mutations. However, molecular clock analysis, as was done in chapter 2, is needed to confirm that this variant was selected during treatment and not generated de novo after discontinuation of treatment. Since resistance profiles of protease inhibitors are more or less similar, retreatment of patients with the same class or even with second generation protease inhibitors may have severe limitations. Despite the higher observed SVR-rates, drawbacks of first generation protease inhibitors are the 1) side effects, 2) three times daily dosing and 3) they are genotype specific [65–67].

With new insights and development of novel drugs, resistance will likely become less of a problem. Second generation protease inhibitors with lower chances of developing resistance will be licensed in the (very) near future [54,55,68]. Second generation protease inhibitors have shown to be highly effective in patients who have shown to be difficult to treat before, including patients with cirrhosis and previous non-responders [69]. Combination of these protease inhibitors, together with NS5B polymerase and NS5A inhibitors, have led to impressively successful response rates of almost 100%, even in pegIFN free regimens [70]. In the future, such treatment regimens may likely lead to further improved success rates thereby significantly reducing the disease burden of HCV.

In chapters 5 to 9, acute HCV infections in a group of difficult-to-treat patients, i.e. HIV-infected MSM, were discussed. Previous studies have reported that the SVR-rate in this population is much lower (29%) compared to those that were infected with HCV only (SVR of 50-60%) [40,41,71]. With the current regimens of antivirals against HCV, this should and will no longer be a major problem since SVR-rates of up to 84% can be achieved also in HIV co-infected patients [72]. With the current future perspectives of IFN-free regimens and shorter treatment durations, it should be possible to further reduce the disease burden also for these patients.

In this new era of treatment with DAAs, it might theoretically be possible to completely eliminate HCV within a few years. However, as at least 12 out of 20 countries with the largest burden of HCV-infected individuals are from low- or middle income regions [73], the greatest obstacle in achieving this might well be the high costs of these antivirals. Indeed, the costs of achieving an SVR with a DAA is almost three times as high compared to the previous standard of care (pegIFN+RBV) [74]. In 2013, the addition of a DAA to the standard of care for HCV genotype 1 infections increased the cost of treatment to about $189,000 per SVR. In addition, adverse events were higher which raised the costs even more [75]. Fortunately, the prospects of reducing the manufacturing costs for these treatments seems feasible as they can be lowered to $100-$250 per 12-week treatment course [73]. Combined with the shorter dosing regimen, these cost reductions might make it possible for IFN-free HCV treatment regimens to be available for everyone in need [69,73,74,76]. However, such cost reductions will unfortunately unlikely be achieved in the near future as we have learned from the HIV-field where it has taken almost 20 years to lower the costs of highly active antiretroviral treatment (HAART) regimens from $10,000 to $66 per person per year [73,77,78].

Given the costs of DAA treatment and the likely long term before they will be lowered [73], the availability of a therapeutic vaccine that not necessarily prevents HCV infection but does prevent viral persistence [79] represents an alternative approach. Studies have shown that clearance of infection is strongly associated with an early neutralizing antibody (nAb) response and a strong sustained broad HCV-specific CD4+ T-cell response [80–85]. In chapter 9 we found that the breadth of nAbs in succesfully treated patients was correlated with the amount of CD4+ T-cells prior to HCV acquisition. It has been observed that chronically infected patients develop poorly neutralizing antibodies during the first stage of infection, but develop highly and broadly neutralizing antibodies in later stages of the disease [80]. Next to the continuous viral escape, chronically infected patients show a poor cellular T-cell response [86,87]. Therapeutic vaccines can include immune-stimulatory molecules that enhance or even restore cellular immunity in these patients resulting in eventual viral clearance [88,89].

Can hepatitis C virus infections be prevented?

The first step towards prevention is awareness. Thus far this has proven to be successful in the Netherlands, as we have a substantially lower rate of new HCV infections among IDUs compared with the USA [90,91]. Blood screening and harm reduction strategies such as methadone dose and needle exchange programmes are well-organized in the Netherlands [92,93]. However, these awareness and harm-reduction strategies are not effective for all groups at risk as exemplified by the reported high incidence of HCV reinfections in HIV-infected MSM [94,95].

Obviously, an effective vaccine remains the ideal approach for prevention. However, observations of reinfections in patients indicate the absence of broad sterilizing immunity after viral clearance [94–102].

