Diagnosis and Treatment of Acute Hepatitis C Among HIV Positive Men Having Sex with Men

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INTRODUCTION

The hepatitis C virus (HCV) is a single stranded RNA virus that is able to infect humans and chimpanzees. HCV is mainly transmitted by blood-blood contact and thereafter infects hepatocytes of the liver. When HCV has reached the liver, the immune system reacts on the presence of the virus, which results in inflammation. This immunological and inflammatory reaction can result in spontaneous clearance or can be insufficient leading to chronic persistence of the virus.

The worldwide epidemic of HCV probably originated by the use of non-sterile medical equipment and blood transfusions between 1940 and 1960(1). The evolution of the virus in humans before that period is largely unknown(2). Between 1970 and 1989 the disease was identified as non-A, non-B hepatitis and thereafter named HCV(3, 4). Currently, HCV is a widespread infectious disease throughout the world, with about 170 million chronic carriers being theoretically able to transmit the virus(5).

HCV is a disease with potential severe complications like cirrhosis, hepatocellular carcinoma and hepatic decompensation(6). Besides liver related complications, other effects on different organs have been related to the presence of HCV like diabetes, cryoglobulinemia and kidney disease(7). However, most patients do not develop these complications and many are not aware that they are infected(8).

A recently acquired HCV infection is mostly asymptomatic, or at least stays undetected, and leads to chronicity in 74% (95% CI 72% - 79%) of reported cases(9). Therefore, patients are mostly diagnosed in the chronic phase during general medical screening or when liver related complications occur. This results in a paucity of studies evaluating viral and immunologic characteristics of the first stage of disease, also called acute HCV. Furthermore, due to suboptimal study design (often retrospective cohorts) and small numbers of enrolled patients per study, many issues on acute HCV remain pending. The aim of the work presented in this thesis was to acquire further insight into the diagnostic methods and treatment in HIV infected patients with an acute HCV co-infection.

Epidemiology

Three major populations at risk for new HCV infections can be identified, each with differing modes of transmission, occurring with different frequencies in different

parts of the world: injecting drug users, patients at risk of nosocomial infection and HIV positive men having sex with men (MSM)(5).

Sharing of injecting equipment among drug users is a major risk factor for acute HCV and IDU accounts for most acute HCV infections worldwide. Currently, it is estimated that there are 0.75-1.0 million active IDU in Europe alone(10). Acute HCV incidence within this group varies between 2.7 and 66 per 100 person years of follow up (PYFU) (11). This results in an anti-HCV prevalence of above 50% in Europe and around 67% worldwide(12, 13). A remarkable example of the ongoing epidemic in IDU outside Europe is within young adult IDU in the United States with reported incidences as high as 27 per 100 PYFU(14, 15). During the last decade this epidemic has expanded to rural areas and has been associated with increased prescription opiate use(16, 17). In the IDU population a variety of interventions have been implemented that resulted in a reduced acute HCV incidence. Most implemented interventions are opiate substitution and needle exchange programs of which high coverage of combined programs is associated with a reduction in acute HCV incidence(18, 19). However, HCV incidence within IDU does not seem to decline to the very low levels that have been observed for HIV(20). This difference might be attributable to the virological characteristics of the virus, as HCV is about 10 times more likely to be transmitted through a needle puncture than HIV(21). Furthermore, HCV transmission might also occur through sharing of drug use equipment other than needles such as tattooing or using snorting straws.

Within health care systems of developed countries HCV transmission occurs infrequently. However, small transmission clusters are described occasionally. These outbreaks occur because of inadequate sterilization of medical devices or are driven by infected health care practitioners who spread the virus. For example, according to the national surveillance system in France, invasive medical procedures account for up to 25% of all acute HCV infections diagnosed(22). Additionally, in a recent review, 13 outbreaks were described between 1995 and 2012 in Europe and the North America in which health care practitioners transmitted HCV to their patients(23). Accordingly, individual HCV transmission from patient to clinician was still reported in the Netherlands in 2013 and 2014(24). So, transmission of HCV still occurs in developed countries but not structurally.

In contrast, high rates of acute HCV infections within health care systems occur in developing countries. There are several developing countries that do not screen blood donors for HCV because of limited healthcare budget, leading to transmission of HCV. The most dramatic example is Egypt, in which the iatrogenic transmission of HCV during the era of parenteral anti-schistosomal therapy mass-treatment between 1960 and 1980 led to a nationwide epidemic. As a result, 10-20% of the total Egyptian population is currently infected, most of them with genotype 4. Inadequate sterilization of healthcare equipment and the high prevalence in the general population account for an ongoing spread of HCV in Egypt(25) resulting in an annual number of new HCV infections is estimated to be around 150,000(26). Intra-familial transmission (3%) and heterosexual transmission (0.07%) seem to be of limited importance(25, 27).

The third risk group for HCV infection is MSM in whom HCV is mainly a sexually transmitted disease. More specifically and for partially unknown reasons, this acute HCV epidemic has been limited largely to HIV positive MSM(28). In Europe, during the past decade incidence was rising from 0.08/100 PYFU up to 1.75/100 PYFU(29-33). Currently, the epidemic appears to be leveling off in the Netherlands(34). Outside Europe (the USA and Japan), HCV infections are still on the rise, with recent reported incidences between 0.21/100 PYFU and 2.49/100 PFYU(35, 36). The acute HCV reinfection rate is even higher with reported rates of 7.8 and 15.2 per 100 PYFU(37, 38). A supposed reason for this epidemic is the emergence of national and international networks of HIV positive men that serosort on HIV positivity and have unprotected sex(39). The occurrence of such networks may be explained by the successful HIV treatment, widespread internet use and low-budget travel possibilities(30, 40). Determinants for HCV transmission in this risk group are recreational drug use, injecting drugs, sharing of snorting straws, receptive fisting, receptive unprotected anal intercourse, ulcerative sexually transmitted infections such as syphilis, group sex and rectal trauma with bleeding(32, 41-44). There are also a number of potential mechanisms related to HIV that might result in enhanced infectivity and susceptibility to HCV, including increase HCV loads in serum and semen as well as defects in the gastrointestinal immune system(45).

Despite adequate surveillance systems, there is still a significant fraction (up to 45%) of acute HCV infections for which the mode of transmission cannot be identified.

Suggested explanations for these unknown transmissions include denial of risk factors, transmission by acupuncture, tattooing, piercing or shaving by barbers(25, 46).

Diagnosis

Case definitions of acute HCV infection differ widely between epidemiologic, immunologic and intervention studies. Also, within these subgroups a wide range of criteria is used(47).

Detection of seroconversion remains the most important diagnostic method of acute HCV infections(48). Detection of seroconversion and demonstration of genoconversion by nuclear acid amplification assays, relies on the availability of a recent pre-diagnostic sample. Although sample storage is common practice within the HIV co-infected patients in tertiary-care centers in Europe, this is rarely done in other risk populations, who do not regularly visit outpatient clinics. Therefore, most acute HCV infections will remain undiagnosed. Alternatively, the combination of HCV antibody negativity and positive HCV RNA at a single time point may be indicative of an acute infection. However, in immunocompromised patients seroconversion takes time and remains absent in 2%-5% at one year after infection, making this combination less useful in HIV positive patients(49, 50). If a patient is already anti-HCV and HCV RNA positive at the time of first sampling, IgG avidity testing may give an estimation of infection duration(51). In case of reinfection, HCV genome amplification is the gold standard. Additionally, HCV antigen, IgG avidity and IgG titer measurements are promising alternatives(50, 52).

Although elevation of alanine transaminase (ALT) is still used in several definitions of acute HCV, cut-off points differ widely across studies from any value above the upper limit of normal to 20 times the upper limit of normal(47). Interestingly, the presence of normal ALT values in the acute phase of infection has been reported, in association with fluctuating viremia(53). Thus, excluding a diagnosis of acute HCV infection in a high-risk individual by relying on a normal ALT may not be appropriate.

Virology

Within the acute phase of a HCV infection, three patterns of HCV RNA kinetics can be identified. The first pattern is seen in spontaneous clearance, which occurs probably more frequently in HCV mono-infected than in HIV co-infected patients(9). This means that there is an initial rise in HCV RNA followed by a steady decline to undetectable

levels of HCV RNA. Clinical observations show that this generally occurs within the first year after transmission, but may take up to two years (54). However, spontaneous clearance can occasionally also be observed in the chronic stage of disease when a patient's immunity is restored or boosted by an infection with another hepatitis virus or when combined antiretroviral (cART) therapy is started(55-57). Second, a pattern of fluctuating viremia can be observed during an acute HCV infection. This pattern is defined by rapidly changing viral loads that can differ with a factor 10 to 100 with a few weeks(53, 58). This is an interesting virological pattern because currently we do not know whether it is preferable to observe the outcome during this phase or to start peginterferon (pegIFN) based treatment as early as possible to maximize treatment efficacy. The last pattern of HCV RNA kinetics during an acute HCV can be described as an early chronic pattern. In these patients a stable high viral load is seen. This may be observed because the fluctuating stage of disease is very short and therefore often missed, or because the immune system becomes exhausted shortly after infection(53).

Predicting clearance

The process leading to either spontaneous clearance or chronicity is dependent on specific viral and host characteristics. These determinants are heavily debated because they are based on studies in small numbers of patients or in different acute HCV cohorts: IDU, HIV co-infected, nosocomial infected or combined. In a recent multicenter study, patients with a genotype 1 infection had an adjusted hazards ratio (AHR) of 1.56 compared to other genotypes in favor of clearance(54). The chances for spontaneous clearance can also be partly explained the diversity in quasispecies which can be a result of host, viral and external factors. Increased quasispecies heterogeneity equates with longer estimated duration of infection and higher serum HCV RNA levels. The heterogeneity in quasispecies can be partially explained by the route of transmission (IDU versus sexual)(59), immune exhaustion and the imperfect function of the viral RNA polymerase combined with a production of 10^12 virions a day(60).