Nevertheless, there is a window for optimism as there seems to be partial-protective immunity after spontaneous viral clearance [80,103,104]. As mentioned earlier, studies have shown that clearance of infection is strongly associated with an early neutralizing antibody (nAb) response and a strong sustained and broad HCV-specific CD4+ T-cell response [80–85]. This opens the opportunity for at least two prophylactic vaccine approaches based either on inducing nAbs targeting the envelope glycoproteins or inducement of T-cell responses [79,105–107].

In chapter 6, we observed that patients infected with HCV genotype 1a were less likely to be reinfected with the same subtype, suggesting the presence of partial immunity against the primary genotype. This was in concordance with results in chapter 9, in which we investigated the presence, breadth, potency and dynamics of nAbs after treatment induced viral clearance, and found that nAbs present were directed more at the primary genotype. Cross-reactive protective immunity in patients has also been described in previous reports [80,108]. Cross-neutralizing nAbs have even been observed in patients immunized with envelope glycoproteins of HCV genotype 1a, suggesting that a single strain can elicit such antibodies [109]. The relative potency and specificity for different HCV geno(sub)types and even different variants within a single patient remains unclear. However, our results combined with previous reports [80,108,109] suggest that cross-protective nAbs can be developed, but that these will be more potently directed at the primary genotype. This implies that the strain used for a prophylactic vaccine approach based on immune induced nAbs should be carefully considered [110]. In addition, with the observed decline in nAbs at 6 months after treatment induced viral clearance (chapter 9), it remains unclear whether a prophylactic vaccine induces long lasting protection.

A major challenge using an envelope-immunization approach is the genetic diversity of HCV. Given the adaptive properties of HCV, viral escape variants can emerge as result of the selective pressure exerted by nAbs [124,125] or glycan shifting can occur where shifts in glycosylation sites decrease the efficacy of nAbs [113]. In chapter 9 indications for the presence of immune escape was observed. Cluster-analysis using sera from of HCV1a-infected HIV-infected MSM and HCV pseudo-particles (HCVpp) with different HCV envelopes demonstrated two different antigenic clusters: one sensitive and one resistant to neutralization with patient sera. The HCVpp designated to the resistant cluster harboured putative escape mutations which might explain the observed nAbs response in patients.
An alternative prophylactic immunization approach might be the use of non structural proteins of HCV triggering a broad cellular immune response [110]. The major advantage of using this approach is that it will be able to target multiple genotypes as non structural regions are more conserved among genotypes [16,114]. However, as for the humoral antibody response, T-cell escape may also occur [112].
Ultimately, as both T-cells and nAbs are important for viral clearance, the combination of a T-cell targeted approach with an approach that elicits broadly nAbs might be the most promising strategy. In mice, combining HCV non structural proteins and recombinant E1E2 glycoproteins expressed by adenovirus vectors have shown to induce both nAb and T-cell responses [115]. Perhaps the most promising strategy is to target such vaccines at genotypes circulating within a specific population, for example at genotypes 1a and 4d which represent the predominant genotypes in HIV-infected MSM in Amsterdam (chapters 5 and 6).

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Part IV

Appendixes

Nederlandse Samenvatting

Hepatitis C virus (HCV) is een klein (55-56 nm groot) virus met een enkelstrengs RNA genoom van positieve oriëntatie wat omhuld is door een eiwitmantel. Het behoort tot het *Hepacivirus* genus in de *Flaviviridae* familie. Transmissie van HCV vindt plaats voornamelijk door middel van bloed-bloed contact, maar seksuele overdracht is ook beschreven in HIV-geïnfecteerde mannen die seks hebben met mannen (MSM). Wereldwijd is het genotype 1 het meest voorkomend, gevolgd door genotypen 2 en 3. Mede vanwege het asymptomatische karakter, worden acute HCV infecties zelden opgemerkt. Milde symptomen zijn vermoeidheid, misselijkheid, griepachtige symptomen, pijn in de onderbuik en incidenteel geelzucht. HCV infectie wordt vaak in een laat stadium vastgesteld, met een vergrootte kans op chronische ontsteking en progressieve leverfibrose wat uiteindelijk kan leiden tot levercirrose. De meeste patiënten (80%) kunnen hun infectie niet spontaan klaren met een chronische infectie tot gevolg. In verschillende studies is aangetoond dat een sterk en stabiel CD4+ cel aantal, CD8+ T-cel respons en een vroege aanwezigheid van neutraliserende antilichamen geassocieerd zijn met het klaren van HCV. Helaas leidt het klaren van een primaire infectie niet tot steriliserende immuniteit, aangezien er in hoog-risico populaties zoals HIV-geïnfecteerde MSM en intraveneuze drugsgebruikers, re-infecties met dezelfde of andere genotypen zijn beschreven. Daarentegen is de duur en hoogte van de viremie bij een secundaire infectie korter en lager vergeleken met de primaire infectie, wat zou betekenen dat er weldegelijk een mate van beschermende immuniteit aanwezig is in patiënten. Er is nog geen vaccin beschikbaar dat HCV infecties kan voorkomen, maar de huidige antivirale middelen kunnen >90% van hepatitis C infecties genezen. Deze indrukwekkende resultaten worden bereikt door behandeling met middelen die direct aangrijpen op het virus (DAAs). Met de huidige kosten voor DAAs, kan niet iedereen zich de behandeling veroorloven. Om deze reden blijft een profylactische vaccin dat nieuwe infecties voorkomt of een therapeutisch vaccin dat de ziektelast van patiënten vermindert nodig.

Dit proefschrift beschrijft de epidemiologische, immunologische en virologische aspecten van hepatitis C infecties in zowel acuut als chronisch geïnfecteerde patiënten. Deel I beschrijft studies in chronisch hepatitis C geïnfecteerde patiënten en Deel II beschrijft studies in acute HCV –infecties in HIV-geïnfecteerde MSM.

Genetische diversiteit van hepatitis C virus tijdens chronische infectie

NS3/4A protease remmers zoals telaprevir, in combinatie met gepegyleerd interferon (pegIFN) en ribavirine (RBV) hebben voor een significante verhoging van HCV-klaring percentages (SVR) gezorgd in vergelijking met de standaardtherapie in genotype 1 geïnfecteerde patiënten. In patiënten die gefaald hebben op therapie, is het ontstaan en/of opkomen van drug-resistente varianten tijdens therapie beschreven. Het is onduidelijk in hoeverre deze varianten al voor behandeling aanwezig waren en vervolgens uitgeselecteerd worden tijdens behandeling en of persisteren in onbehandelde patiënten. In **hoofdstuk 2** hebben we door middel van ultra-deep pyrosequencing onderzocht of er na vier jaar een verhoogde frequentie van resistente varianten aanwezig was na een 14-daagse behandeling met telaprevir. In deze studie werden de met resistentie geassocieerde mutaties voor behandeling met lage frequenties gedetecteerd. In het algemeen was de prevalentie van resistente mutaties na behandeling niet verhoogd vergeleken met het tijdstip voor de start van de behandeling. Slechts één patiënt had een kleine maar statistisch significante verhoging van de V36M en T54S varianten vier jaar na telaprevir

therapie. Aan de hand hiervan hebben we geconcludeerd dat persistentie van resistente varianten in patiënten die 14 dagen met telaprevir monotherapie behandeld zijn, zeldzaam is.

In **hoofdstuk 3** hebben we de dynamiek van de evolutie van NS3 resistente varianten geanalyseerd door het klonaal analyseren van NS3 protease sequenties van 32 HCV genotype 1 geïnfecteerde patiënten behandeld met narlaprevir. Herbehandeling met narlaprevir zorgde voor een 2.58 en 5.06 log10 IU/ml daling van virale load in patiënten met en zonder mutaties (P=<0.01), respectievelijk. Een vertraagde virale load daling werd gezien in patiënten met resistent-geassocieerde mutaties. Deze mutaties waren niet meer detecteerbaar binnen zes maanden na einde van de behandeling met uitzondering van de R155K mutatie die persisteerde in twee patiënten tot ongeveer drie jaar na narlaprevir therapie.

Er zijn maar liefst zeven HCV genotypen, >100 subtypen. Daarnaast zijn ook intra- en inter-genotypische recombinante virussen beschreven. Sinds de initiële identificatie in St. Peterburg, Rusland, is de recombinant HCV 2k/1b aangetroffen in verschillende landen in Europa en Azië. Voor zover bekend is de 2k/1b variant is de enige HCV recombinant die zich heeft kunnen verspreiden, wat leidt tot de vraag naar de oorsprong van deze recombinant. In **hoofdstuk 4** hebben we de epidemiologische achtergrond en oorsprong van deze 2k/1b recombinant onderzocht. We hebben geschat dat de oorsprong van de 2k/1b variant lag tussen 1923 en 1956, substantieel voor de eerste detectie van de recombinante variant in 1999. De tijdsschaal en geografische verspreiding van de 2k/1b suggereert dat de oorsprong ligt in de voormalige Sovjet Unie, ongeveer toen de eerste nationale bloedtransfusie en opslagdiensten ter wereld opgericht werden. Ook hebben we de epidemiologische geschiedenis van de 2k/1b gereconstrueerd door gebruik te maken van “coalescent theory”-gebaseerde methoden, in overeenstemming met eerder beschreven modellen voor andere epidemiologische HCV subtypen.