Important host protective factors that have been associated with spontaneous clearance are: nonblack race (Odds Ratio(OR) 5.15), female gender (AHR 2.16) (with a protective effect of oestrogen) and host interferon lambda gene 28B (IL28) C/C genotype (AHR 2.26)(54, 61, 62). Differences in host IL28 polymorphisms seem to constitute one of the few genetic predictors of viral clearance, with CC being the most favorable

genotype, followed by CT and TT(63). The closely linked rs12979860 and rs8099917 subtypes are associated with clearance, treatment effect and progression to cirrhosis in chronic HCV. For example within a study among 190 women, spontaneous clearance was more common in patients with the C/C genotype (43/67; 64%) compared with C/T (22/90; 24%) or T/T (2/33; 6%) (P < .001)(64). The presence of hepatitis B antigens seems to be protective for chronicity (OR 2.91)(65). In contrast, infection with HIV is associated with progression to chronicity (OR 2.19)(61). However, when the immune status of a patient can be restored by combined antiretroviral therapy, the chance of spontaneous clearance seems to increase(66).

In the past, several models have been developed to prevent unnecessary peginterferon-based treatment for patients who would have cleared HCV spontaneously(54, 67-70). These prediction algorithms rely on pathophysiological causative mechanisms but also on clinical or biochemical symptoms like jaundice, bilirubin or the IP-10 level, which may be a reflection of well-functioning immune system. These algorithms reliably predict spontaneous cure rates but have not been prospectively validated.

Immunology

Cellular immune response

Mounting an effective T cell response is a key immunological element of spontaneous viral clearance(71). Specifically for acute HCV mono-infections, several studies have demonstrated that a broad and vigorous HCV-specific CD4+ and CD8+ T cell response is associated with clearance of the infection(72-75). Similar observations linking T cell responses to spontaneous viral clearance have been made in HIV-infected patients with an acute HCV infection. However, comparisons between acute HCV mono-infected and HIV/ acute HCV co-infected patients have revealed impaired responses in the latter group, which may account for the observed reduced clearance rates in this patient group(75, 76).

The innate immune response also plays a role in the acute stage of the disease though its exact contribution is unclear. HCV RNA is sensed through either the helicase RIG-I, which forms a complex, or Toll-like receptors, ultimately leading to production of interferon alfa (IFN-alfa) and upregulation of interferon-stimulating genes (ISG)(77, 78). Several HCV proteins have been shown to interact with this intracellular signal-

ing cascade, e.g. HCV protease, which cleaves the mitochondrial antiviral-signaling protein downstream of RIG-1(78, 79). Differences in production of IFN-alfa, both in plasma and in the liver, have been attributed to the observed differences in spontaneous viral clearance rates between acute HCV mono-infected and HIV/ acute HCV co-infected patients(80, 81).

Humoral immune response

The role that antibodies play in the spontaneous clearance of acute HCV is less well understood(82). Early in vitro studies showed that highly specific neutralizing antibodies (NAs) against HCV envelope proteins E1 and E2 were frequently present, with the hypervariable region 1 (HVR1) in the E2 being an important target for NAs. However, technical limitations were encountered, e.g. use of pseudoparticle cell systems enabling only a limited number of viral genotypes together with the use of the general reference strain H77c. Despite the development of JFH-1 cell culture system, which accelerated NA research, how HCV evades neutralization still requires further elucidation. Partly, this can be explained by cell-to-cell spread via tight junctions(83), partly because of the high specificity of these antibodies for variants that can subsequently mutate under selection pressure. Despite evasion of the immune system, there is clinical evidence for a significant role of NAs in protection against HCV. For example, from early hepatitis B immunoglobulin vaccination studies in liver transplant patients it became apparent that lower HCV recurrence rates were seen in those patients receiving hepatitis B immunoglobulin(84). Furthermore, in acute HCV infected patients, high NA titers corresponded with spontaneous viral clearance(85). Similar to the pattern of T cell responses, broadly NAs are associated with resolving infections(85). Such NAs may recognize conformational epitopes most often around the CD81 binding site, one of the co-receptors for HCV cell entry(86). Additionally, although it is a rare occurrence, NA binding to linear epitopes has been described(87), leading to the discovery of monoclonal antibodies such as AP33, with potent neutralization capacity(88). Taken together, the cellular and humoral immune system respond to the presence of a HCV infection. In most of the cases these responses are inadequate because they are interrupted by HCV itself or evaded by the high mutation frequency of HCV.

Vaccination

One question in the era of direct acting antivirals (DAAs) is whether we still need a vaccine. It could be reasonably argued that, on a population level, vaccination is the

most effective way of eradicating HCV. Moreover, on an individual level, adequate treatment gives no protection against reinfection(38). However, the correlates of immunity for HCV are still largely unknown hampering vaccine development(89). Based on the chimpanzee HCV model a limited number of vaccine candidates have advanced into human clinical testing with varying degrees of success(90). Different approaches have been tried; vaccines based on recombinant proteins or peptides, DNA, and viral vectors. Combining two of these, e.g. a viral vector with a protein, seems attractive, potentially providing both humoral and cellular immunity(91). For example, the combination of an adenoviral vector with a recombinant HCV E1E2 protein was demonstrated to elicit a strong antibody and T cell response in mice and guinea pigs(92). A recent phase 1 human study showed that very high levels of CD4+ and CD8+ T cells can be induced using viral vectors based on rare adenovirus serotypes: currently, a phase 2 study using such vectors is underway to investigate the efficacy of this prime-boost T cell vaccine approach in an injecting drug using community in Baltimore(93).

Treatment

Until recently, acute and chronic HCV infections were treated with subcutaneous IFN-alfa and oral ribavirin. The pegylated form of interferon is preferably used because it can be administered once a week subcutaneously. Interferon is a signal protein that can also be produced by many human cells in reaction to many different pathogens, in particular after a viral infection. Interferon has a broad range of actions, which result in general activation of the immune system. When combined with ribavirin, a broad spectrum antiviral drug with a not-well understood mode of action, interferon is a moderately effective treatment for chronic HCV and is a very effective treatment for acute HCV(62). However, the use of pegylated-interferon (pegIFN) and ribavirin is associated with several substantial side-effects like influenza-like symptoms, cytopenia and depression.

Optimal timing of acute HCV therapy

In clinical practice, the optimal timing of the treatment of an acute hepatitis C infection is controversial. It remains unclear until what time point after infection the chances of spontaneous clearance continue to outweigh the high cure rates of pegIFN based treatment for an acute HCV infection. A model to predict efficacy of treatment in comparison with the chance of spontaneous clearance proposes to treat within the first 2 months or after 4 months if the moment of transmission is

known(68). However, this model assumes a reliable transmission date, which is often not available. As a result, most physicians refrain from applying treatment during at least the first month after diagnosis to await spontaneous clearance. This is in line with the current European AIDS treatment network (NEAT) and European Aids Clinical Society (EACS) guidelines recommending a 4-week period to observe a potential HCV RNA decline of at least 2-log, after which the chance of spontaneous clearance becomes substantially higher(94-96). Without this 2-log decline, treatment can be initiated. However, observations from a large international study of 632 participants with acute HCV mono-infection did not support this approach. Among patients with spontaneous clearance, 33% became HCV RNA negative later than 6 months after the estimated date of infection(54). These data suggest that refraining from therapy for a longer time also has clear advantages. As a result, current EASL and AASLD guidelines are inconclusive about the observation period(48, 97). In 130 mono-infected patients who were treated after a 12 week delay, similar sustained viral response (SVR) rates were found as in studies using a 4 week observation period (98). In contrast, data from the German HepNET III study on acute HCV mono-infection did not support a 12-week delay in treatment after diagnosis, as this was associated with a lower SVR rate than immediate treatment (SVR, 54% versus 67%)(99). Since the difference in SVR rates was largely driven by the fact that more patients in the delayed treatment group were lost to follow up, the authors concluded, however, that delayed treatment could be as effective when protocol and treatment adherence can be ensured. Nowadays, in the DAA era with cure rates of 90% and higher, timing of treatment initiation for acute HCV infections remains debatable.

Rationale for treatment

Patients with acute HCV have historically been treated with shorter regimens, yielding substantially higher SVR rates, than those longer regimens used in patients with chronic infection. At the moment, it is still unclear why there is an increased response to interferon during acute infections. It is hypothesized that the early disappearance of HCV antigens prevents exhaustion of the immune system(100). An alternate explanation could be that early treatment prevents the development of stable quasispecies strains that are able to evade the immune response.

With new DAAs being licensed, the treatment of chronic HCV infection has become safe and very effective. Therefore, early treatment may have no advantage with respect to SVR rates. As a result, current AASLD guidelines advocate waiting until

infection becomes chronic and selecting treatment regimens without pegIFN(97). However, there are several reasons to treat in the acute phase, e.g. to reduce viral transmission or to treat symptomatic patients. In many countries, the most important reason for treating with pegIFN-based regimens during acute infection is the lack of insurance coverage of new DAAs in patients without an urgent medical need for HCV therapy. This is certainly not restricted to resource-limited settings. In several European countries, DAAs can only be used in patients with advanced fibrosis or cirrhosis. Even when DAAs can be used regardless of fibrosis grade, DAAs are not EMA nor FDA registered for the treatment of acute HCV, as their efficacy and safety in patients with acute HCV has not yet been studied. Therefore, despite its side effects, pegIFN-based treatment for acute HCV will remain the only available option in many countries for several years to come.

Treatment regimen for acute HCV infection

Currently, pegIFN-alfa as monotherapy, or in combination with ribavirin (in patients with HIV co-infection), is still the only treatment that has been adequately studied in patients with acute HCV infection and is approved for this indication. In acute HCV mono-infected patients, cure rates between 71% and 94% have been reported with pegIFN-alfa monotherapy(101, 102). As the therapeutic mechanism for pegIFN-alfa and standard IFN-alfa is similar, starting the treatment with high-dose standard interferon-alfa 2a or 2b is common in resource limited setting in which pegIFN-alfa is not available or more costly.

The wide variety of cure rates within the HIV co-infected population makes it difficult to interpret the effects of different treatments. Small, mostly underpowered, cohort studies with a variable genotype distribution have shown an average cure rate of 61%(standard deviation of 17)(95). Although its role remains controversial, ribavirin is often added to pegIFN-alfa in HIV co-infection resulting in higher SVR rates in general. Most physicians start with pegIFN-alfa and ribavirin, with tapering or discontinuation of ribavirin if adverse events occur(48, 95). However mono-therapy could be suitable in a subset of co-infected patients treated within the first weeks after diagnosis of acute HCV infection(103).

Duration of treatment

In most clinical studies and treatment guidelines, the duration of pegIFN-alfa therapy in HCV mono-infected patients has been 24 weeks. However, 12 weeks of therapy

has also been successfully used in observational studies with cure rates of 72% and 74%(104, 105). In a recent large randomized controlled trial no difference in response rates between 24 weeks of pegIFN-alfa and 12 weeks of pegIFN-alfa (with or without ribavirin) was observed(98). As this study concluded that the only predictor of SVR was a rapid viral response at week 4, it is remarkable that this decision rule is not integrated in current EASL guidance(48).

For HIV co-infected patients, the 2015 EACS acute HCV guideline suggests to base treatment duration on the week 4 HCV RNA load. If HCV RNA is undetectable at week 4, treatment with pegIFN and ribavirin until week 24 is recommended and if not, treatment duration has to be expanded to week 48 (96).

DAAs in the treatment of acute HCV

Several new classes of DAAs have shown cure rates above 90% in patients with chronic HCV, for both HIV negative and HIV positive patients (106-108). These DAAs inhibit three separate parts of the HCV replication cycle instead of boosting the host immune system with pegIFN-alfa and ribavirin. First, NS3/4a inhibitors, inhibit the viral protease which is responsible for cleavage of the HCV polyprotein into four different proteins. Second, NS5a inhibitors inhibit the broad function of NS5a that partly promotes HCV transcription and has also a function in modulating the host cell environment in favor of the virus (109, 110). NS5b inhibitors are (non) nucleoside analogues and inhibit the HCV polymerase (111). The HCV polymerase is an essential subunit of the replication complex to multiply the HCV genome(112). Besides the high effectivity of these DAAs, very few side effects of different regimens have been reported. For the newest combinations, i.e. grazoprevir and elbasvir or sofosbuvir and velpatasvir, the number of clinical side effects in the intervention group were equal to the number in the placebo arm(113, 114). However, rare but serious side-effects can only be excluded after marketing approval and therefore phase 4 studies and post-marketing data have to be awaited.

For the treatment of acute HCV, only telaprevir, a first generation HCV protease inhibitor has been tested in a pilot study in 19 co-infected patients. It was given for only 12 weeks in combination with pegIFN-alfa and ribavirin and a promising 84% SVR rate was seen(115). If these results can be confirmed by other ongoing study's, the use of first generation protease inhibitors for the treatment of acute HCV could become an effective and attractive treatment strategy(116, 117). Currently,

Table 1: Current and future studies with direct acting antivirals in acute HCV

Study name	Coordinator	Therapy	HCV genotype	Duration (weeks)	HIV status	Number of patients
DAHHS2	EMC	GZP+EBV	1+4	8	-/+	80
REACT	Kirby Institute	SOF/VEL	All	6-12	-/+	250
TARGET3D	Kirby Institute	DSV+PTV/r+ OBV	1	8-24	-/+	60
DARE-C2	Kirby Institute	SOF+RBV	All	8-6	-/+	20
SWIFT-C	ACTG	SOF+LDV	All	8	+	50
SOL	UKB	SOF+LDV	1,4	6	+	26
Hep-Net Acute HCV	MHH	SOF+LDV	1	6	-	20

HCV: hepatitis C virus; EMC: Erasmus Medical Center, The Netherlands; UKB: Universitätsklinikum Bonn, Germany; Kirby Institute, Australia; ACTG: Aids Clinical Trials Group, the United States of America; MHH: Medizinische Hochschule Hannover, Germany; RBV: ribavirin, SOF: sofosbuvir, LDV: ledipasvir; GZP: grazoprevir; EBV: elbasvir; VEL: velpatasvir; DSV: dasabuvir; PTV/r: paritaprevir/ritonavir; OBV: ombitasvir

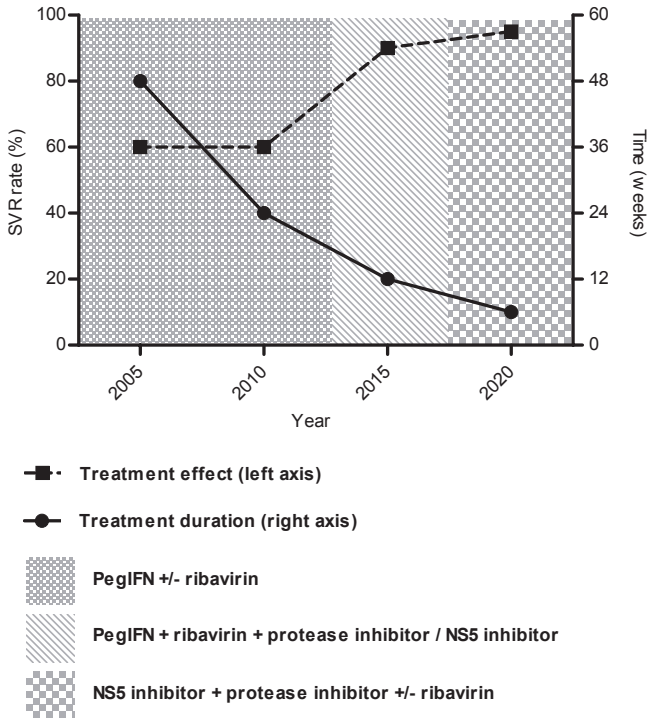

Figure 1: ACUTE HCV treatment and response prognosis

also interferon free DAA containing studies are being set up (table1)(118). However, so far, no DAA containing regimen has been extensively studied in patients with an acute HCV infection. Therefore, pegIFN based therapy is likely to remain part of the treatment armentarium for acute HCV during the next several years. DAAs will be added consecutively, with decreasing treatment duration as a result (figure 1).

CONCLUSION

The field of acute HCV remains complex because of limited research into prevention, adequate diagnosis, treatment initiation and treatment regimens. Despite all efforts, there is still no conclusive evidence for a decline in spread of the virus within major risk populations. Even with high incidence rates, the identification and inclusion of substantial numbers of individuals with acute HCV for trials with sufficient power remains challenging.

The availability of new DAAs will result in many more patients being cured. The high rates of adverse events and discontinuations of pegIFN-based therapy have led to delays in treatment initiation in acute infections, with possible resultant onward transmission of infection. DAAs will probably be also effective for the treatment of acute HCV infections but this has to be demonstrated by adequate studies. Because of the projected significant costs and, therefore, limited worldwide availability of DAAs, it remains to be seen whether more advanced treatment approaches will have a substantial effect on a population level.

AIMS AND OUTLINE OF THIS THESIS

The general aim of this thesis is to study different aspects of an acute HCV infection in HIV positive MSM. The primary aim is to study the effectivity and safety of boceprevir in addition to pegIFN and RBV for the treatment of acute HCV infections within the Dutch Acute HCV in HIV study (DAHHS). Subsequently, the DAHH-Study provided an unique opportunity to study the epidemiologic, clinical, immunologic and virologic characteristics of acute HCV infections among HIV positive MSM in the Netherlands.

In **Chapter 2**, the epidemiology of acute HCV infections in the Netherlands is studied based on an incidence measurement during the DAHH-Study in 2014. Because an acute HCV infection is sometimes difficult to diagnose, the additional value of HCV antigen testing in the diagnosis of acute HCV is studied in **Chapter 3**.

The results of the DAHH-Study are presented in **Chapter 4** and address the primary aim of this thesis. In **Chapter 5** potential drug-drug interactions with boceprevir and rilpivirine, a component of cART, are studied.

Because of the high effectivity combined with high costs of DAAs there is increased interest in the benefits and costs on a societal scale. The economic as well as the epidemiological effects of mass-treatment of all HCV co-infected MSM are studied in **Chapter 6**.

A major threat to the effectivity of DAAs could be the emergence of resistant HCV variants. In **Chapter 7**, the presence of resistance associated substitutions in the DAHHS cohort is studied to determine whether resistance for different DAAs plays a role in patients with infections acquired in 2013 and 2014.

The immunological profile of acute HCV infections is studied in the last part of this thesis. In **Chapter 8**, the frequency and functionality of mucosal-associated invariant T cells is evaluated between healthy volunteers, HIV-infected, chronic and acute HCV infected patients. In **Chapter 9** the biomarker profile of acute HCV infected patients is studied during their first and consecutive HCV infections.

In **Chapter 10,** the outcomes of this thesis are discussed and summarized. For laymen, a Dutch summary is provided in **Chapter 11**.