Acute hepatitis C virus infecties in HIV-geïnfecteerde mannen die seks hebben met mannen

Recente data suggereert dat het aantal nieuwe infectie van seksueel overgedragen HCV in MSM in Amsterdam zich stabiliseert, maar er is weinig bekend over de incidentie van re-infecties in MSM die een eerdere HCV infectie geklaard hebben. Daarom hebben we in **hoofdstuk 5** de incidentie van HCV re-infecties in, voor HCV, succesvol behandelde HCV/HIV-co geïnfecteerde MSM bestudeerd. In deze studie observeerden we een alarmerend hoge incidentie van HCV re-infecties en concludeerden dat huidige preventiemaatregelen opnieuw bekeken moeten worden en dat na succesvolle behandeling nog regelmatig HCV RNA testen moet worden gedaan. In geval van verdenking op een virale relapse, moet er typering gedaan worden om het verschil te kunnen zien tussen re-infectie en virale relapse. Zonder de genetische analyse van het recidiverende virus (en andere factoren zoals het interval tussen testen), kunnen re-infecties gemisclassificeerd worden als een virale relapse met als gevolg een onderschatting van de echte incidentie van nieuwe HCV infectie in HIV-geïnfecteerde MSM.

In **hoofdstuk 6** hebben we de incidentie van HCV super- en re-infecties onderzocht in een cohort van acute HCV in HIV-geïnfecteerde MSM door het systematisch sequensen van virus gedurende de viremische periode. Door gebruik te maken van een Bayesian coalescent framework, konden we de intrahost evolutie snelheid berekenen en konden we nieuwe infecties detecteren door te laten zien dat de verandering in het genoom over de tijd te groot was om verklaard te worden door intrahost evolutie. Deze nieuwe infecties zouden zonder deze genetische analyse als virale relapse gediagnostiseerd zijn. In deze studie observeerden we dat patiënten met een primaire HCV-1a infectie een gereduceerd risico hadden voor het oplopen van een tweede infectie met HCV-1a.

In **hoofdstuk 7** hebben we onderzocht of we de datum van de meest recente voorouder (TMRCA) konden schatten door gebruik te maken van een Bayesian-coalescent model en deze te gebruiken als het geschatte moment van infectie. De voornaamste bevindingen in deze studie waren dat 1) voor 58 patiënten het mediane verschil tussen TMRCA en geschatte moment van infectie 113 dagen was en 2) lineaire regressie analyse een goede correlatie tussen deze twee data gaf. Het censureren van sequenties aan begin van de infectie zorgde voor een proportionele verschuiving van TMRCA richting het heden. Aan de hand hiervan concludeerden we dat het Bayesian coalescent-based framework een robuuste methode is voor het analyseren van longitudinale sequenties en het schatten van TMRCA. Daarentegen, het betrouwbaar schatten van infectie-datum voor sero-prevalente gevallen, is in de afwezigheid van samples afgenomen vroeg na het oplopen van de infectie lastig. Een praktische applicatie van deze methode kan de identificatie van co-infectie of superinfecties zijn.

Een daling in HCV antilichaam titers (anti-HCV), resulterend in seroreversie, is eerder beschreven na het klaren van hepatitis C infectie in zowel acute als chronische infecties. Desondanks is de frequentie van seroreversie in HIV/HCV geco-infecteerde patiënten onbekend. In **hoofdstuk 8** beschrijven we de anti-HCV dynamiek in HIV-geïnfecteerde MSM na acute HCV infectie en re-infectie. In deze studie observeerden we in individuen die HCV RNA geklaard hadden (n=36) een significante daling in anti-HCV titers (P=<0.001). Anti-HCV werd ondetecteerbaar in acht van de 31 succesvol behandelde patiënten. De cumulatieve incidentie van seroreversie binnen drie jaar na seroreversie was 37%. Achttien deelnemers werden gere-infecteerd tijdens de nabehandelingsperiode, wat gelijk op ging met een verlaging in anti-HCV titers. Aan de hand van deze resultaten concludeerden we dat een daling in het anti-HCV signaal geassocieerd was met het klaren van HCV RNA en dat seroreversie normaal was na een SVR. Bij het optreden van een re-infectie, stegen anti-HCV titers weer. Daarom kan het monitoren van anti-HCV titers een effectieve en alternatieve manier zijn voor het vinden van HCV re-infecties.

De frequentie van geobserveerde re-infecties in voor HCV behandelde HIV-geïnfecteerde MSM suggereert dat behandeling-geïnduceerde klaring niet leidt tot steriliserende immuniteit tegen HCV. Daarom hebben we in **hoofdstuk 9** longitudinaal de aanwezigheid, breedte, potentie en dynamiek van HCV-specifieke neutraliserende antilichaam responsen in HIV-geïnfecteerde MSM met behandeling-geïnduceerde klaring van acute genotype 1a infecties onderzocht.

De dynamiek van de neutralisatie respons verschilde per individu, waarbij maximale breedte van de response niet bij iedereen op hetzelfde tijdstip viel. Echter, in alle patiënten was de respons zes maanden na klaren sterk gereduceerd.. Tijdens viremie nam de potentie van neutraliserende respons toe, en bereikte een hoogtepunt tijdens de laatste RNA positieve meting. Het klaren van viremie ging gepaard met een afname van de potentie en was het laagst 6 maanden na klaren. De meest intrigerende bevinding was dat neutraliserende antilichamen potenter waren tegen HCV genotype 1 pseudo-partikels dan tegen non-genotype 1, in deze genotype 1 geïnfecteerde patiënten. Bovendien was zowel de breedte als potentie van de neutralisatie respons in gecorreleerd met CD4+ T-cel aantallen in deze HIV-geïnfecteerde individuen.

About the Author

Xiomara Varissa Thomas was born on the 15th of February 1986 in Paramaribo, the Republic of Suriname. When she was about three years old, she and her mother moved to Curaçao, a small island in the Caribbean with no more than 150,000 inhabitants. There she attended the Johan van Walbeeck primary school, and later on the Roman Catholic Radulphus College, a secondary school (H.A.V.O) from which she graduated in 2003. Thereafter, she moved to the Netherlands to study Life Sciences at the Hogeschool Utrecht, with majors in Medical Microbiology. Her primary interest in scientific research was awakened in 2006, during her internship at the Academic Medical Centre (AMC) in Amsterdam, were she studied human parechoviruses in stool samples of small children under the supervision of dr. Kimberley Benschop and dr. Katja Wolthers. As part of her study, she did her second internship in a medical bacteriologic laboratory at the Centraal Bacteriologisch Serologisch Laboratorium (CBSL) at Tergooiziekenhuizen in Hilversum. Soon after her graduation in 2008, she was offered a job at the AMC to work on hepatitis C virus. Given her interest in science, she did not hesitate to take the offer and worked as a research-technician on hepatitis C virus, investigating viral resistance to new upcoming antivirals for 1,5 years. In 2009 she was given the opportunity to start her PhD -training under the supervision of prof. dr. Menno de Jong (promotor), dr. Richard Molenkamp and dr. Janke Schinkel (co-promotors). After finishing her four years of PhD-training, studying aspects of hepatitis C virus in acute and chronically infected patients (this thesis), she worked again for dr. Katja Wolthers on rhinoviruses.

List of publications

1. Benschop K, Thomas X, Serpenti C, Molenkamp R, Wolthers K. High prevalence of human Parechovirus (HPeV) genotypes in the Amsterdam region and identification of specific HPeV variants by direct genotyping of stool samples. J Clin Microbiol 2008;46:3965-3970.

2. Lambers FA, Prins M, Thomas X, Molenkamp R, Kwa D, Brinkman K, van der Meer JT, Schinkel J. Alarming incidence of hepatitis C virus re-infection after treatment of sexually acquired acute hepatitis C virus infection in HIV-infected MSM. AIDS 2011;25:F21-F27.

3. Markov PV, van de Laar TJ, Thomas XV, Aronson SJ, Weegink CJ, van den Berk GE, Prins M, Pybus OG, Schinkel J. Colonial history and contemporary transmission shape the genetic diversity of hepatitis C virus genotype 2 in amsterdam. J Virol 2012;86:7677-7687.