Chapter 2

Acute Hepatitis C in the Netherlands; characteristics of the epidemic in 2014

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ABSTRACT

Within the Dutch Acute HCV in HIV Study, a surveillance system was initiated to estimate the incidence of hepatitis C virus infections in 2014. Following the Dutch HIV treatment guidelines, HIV-positive men having sex with men (MSM) in 19 participating centers were screened. Ninety-nine acute HCV infections were reported, which resulted in a mean incidence of 11 per 1000 patient-years of follow-up. Unfortunately, the HCV epidemic among Dutch HIV-positive MSM is not coming to a halt.

INTRODUCTION

Acute hepatitis C virus (HCV) infections remain a major problem throughout the world within the populations of injecting drug users and patients undergoing non-sterile medical procedures. From 2001 the first reports on sexually transmitted acute HCV among men having sex with men (MSM) appeared(119). Although acute HCV can be successfully treated with interferon-based therapy in the majority of patients, this new epidemic has now become endemic in HIV-positive MSM(34). As acute HCV is often a clinically silent disease(120), the exact incidence can only be reliably measured when an active surveillance policy is followed.

METHODS

To measure the incidence of acute HCV in HIV-positive MSM across the Netherlands, a surveillance system was initiated within the Dutch Acute HCV in HIV study (DAHHS). The DAHHS is a study on the efficacy of 12 weeks' therapy with boceprevir, peginterferon and ribavirin for the treatment of acute HCV genotype 1 infections in HIV-infected patients(117). All 27 Dutch HIV centers were invited to report all acute HCV infections to the study coordinator and report characteristics of patients anonymously. Only patients who had not opted out for anonymous data registration in the Dutch ATHENA cohort were included(121). Participation of a center was confirmed if at least one acute HCV patient had been reported or a statement of ' absence of acute HCV diagnosis in 2014' was sent to the study coordinator.

According to the guidelines within the Dutch HIV care system, all HIV-positive MSM are screened for hepatitis using an alanine aminotransferase (ALT) measurement at least twice a year. When a rise in ALT above reference value is observed, the treating physician decides whether or not to test for HCV RNA. At the physician's discretion, patients are also tested for HCV RNA when they report a HCV exposure or present with another sexually transmitted disease. Within this study an acute HCV infection was defined as a first positive HCV RNA measured in 2014, preceded by a negative HCV RNA or HCV immunoglobulin (Ig) G serology within one year before HCV RNA positivity. When a stored plasma sample from within the previous 12 months was not available to document the absence of HCV, a normal ALT preceding the first positive HCV RNA test together with a documented negative HCV IgG test at any time in the

past was used to exclude a chronic HCV infection. In patients without historical HCV test available to exclude chronic HCV, the combination of a positive HCV PCR with a negative HCV IgG finding was used to diagnose an acute HCV infection. The incidence rate was calculated using data from the Dutch Stichting HIV Monitoring (HIV Monitoring Foundation, SHM) (121), which provides detailed information about the number of HIV-positive MSM in care in the different treatment centers in the Netherlands.

RESULTS

From January 1, 2014 until January 1, 2015 a total number of 99 acute HCV diagnoses were reported (table 1). Seventy-eight per cent of the acute HCV diagnoses were based on retesting of stored plasma to prove the absence of HCV RNA. Altogether, 19 of the 27 HIV treatment centers participated, and at the end of 2014 at total of 9487 HIV-positive MSM were in care in these 19 centers. To determine the number of person-years at risk in 2014, the number of MSM in care in the study centers was adjusted for the proportion of MSM already known to be chronically infected with HCV (4.8%) and for the increase in the number of HIV-positive MSM in care during

Table 1. Characteristics at the time of acute HCV diagnosis

		N	Median/%	IQR	Unit
Male		99	100%		
Age		99	43	36-48	years
CD4		95	0.61	0.43-0.82	E9/l
cART	yes	90	90%		
	no	5	5%		
	unknown	4	5%		
HCV genotype	1	78	79%		
	2	2	2%		
	3	0	0%		
	4	18	18%		
	unknown	1	1%		
Subtype within gen 1	a	74	95%		
	b	4	5%		
Reinfection	no	74	75%		
	yes	25	25%		
ALT at diagnosis		91	191	93-520	U/L
HCV RNA at diagnosis		97	601	74-4925	E3 IU/ml

cART: combination antiretroviral therapy, HCV: hepatitis C virus, ALT: alanine amino-transferase, IQR: interquartile rage

2014 (n=384)(121). The number of HCV RNA-negative, HIV-positive MSM at risk for acute HCV between January 1, 2014 and January 1, 2015 was therefore estimated at 8849 person-years. Using the 99 acute HCV infections, a mean incidence rate of 11 per 1000 person-years of follow-up (PYFU) (95% confidence interval 9-14) was calculated.

Spontaneous resolution was observed in 12% of the patients, 36% were treated with 12-weeks' therapy with boceprevir, peginterferon-alfa and ribavirin (DAHHS protocol) and 20% were treated with another combination (mostly 24 weeks' therapy with peginterferon with ribavirin). Thirty-one per cent of the patients did not initiate treatment.

DISCUSSION

The calculated incidence of 11 per 1000 PYFU is a reliable representation of the Dutch acute HCV epidemic within HIV-positive MSM, as we included a population that represents 86% of all HIV-positive MSM in care in the Netherlands. Hence, it is clear that the acute HCV epidemic is no longer limited to a few large cities but has become a nationwide problem (median incidence per center 8.7/1000 PYFU, interquartile range 4,7-12). The number of genotype 1 infections, at 79%, is higher than previously reported (48-67%) and may indicate a genotype shift(34, 122, 123).

The incidence rate we found is comparable with international data from the United States (16/1000 PYFU) and Japan (20/1000 PFY) but not with incidence rates from Taiwan (4.9/1000 PYFU) and Switzerland (41/1000 PYFU) (32, 36, 122, 124). However, the incidence rate we found should be seen as a minimum incidence. Cases could have been missed because different ALT cutoff points were used in participating centers, as the exact threshold was not predefined and a diagnosed case can always remain unreported. Additionally, 10% (9/91) of the patients with a first positive HCV RNA did not present with a raised ALT above 44 U/L. This illustrates that a small but unknown number of additional acute HCV cases may remain undiagnosed. In our study the percentage of patients with a normal ALT at the time of the first positive HCV RNA was relatively low, in comparison to the 24% published by Thomson and colleagues(49). This may be explained by the fact that in Thomson's study the date of the first ALT measurement was done earlier, at only 96 days after the date of the

last negative HCV RNA test. Interestingly, we observed that patients who cleared the infection spontaneously had a higher ALT than those who did not (ALT 1249 U/l versus 348 U/l, p=0.02).

It is clear that HCV infections continue to be a significant problem within HIV-positive MSM across the Netherlands as long as a significant proportion of HCV infections remains untreated. Fortunately, in the Dutch setting prompt identification seems possible. This well-defined population of HIV-positive MSM at risk may be an ideal population in which eradication strategies can be tested or implemented. Unfortunately, the efficacy of the interferon-free therapies has never been investigated in the context of acute HCV. Therefore these therapies are not registered for the treatment of acute HCV. Furthermore, given the high costs of interferon-free HCV therapies, in the Netherlands, as well as in many other European countries they are only reimbursed for patients with Metavir F3 fibrosis or cirrhosis. Given the risk of ongoing HCV transmission if patients remain untreated, a restrictive reimbursement policy may be an expensive long-term strategy. Studies on the cost-effectivity of early HCV therapy that also take into account the benefits of a reduced risk of sexual HCV transmission are therefore needed.

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Chapter 3

HCV antigen instead of RNA testing to diagnose acute HCV in HIV positive patients

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Under review

Chapter 4

Boceprevir, Peginterferon and Ribavirin for acute hepatitis C in HIV infected patients

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ABSTRACT

Introduction

Acute hepatitis C virus infections (HCV) are prevalent among HIV positive men having sex with men and generally treated with peginterferon-alfa (pegIFN) and ribavirin (RBV) during 24 weeks. The addition of a protease inhibitor could shorten therapy without loss of efficacy.

Methods

We performed an open-label, single arm study to investigate the efficacy and safety of a 12-week course of boceprevir, pegIFN and RBV for acute HCV genotype 1 infections in 10 Dutch HIV treatment centers. Primary endpoint of the study was achievement of sustained virological response rate at week 12 (SVR12) in patients reaching a rapid viral response at week 4 (RVR4) and SVR12 in the intent to treat (ITT) entire study population was the most relevant secondary endpoint.

Results

One hundred twenty-seven acute HCV patients were screened in 16 months, of which 65 acute HCV genotype 1 patients were included. After spontaneous clearance in six patients and withdrawal before treatment initiation in two, 57 started therapy within 26 weeks after infection. RVR4 rate was 72%. SVR12 rate was 100% in the RVR4 group. SVR12 rate in the ITT group was 86% and comparable to the SVR12 rate of 84% in 73 historical controls treated for 24 weeks with pegIFN and RBV in the same study centers.

Conclusion

With the addition of boceprevir to pegIFN and RBV, treatment duration of acute HCV genotype 1 can be reduced to 12 weeks without loss of efficacy. Given the high drug costs and limited availability of interferon-free regimens, boceprevir, pegIFN and RBV can be considered a valid treatment option for acute HCV.

INTRODUCTION

During the last decade hepatitis C has emerged into a sexually transmitted disease within HIV positive men having sex with men (MSM). Incidence rates of hepatitis C virus (HCV) infections are estimated to be around 12/1000 person-years and the large majority of infections are with genotype 1 and 4(34). Acute HCV infections are generally defined as the period of infection in which the immune system attempts to eradicate the virus. This may lead to viral clearance but more often results in chronic disease(9, 126). An acute HCV infection in HCV mono-infected patients is treated with pegylated interferon-alfa (pegIFN) for 12 or 24 weeks, resulting in sustained virological response (SVR) rates of about 54-98%(98, 101, 133-135). In HCV/HIV coinfected patients it is believed that the addition of ribavirin (RBV) is necessary to achieve comparable SVR rates with 24 weeks of therapy(95, 136). Recently, the addition of a direct-acting antiviral agent (DAA) to pegIFN and RBV increased cure rates both in chronic HCV mono- and HIV/HCV coinfected patients(137, 138). Also for acute HCV this was first demonstrated by Fierer and colleagues in a pilot study with 12 weeks telaprevir, pegIFN and RBV, which showed a cure rate of 84%(115). Thus, DAAs could also become a valuable addition in the treatment of acute HCV. Because HCV is a sexually transmitted disease in MSM, early treatment is crucial if one of the goals of treatment is the prevention of transmission. However, due to the very high drug costs, the availability of the recently approved pegIFN-free regimens for the treatment of chronic HCV is currently limited to patients with severe liver fibrosis or cirrhosis in most countries. Therefore, in patients with acute HCV, waiting until the infection has become chronic is not (yet) an option if treatment as prevention is aimed for. In the Dutch Acute HCV in HIV Study (DAHHS), our hypothesis was that addition of a protease inhibitor could shorten therapy duration without loss of efficacy. Therefore, we performed a single arm open-label study of a 12-week course with boceprevir, pegIFN and RBV in HIV coinfected patients with an acute HCV genotype 1.

METHODS

This investigator-initiated study was designed as a single arm, open-label multicenter intervention trial in 10 HIV treatment centers in the Netherlands. Other (non-study) Dutch HIV treatment centers were able to refer their patients to one of these study centers for treatment. We included adult, HIV-infected patients, who had to be on

combination anti-retroviral therapy (cART) at the start of HCV treatment. Patients that were included had to start treatment within 26 weeks after the assumed date of infection and had to be infected with HCV genotype 1 with a minimum viral load of 1000 IU/ml at screening. Patients were excluded if they presented with a non-genotype 1 infection, were unable to switch to a cART regimen consisting of nucleoside/tide reverse transcriptase inhibitors and raltegravir or rilpivirine, had a hepatitis B infection complicated by liver fibrosis (METAVIR>F1), had low platelets (<100E9/L), reduced kidney function (clearance <50 mL/min) or neutrocytopenia (<0.75E9/L). All patients provided written informed consent. The study was done according to Good Clinical Practice guidelines in accordance to the declaration of Helsinki and was approved by the ethics committee of the Erasmus Medical Center (ClinicalTrials.gov, number NCT01912495).

In the participating hospitals, HIV-positive patients were tested for HCV RNA if they presented with an alanine aminotransferase (ALT) rise above the upper limit of normal. ALT measurements were performed at least twice a year during regular HIV monitoring visits or more often at the discretion of the treating physician.

If a patient had detectable HCV RNA (CAP/CTM, V2, Roche diagnostics or Abbott Realtime M2000) genotyping was performed and the most recent stored plasma sample from previous visits to the outpatient clinic (up to one year before diagnosis) was tested for HCV RNA to determine whether infection was recent(49, 136). If no historical sample was available, the most recent (up to one year before diagnosis) negative HCV IgG test or ALT level below the upper limit of normal was used to exclude chronicity.

Genotype 1 infected patients with no 2-log HCV RNA drop four weeks after diagnosis had to initiate therapy within 26 weeks after the presumed date of infection (midpoint between first positive HCV RNA and last negative laboratory test). If patients had a 2-log drop in HCV RNA one month after diagnosis, a consecutive HCV RNA measurement was performed 2 months after diagnosis(139). If there was no further significant decrease in HCV RNA at that time, treatment was initiated.

Treatment consisted out of boceprevir (BOC) 800 mg thrice daily, weight based pegIFN alfa-2b (1.5 µg/kg/week) and weight based RBV (<65 kg: 400 mg twice-, 65–80 kg: 400 mg once- and 600 mg once-, 81–105 kg: 600 mg twice-, >105 kg 600 mg

once- and 800 mg once-daily). Treatment was started without a lead-in of pegIFN and RBV and continued for 12 weeks. HCV RNA was measured at week four and if positive every two weeks thereafter until undetectable. An undetectable HCV RNA was defined as HCV RNA <15 IU/mL (Roche) or <11 IU/ml (Abbott), with the PCR target not detected. For patients with HCV RNA still detectable at week 8, treatment could be prolonged to 24 weeks (response guided therapy).

The primary endpoint of the study was sustained virological response at week 12 after therapy (SVR12) within the patient group with an undetectable HCV RNA at week four (RVR4).

Secondary endpoints were SVR12 in the whole intention to treat (ITT) population, SVR12 in patients that started therapy within 12 weeks after the presumed date of infection and RVR4 and SVR12 according to IL28 genotype. All patients were included in safety analysis of adverse events and quality of life was assessed by RAND-36 at baseline, week 4, 12 and 24. Utility score estimates were derived from the RAND-36 using the ordinal health state evaluation model(140). A utility score of 1 is defined as the highest level of well-being and a score of 0 as death.

To put the cure rate found in this study in perspective, historical controls were retrospectively selected from the 10 study centers. Partly these controls were selected from the MOSAIC (MSM Observational Study of Acute Infection with hepatitis C) cohort. The MOSAIC cohort is an open, on-going, prospective, observational cohort, initiated to study determinants and sequelae of acute HCV infection among HIV-infected MSM. The MOSAIC is a collaboration between the Public Health Service of Amsterdam, five large HIV outpatient clinics in the Netherlands, which all also participated in the DAHHS, and the Stichting HIV Monitoring (SHM)(141). Study centers that did not participate in the MOSAIC cohort selected controls out of the Dutch ATHENA cohort, if available(142). Each center provided data of as much acute HCV genotype 1 controls as possible. These controls had to have received at least one dose of pegIFN and RBV and started therapy between 2007 and 2013 at a time when the standard treatment duration was at least 24 weeks(136). ITT SVR12 data were obtained from these controls. Since it was not part of standard care to measure a HCV RNA at week 12 after therapy, SVR12 in the historical controls was defined as the first HCV RNA measured from week 10 after therapy, but no later than week 48 after the end of therapy.

Statistical analysis

For the primary and secondary endpoints, a 2-sided confidence interval using the Wilson score method without continuity correction of the SVR12 was calculated. Due to the retrospective nature of the historical control group, no formal non-inferiority statistical comparison between the control group and the prospective ITT study population was planned. However, to compare SVR12 rates the chi- square test was used. The paired t-test was used for the comparison of utility scores between baseline and treatment time points.

Role of the funding source

MSD provided BOC as study drug and financially supported this investigator-initiated study. MSD had no role in the conduct and analysis of the data.

RESULTS

From September 2013 until December 2014, 127 patients with a recent HCV infection were reported by 19 of the 27 existing HIV treatment centers across the Netherlands. Sixty-two patients were excluded because of genotype 4 (n=22), genotype 2 (n=1), infection > 6 months old (n=17), refused (n=13), spontaneous clearance without inclusion (n=8), hepatitis B with cirrhosis (n=1)(figure 1). Sixty-five patients with an acute HCV genotype 1a/b infection were referred to or already in care in one of the 10 study centers and were included. Eight patients did not initiate treatment because of spontaneous clearance (n=6), refusal after inclusion (n=1) and a serious adverse event before initiation of therapy (n=1). Finally, 57 patients initiated treatment and received at least one dose of pegIFN, RBV and BOC. In 48 of these 57 patients the diagnosis of acute HCV was based on a negative HCV RNA test result on stored plasma that was taken during the previous outpatient visit. To avoid drug-drug interactions with BOC, 26 patients switched from cART regimen before initiation of therapy. Nine patients switched to a rilpivirine and 17 to a raltegravir containing regimen. All patients remained HIV RNA undetectable after this cART switch. Except for a single patient who discontinued cART at his own initiative during HCV therapy.

Seventy-three historical controls from the same treatment centers, who initiated pegIFN and RBV based treatment during 2007-2013, were included. Baseline characteristics are shown in table 1.

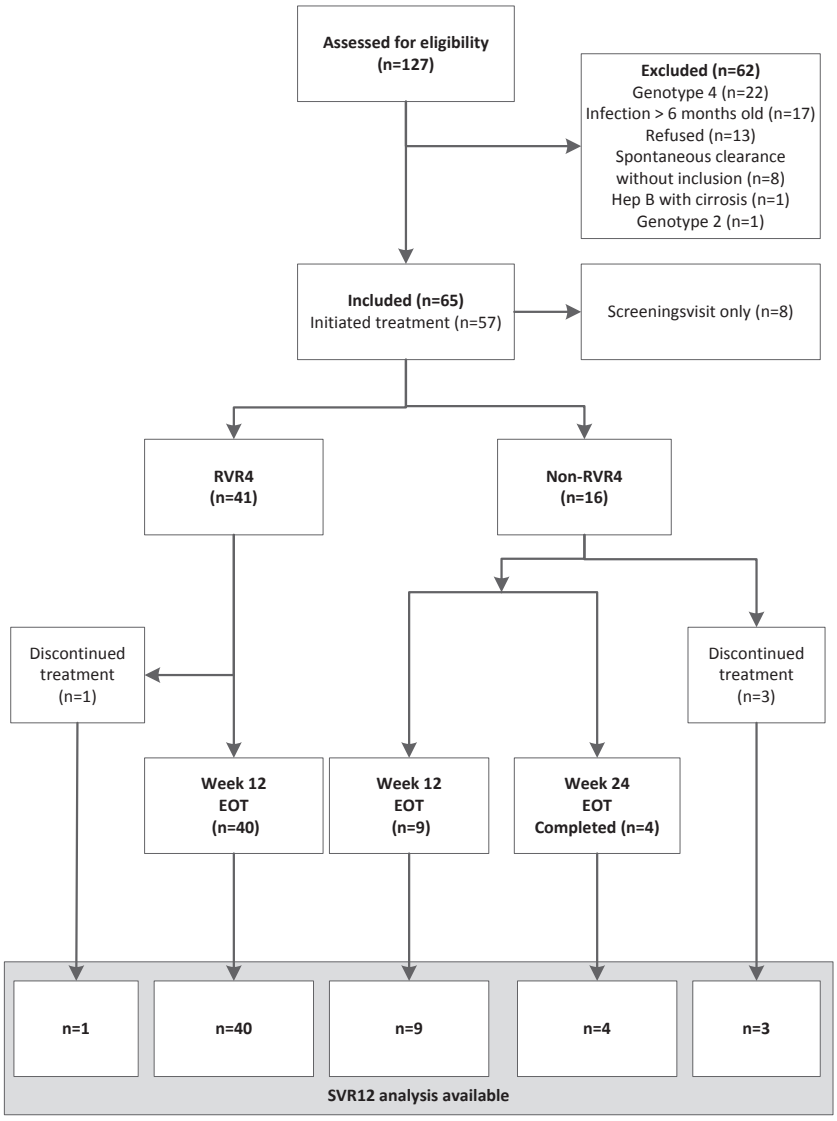


Figure 1: Patient flow diagram. RVR4: rapid virological response at week 4, EOT: end of therapy