4. Raghwani J, Thomas XV, Koekkoek SM, Schinkel J, Molenkamp R, van de Laar TJ, Takebe Y, Tanaka Y, Mizokami M, Rambaut A, Pybus OG. Origin and evolution of the unique hepatitis C virus circulating recombinant form 2k/1b. J Virol 2012;86:2212-2220.

5. Thomas XV, de Bruijne J, Sullivan JC, Kieffer TL, Ho CK, Rebers SP, de Vries M, Reesink HW, Weegink CJ, Molenkamp R, Schinkel J. Evaluation of persistence of resistant variants with ultra-deep pyrosequencing in chronic hepatitis C patients treated with telaprevir. PLoS One 2012;7:e41191.

6. de Bruijne J, Thomas XV, Rebers SP, Weegink CJ, Treitel MA, Hughes E, Bergmann JF, Knegt RJ, Janssen HLA, Reesink HW, Molenkamp R, Schinkel J. Evolutionary dynamics of hepatitis C virus NS3 protease domain during and following treatment with narlaprevir, a potent NS3 protease inhibitor. JVH 2013.

7. Grady BP, Schinkel J, Thomas XV, Dalgard O. Hepatitis C virus reinfection following treatment among people who use drugs. Clin Infect Dis. 2013 Aug;57 Suppl 2:S105-10.

8. Vanhommerig JW, Thomas XV, van der Meer JT, Geskus RB, Bruisten SM, Molenkamp R, Prins M, Schinkel. Hepatitis C Virus (HCV) Antibody Dynamics following Acute HCV Infection and Reinfection among HIV-Infected Men who have Sex with Men. Clin Infect Dis. 2014 Sep 3. pii: ciu695.

AMC Graduate School for Medical Sciences PhD Portfolio

PhD period: September 2009-September 2013
Name PhD supervisors: Dr. Janke Schinkel, dr. Richard Molenkamp and prof. dr. Menno de Jong

1. PhD training

	Year	Workload (Hours/ECTS)
General courses		
The AMC world of science	2009	0,7
Scientific writing in Englich for Publication	2010	1,5
Practical Biostatistics	2010	1,1
Web of science	2010	0,7
Infectious diseases	2010	1,3
Evidence based searching	2010	0,1
Project management	2010	0,6
Career development	2011	0,6
Specific courses		
International bioinformatics workshop on virus evolution and molecular epidemiology	2011	2,4
Seminars, workshops and master classes		
Weekly department seminars	2009-2013	4
Poster presentations		
Analysis of resistance-Associated mutations in patients treated with narlaprevir and subsequently standard of care, presented at AASLD 2010	2010	0,75
Hepatitis C virus reinfection in HIV-positive MSMs, presented at CROI 2011	2011	0,75
Long-term follow-up of patients with chronic hepatitis C treated with telaprevir: Evaluation of persistence of resistant variants by ultra-deep sequencing, presented at EASL 2011	2011	0,75
HCV reinfection in HIV-infected MSM: Evidence for partial immunity, presented at 19th international symposium on hepatitis C viruses and related viruses 2012	2012	0,75
HCV reinfection in HIV-infected MSM: Evidence for partial immunity, presented at NCHIV 2012	2012	0,75
Oral presentations		
Analysis of Resistance-Associated Mutations in Patients treated with Narlaprevir and subsequently Standard of Care, presented at: the Oxford Virology Symposium 2010	2010	0,75
Analysis of Resistance-Associated Mutations During a Phase I Trial with a Potent New Hepatitis C Virus NS3 Protease Inhibitor, presented at: Scientific Spring Meeting 2010	2010	0,75

Analysis of Resistance-Associated Mutations in Patients treated with Narlaprevir and subsequently Standard of Care, presented at : 1st Antiviral Congress 2011	2011	0,75
Evaluation of persistence of resistant variants with ultra-deep pyrosequencing in chronic hepatitis C patients treated with telaprevir, Presented at Scientific Spring Meeting KNVM & NVMM 2012	2012	0,75

2. Teaching

	Year	Workload (Hours/ECTS)
Supervising		
Jeanette Roozendaal, HCV in HIV-infected MSM	2010	1
Mehmet Yilmaz, Molecular evolution of HCV in narlaprevir treated patients	2011	1

3. Parameters of Esteem

	Year
Grants	
Travel grant AASLD 2010	2010
Travel grant EASL 2011	2011

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