Table 1. Baseline characteristics

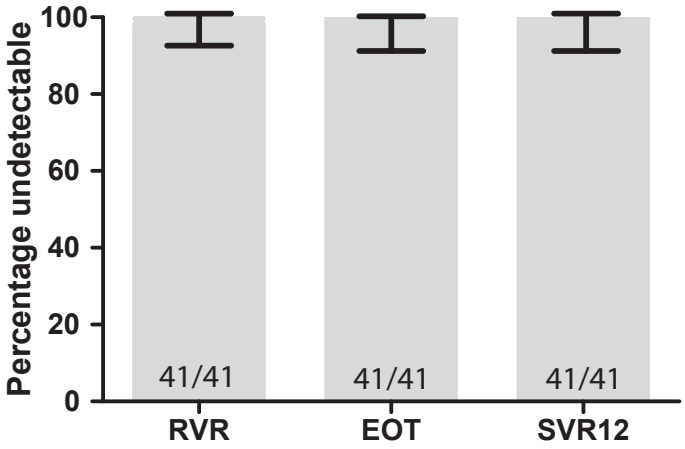
Baseline characteristics	DAHHS (n=57)	Retrospective cohort (n=73)
Sex, male	57 (100%)	73 (100%)
Age, years, median (IQR)	40 (34-47)	44 (39-49)
CD4 count, E9/L, median (IQR)	0·66 (0·45-0·79)	0·48 (0·40-0·68)
ALT, U/L, median (IQR)	160 (67-416)	NA
Bilirubin. umol/L, median (IQR)	10 (6·5-13)	NA
HCV genotype 1		
subtype a	54 (95%)	61 (83·6%)
subtype b	3 (5%)	8 (11%)
unknown	0	4 (5·5%)
HCV RNA IU/mL, median (IQR)	222.000 (5615-1.235.000)	120.000 (2125-1.215.000)
Treatment duration, weeks, median (IQR)	12 (12-12)	24 (23-26)
Interval infection-treatment, weeks, median (IQR)	22 (16·5-25)	23 (17-30)
Il28B genotype		
RS 1297, CC	25 (42·5%)	NA
RS 1297, non-CC	30 (57·5%)	NA

IQR: interquartile range, ALT: alanine-aminotransferase, NA: not available

Primary endpoint

All patients in the intervention group reached week 4 with 41/57(72%) achieving RVR4. All RVR4 patients had an undetectable viral load at end of therapy. One patient discontinued treatment at week 10 due to adverse events while subsequently achieving a SVR12. All 41 (100% with 95% confidence interval (CI), 91-100%) patients with a RVR4 achieved a SVR12 (figure 2).

A



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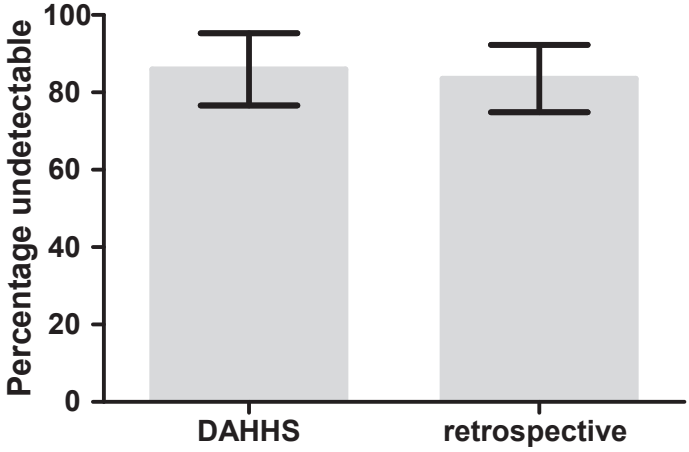

Figure2: Virological responses. (A) Virological response in RVR4 patients. (B) Virological response in ITT population. RVR4: rapid virological response at week 4, EOT: end of therapy, SVR12: sustained virological response at week 12

Secondary endpoints

SVR12 in ITT population

Next, we analyzed the ITT population, regardless of RVR4 result. A total of 49 patients were treated 12 weeks, four were treated 24 weeks and four patients discontinued therapy before week 12 (figure 1). Of the 16 patients without a RVR4, six had a detectable but non-quantifiable HCV RNA load (<15 IU/l, target detectable) at week 4. Subsequently, nine of these 16 were undetectable at week 8 and continued therapy until week 12. Three discontinued before week 12. Out of the four patients that continued therapy until week 24, two had a detectable viral load at week 8 and were treated with pegIFN/RBV/BOC resulting in a SVR12. One patient had a detectable load at week 8 and was unsuccessfully treated with 12 weeks pegIFN/RBV/BOC followed by 11 weeks pegIFN/RBV. The last patient had an unknown load at week 8 and was treated successfully with 24 weeks pegIFN/RBV/BOC based on decision of the treating physician.

The SVR12 rate in the ITT population was 49/57, 86% (95% CI, 75-93)(figure 2b). In total, 8 patients had a treatment failure of which 3 had a relapse, three discontinued therapy at a time that they still had a detectable viral load and two patients had a viral breakthrough. In all 3 patients with a relapse, phylogenetic analysis showed that the same strain was present at the time of failure, which excluded reinfection. Another patient with an RVR4 and HCV RNA negative 4 weeks after the end of therapy, had detectable HCV RNA 12 weeks after the end of therapy. Phylogenetic analysis showed that he was reinfected with a new HCV strain.

SVR12 with treatment initiation within 12 weeks after infection

Another predefined secondary endpoint was the SVR12 rate in patients that started therapy within 12 weeks after the presumed date of infection. Five out of five patients (100%) treated within 12 weeks after infection had a SVR12 compared to 44/52 (85%) after 12 weeks. Given the small number of patients in the former group, no conclusions can be drawn on this subgroup analysis. No correlation between the IL28 genotype and SVR12 nor RVR4 rates was found (figure 3).

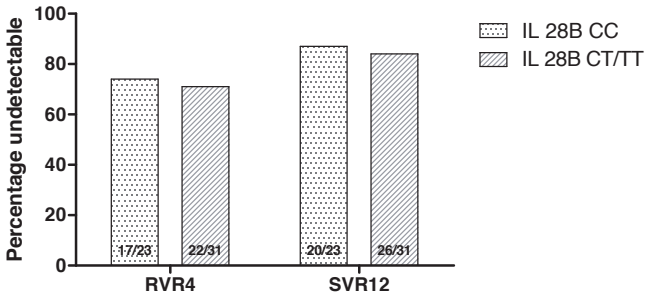

Figure 3: Virological response according to IL 28B genotype. RVR4: rapid virological response at week 4, SVR12; Sustained virological response at week 12, Il 28B genotype RS12979860 was available in 54 patients.

SVR12 in historical controls

In the retrospective cohort, the ITT SVR12 result was 84% (95% CI, 73-90)(figure2b). Median treatment duration with pegIFN and RBV was 24 weeks (interquartile range (IQR) 23-26). As RVR4 testing was not part of the standard of care, results were only available in a minority of cases and therefore not shown. One patient was treated in the historical cohort for a previous acute HCV infection as well as in the prospective cohort.

The SVR12 rates in the prospective study population (86%) and the retrospective controls (84%) were not statistically significant (p=0.71).

Quality of life and safety

As expected, the quality of life decreased during therapy (-0.05 utility score, p<0.001) but was no longer different from baseline at the SVR12 time point (figure 4).

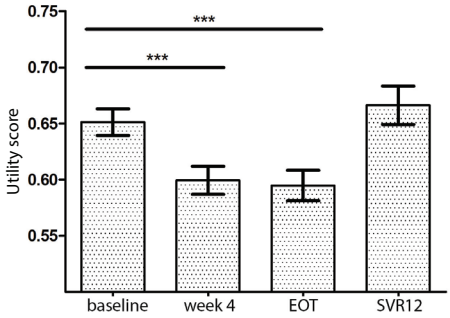

Figure 4: Change in utility score during treatment with peginterferon, ribavirin and boceprevir. EOT= End of Treatment, SVR12; time point of sustained virological response at week 12. For this analysis only available data of patients treated for 12 weeks were used. *** p<0.001

During the study, four serious adverse events (grade 4) were documented. One myocardial infarction in a patient with a high-grade left anterior descending artery stenosis that was stented uneventfully (hemoglobin (Hb) 8.7 mmol/L at the time of the infarction). One suspected transient ischemic attack that resolved spontaneously and with normal MRI findings (Hb 6.6 mmol/L). One anemia (Hb 5.1 mmol/L) for which a transfusion was given and the RBV dose was decreased. All three patients continued their HCV therapy. The fourth SAE occurred in a patient that had not yet started HCV therapy (cerebral hemorrhage). Additionally, five grade 3 events were reported (depression, paradontitis, malaise, personality change, fatigue). At week 12, median decrease in Hb was 2.4 mmol/L (IQR 1.6-3.3). Grade 1 anemia was observed in 83%, grade 2 in 12% and 5% had no anemia at week 12. Dose reduction of RBV was done in six patients because of rapid Hb decline at week 4 or 8, but not because of grade 3/4 anemia. Erythropoietin was not used in any of the patients.

DISCUSSION

In this prospective multicenter study on the treatment of acute HCV in HIV positive patients, 12 weeks of BOC, pegIFN and RBV cured 100% of the patients that achieved a RVR4. Moreover, the ITT analysis showed a high SVR12 rate of 86%. This overall 86% cure rate was comparable to the 84% cure rate in patients treated with 24 weeks of pegIFN and RBV in the same study centers in the years preceding the prospective study. Hence, this study on the addition of BOC to pegIFN and RBV shows that treatment duration can be halved in the majority of patients without loss of efficacy.

Although less conclusive, the absence of a RVR4 was associated with a reduction in SVR12 from 100% to 50% (8/16). More specifically, the SVR12 rate of patients without RVR4 was 56% when treated 12 weeks and 75% when treated for 24 weeks. Therefore, although not formally tested in this study, treatment duration may have to be increased in patients without a RVR4. Also, the other way around, one may decide to discontinue treatment in patients without a RVR4 if side effects of treatment are problematic.

These data are in line with the results of a small single center pilot study by Fierer and colleagues, who reported a SVR12 rate of 84% in 19 patients treated with 12 weeks of telaprevir, pegIFN and RBV(115). However, because of the small sample size

and the overrepresentation of patients with the favorable IL28B CC genotype, these data needed confirmation in a larger and more representative population. As such, our study is the largest study on the treatment of acute HCV with a DAA containing regimen ever performed. Also, the study population had a more representative IL28 genotype distribution(63) (table 1).

Because several antiretroviral drugs cannot be combined with BOC, 46% of the patients had to switch their cART to rilpivirine or raltegravir. In all these patients, who were treatment adherent, the HIV viral load remained undetectable throughout the study. Therefore, the need for a cART switch should not withhold patients or physicians to start HCV therapy.

In the light of the rapid switch to more advanced DAAs in the treatment of chronic HCV in some resource rich countries, one may question whether there is any role for a treatment that consists of pegIFN, RBV and a DAA like boceprevir in the treatment of acute HCV. It could be advocated that nothing is lost when spontaneous clearance of HCV is awaited and HCV is treated with one of the many very effective interferon-free DAA combination therapies if after 12-18 months the infection has become chronic. However in most resource rich and almost all resource-limited countries, these advanced DAAs are not reimbursed for patients without substantial liver disease or not available at all. Given the fact that HCV can be considered a highly infectious disease in intravenous drug users as well as HIV positive MSM, untreated HCV patients will continue to transmit the virus and therefore continue to drive the HCV epidemic. So far, pegIFN based therapy will remain the only treatment available for acute HCV as long as interferon-free treatments are not registered for this indication. This is illustrated by the most recent EACS guideline, which still advices to treat AHCV with 24 weeks pegIFN and RBV(96). Therefore, the 50% reduction in treatment duration with the addition of a first generation HCV protease inhibitor to pegIFN will make acute HCV therapy possible in more patients.

For countries in which DAAs are now available, this study clearly shows that also HIV positive patients with acute HCV can now benefit from a short treatment when a protease inhibitor is added to pegIFN and RBV. We think that second wave protease inhibitors like simeprevir or NS5 inhibitors, all better tolerated than BOC, are likely to lead to comparable results if combined with pegIFN and RBV.

In the end, it is plausible that we will be able to treat acute HCV without pegIFN or RBV. Although this is already advocated by the American Association for the Study of Liver Disease, this statement is without any supporting evidence. Acute HCV, with its extremely fluctuating HCV RNA plasma load, is a disease that immunologically and virologically differs from chronic HCV and the effects of pegIFN free regimens during acute HCV may therefore differ as well. This is illustrated by several abstracts of small pilot studies presented at the 2015 Liver Meeting. An ACTG study reported a SVR12 rate of 59% in patients treated with 12 weeks of sofosbuvir and RBV(143). Even lower SVR12 rates of 32% were seen in a study with 6 weeks of sofosbuvir and RBV(144). In contrast, when sofosbuvir was combined with ledipasvir for 4 weeks or with simeprevir for 8 weeks, the SVR12 rate was 100% (14/14 and 13/13 respectively) (145). When confirmed in larger studies, this suggest high effectivity also during treatment of acute HCV when DAAs are combined. Future will tell if, for the treatment of acute HCV as well, the pegIFN era has come to an end.

Our study has some limitations. First, despite being able to reliably determine the date of HCV transmission by the use of stored plasma samples, which enabled us to treat patients in the early stage of disease, we cannot draw definite conclusions on the effect of a 12-week treatment for late acute/early chronic HCV stage (e.g. >6 months but <12 months after infection). However, a recent study suggested that initiation of treatment with pegIFN and RBV could be delayed at least for some months without loss of efficacy(99). Furthermore in our study, patients started therapy at an estimated median of 22 weeks after infection. Therefore, it is likely that treatment of early chronic HCV (e.g. in the 6th or 7th month after infection) will not be much different. Second, our study design lacked a formal control group because a randomized study was considered unfeasible as in clinical practice new HCV infections are mostly diagnosed at the time they have become chronic. Also, we anticipated that the willingness to undergo a 24-week pegIFN based regimen was poor in the light of pending pegIFN-free regimens for chronic HCV. Therefore, we considered a large randomized non-inferiority trial unachievable even with the multicenter study design in which an estimated 80% of the acute HCV infections diagnosed in HIV positive MSM in the Netherlands in 2014 were included. In our view, we created the most optimal comparator group, with genotype 1 infected patients that had been treated recently in the same study centers. Still, the comparator group may have been biased (e.g. by selecting the most motivated patients willing to undergo a 24-week pegIFN therapy). Additionally, the SVR12 rate of 84% in our control group was much higher than the

60% SVR12 published in a review by Rockstroh and colleagues(95). However, the data of our control group are in line with Dutch data published in 2011 and recent data from the Swiss cohort(123, 141).

In conclusion, 12 weeks of triple therapy consisting of pegIFN, RBV and BOC results in very high cure rates in those patients achieving a RVR4 with acceptable toxicity and therefore is a good treatment option in HIV-infected patients with an acute HCV infection.

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Chapter 5

Safety of rilpivirine and boceprevir co-administration in HIV-infected patients treated for acute hepatitis C virus infection

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ABSTRACT

Treatment of hepatitis C virus infections (HCV) in HIV co-infected patients improved substantially when HCV protease inhibitors became available. CYP3A4 inhibition by boceprevir may influence the metabolism of the certain antiretroviral drugs like rilpivirine. In this study 12 HIV positive patients treated with rilpivirine and boceprevir had blood samples taken together with an ECG measurement before and during acute HCV treatment. Treatment with boceprevir resulted in increased rilpivirine $AUC_{0\text{-}24h}$ from 3.8 mg·h/L to 6.5 mg·h/L, but did not result in increased QTc-intervals.

INTRODUCTION

In 2012, hepatitis C virus (HCV) protease inhibitors were the first direct-acting antivirals (DAAs) that led to an improved treatment response when combined with peginterferon-alfa and ribavirin for the treatment of HCV infections. However, when used in HIV-positive patients, treatment with DAAs is more complicated regarding possible drug-drug interactions (DDI) with combination antiretroviral therapy. The nonnucleoside reverse transcriptase inhibitor rilpivirine is a CYP3A4 substrate and therefore potentially interacts with boceprevir, a CYP3A4 inhibitor. Based on a healthy volunteer DDI study, exposure to rilpivirine increased with 39% when given together with boceprevir (146). This increased exposure did not lead to clinically significant corrected QT (QTc) prolongation during short-term administration in the healthy volunteers. Therefore the product label of boceprevir states that the drug can be coadministered with rilpivirine; this also holds for other HCV protease inhibitors and CYP3A4 inhibitors, such as telaprevir and simeprevir(146-148).

We performed a subanalysis within the Dutch Acute HCV in HIV Study (DAHHS) to describe the pharmacokinetic interaction between boceprevir and rilpivirine in HIV/HCV-positive patients and the possible effects on QTc-interval prolongation(149).

METHODS

In the DAHHS, 57 HIV-positive patients were treated for an acute HCV infection with 12 weeks of weight-based pegylated interferon-alfa (pegIFN), ribavirin and boceprevir 800 mg thrice daily(150). In this substudy patients treated with rilpivirine 25 mg daily combined with two nucleos(t)ide reverse transcriptase inhibitors were included. Patients were instructed to take rilpivirine together with at least a 500 Kcal meal. Blood samples were taken before the start of HCV therapy (T0) and at week 4 of therapy (T4) during the elimination phase of the drug (t=4-24h after administration). Concurrently, with the blood draw, a single 12-lead ECG was recorded in all participants at T0 and T4. Heart rate corrected QT was calculated using Bazett's formula. Rilpivirine plasma concentrations were analyzed by a validated reversed-phase ultra-performance liquid chromatographic method with ultraviolet detection (linear calibration range (0.0063-3.75 mg/L). Concentration-time data were analyzed using nonlinear mixed-effect modeling (NONMEM version 7.2; ICON, Dublin, Ireland). The effect of boceprevir

and alanine-aminotransferase (ALT) on rilpivirine apparent clearance (CL/F) was evaluated. Area under curve $(AUC)_{0\text{-}24h}$ was calculated using individual estimations for CL/F and dose. The correlation between $AUC_{0\text{-}24h}$ and QTc time was analyzed using linear regression.

RESULTS

Twelve male patients (11/12 white people) were included. Median age was 38 years (interquartile range (IQR) 33-43). Patients had a median ALT of 126 U/l (IQR 29-198) with no signs of liver failure at T0 and their median ALT at T4 was 36 U/l (IQR 28-39). In all patients the HIV viral load was fully suppressed at both time points. One out of 12 patients had concomitant medication potentially influencing the rilpivirine metabolism or QTc time (acetylsalicylic acid 80mg daily, prasugrel 10 mg daily, pravastatin 40 mg daily, perindopril 2mg daily)

Median rilpivirine concentration at T0 was 0.16 mg/L and at T4 0.26 mg/L. In the individual patients, the absolute rilpivirine concentration increased at T4 from +0.01mg/L (8%) to +0.10mg/L (63%), when measured at the same time point after intake. Pharmacokinetics of rilpivirine were described using a one-compartment model with fixed values for the absorption rate (k_a=0.7 h^{-1}) and volume of distribution (V_d=152L) based on literature. CL/F decreased from 7.2 to 3.8 L/h when patients were treated with boceprevir (relative SE 14%, p<0.005). In the simulated pharmacokinetic profile, a clear decrease in CL/F and $AUC_{0\text{-}24h}$ is seen (figure 1). This DDI explained 34% of the variability in CL/F. The remaining variability of CL/F was 44% (relative SE 13%). No significant correlation between ALT and CL/F was seen. Measured QTc-intervals were not correlated to the calculated rilpivirine $AUC_{0\text{-}24h}$. Patients without boceprevir had a median rilpivirine $AUC_{0\text{-}24h}$ of 3.8 mg/L/h with corresponding QTc interval of 382 ms (range 355-425 ms) and patients treated with boceprevir had a median rilpivirine $AUC_{0\text{-}24h}$ of 6.5 mg/L/h and a corresponding QTc interval of 384 ms (range 349-425 ms).

DISCUSSION

This study showed that the concomitant use of boceprevir in HIV-positive patients treated with rilpivirine decreased the rilpivirine CL/F, resulting in increased rilpiv-

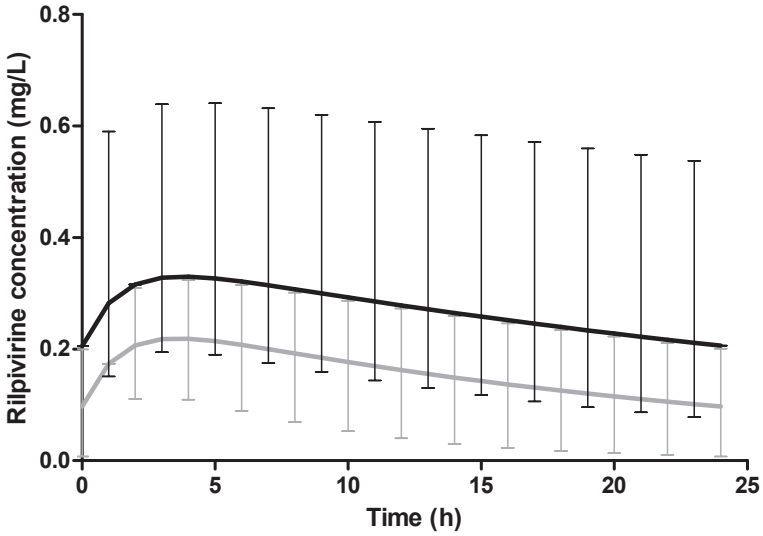

Figure 1: Rilpvirine concentrations with and without boceprevir. Median pharmacokinetic profile and range of rilpivirine in patients treated with (black) and without (grey) boceprevir.

irine $AUC_{0\text{-}24h}$ from median 3.8 mg/L/h to 6.5 mg/L/h. This DDI could explain 34% of the variability in CL/F seen in these patients, but did not result in increased QTc-intervals. The $AUC_{0\text{-}24h}$ values seen in the patients without boceprevir treatment were comparable to values described previously(151).

The increase in rilpivirine $AUC_{0\text{-}24h}$ (71%) we found in this study is higher than previously described in healthy volunteers (39%)(146). This suggests that data from healthy volunteers cannot be translated one on one to HIV-infected patients.

The main limitation of the study was the limited number of samples per patient that were obtained, one before and one during treatment with boceprevir. As a consequence, only CL/F and its variability could be estimated. These values changed after adding the effect of boceprevir treatment, resulting in a significant improvement of the model ($p<0.005$). In literature, the influence of race, gender and food on rilpivirine CL/F is described. Unfortunately, this is not evaluated in this study as gender and race were male and predominantly Caucasian(151-153).

This is the first analysis of rilpivirine DDI interactions with boceprevir in HIV-infected patients. More effective interferon-free treatment options have recently become available for the treatment of chronic HCV. However, they are not approved for acute HCV and given the high costs, their availability is currently limited to a very small number of patients. Therefore, and given the results of the DAHHS in which 86% of the patients could be cured of their acute HCV with a 12-week treatment, it is likely that boceprevir will continue to be used for some time. We show that it can be safely combined with rilpivirine.

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Chapter 6

Immediate or delayed start of direct acting antivirals on hepatitis C in HIV-infected men-who-have-sex-with-men

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Submitted

Chapter 7

Use of whole genome sequencing in the Dutch Acute HCV in HIV study. A focus on phylogenetics, mixed infections and transmitted antiviral resistance

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ABSTRACT

Introduction

Within HIV positive men having sex with men the epidemic of hepatitis C (HCV) is ongoing. Transmission of resistant variants of HCV after failure of treatment with directly acting antivirals (DAA) could be a major threat to the effectivity of therapy. We determined whether HCV resistant variants to DAAs were prevalent amongst patients with an acute HCV infection diagnosed in 2013 and 2014 in the Netherlands.

Methods

Target enrichment for viral nucleic acid separation and deep sequencing were used to recover whole HCV genomes of 55 patients with an acute HCV infection. The genomes were assembled by *de novo* assembly and analysed for known DAA resistance mutations, phylogeny and mixed infections before and during acute HCV therapy.

Results

In acute HCV infected treatment-naïve patients the relevant resistance associated substitutions were in the Q80K (40%) in NS3/4a, the M28V (22%) and Q30H with Y93H (2%) in the NS5A and the M414T (2%) or S556G (2%) the NS5b gene. Patients who failed on boceprevir, peginterferon and ribavirin therapy developed mutations in the NS3 gene at position T54A and R155K.

Conclusion

The presence of resistant variants implies that the combination of DAAs should be carefully selected when these patients are treated with interferon-free DAA.

INTRODUCTION

Since very effective and well-tolerated direct-acting antivirals (DAAs) have become available, hepatitis C (HCV) has progressed from a difficult to treat to an almost universally curable infection. These new DAAs target specific regions in the HCV genome, resulting in very high cure rates when given in combination. Although, the first generation DAAs have increased the cure rates of genotype 1 infection, some continue to fail treatment potentially due to acquired resistance against the DAA and/or transmission of already resistant clades. Recently, HCV became endemic as a sexually transmitted infection amongst HIV positive men who have sex with men (MSM)(119). In this specific patient population, the risk of transmitted DAA drug resistance is clearly present as ongoing sexual transmission of HCV occurs.

Active surveillance of liver enzymes during antiretroviral therapy facilitates the early identification of HCV infections. In 2014, 99 HIV positive patients with a newly acquired HCV infection were identified through active surveillance in a multicenter study in the Netherlands(125). As part of the Dutch Acute HCV in HIV Study (DAHHS), most of these newly identified HCV positive patients were treated with peginterferon (pegIFN), ribavirin (RBV) and boceprevir (BOC)(130). At the same time they consented to donate blood for further analysis and this in turn allowed us to investigate the prevalence of DAA resistance amongst acutely HCV infected patients in an era when the first generation HCV protease inhibitors boceprevir and telaprevir had been in use for 2 years in the Netherlands.

Current methods for phylogenetic and antiviral drug resistance genotyping include RT-PCR based enrichment techniques of small regions of the genome with subsequent Sanger population sequencing or non-enriched direct next generation sequencing (186, 187). Due to the highly variable genome of HCV, especially between genotypes the design of RT-PCR primers that cover the whole genome of all genotypes has been proven to be virtually impossible. Whole genome sequencing (WGS) directly from clinical samples can contribute significantly to the understanding of clinical population structures, including minority populations, mixed infections and resistance mutations. However, with the current methodologies, non-enriched direct next generation sequencing, very high viral loads (> one million international units) are required for full genome deep sequencing analysis. We have previously shown that target enrichment, which uses custom designed 120-mer RNA nucleotides that

span the entire target genome, can recover (by hybridization) low copy numbers of viruses from clinical samples with sufficiently high sensitivity and specificity to enable ultra-deep whole genome sequencing(188, 189). In this study, we apply the target enrichment approach directly to clinical HCV positive specimens. The goals of this study were 2-fold. First we wanted to demonstrate the effectiveness of this sequencing method by deep sequencing 55 HCV genomes and generating close to complete genomes with high read depth. Secondly, using this method we want to examine the prevalence of transmitted DAA resistance among a relatively large group of patients that became HCV infected in the DAA era.

METHODS

Within the DAHHS, 65 patients with an acute HCV genotype 1 were included of which 57 were treated with 12 weeks of pegIFN, RBV and BOC. Patients initiated treatment within 26 weeks after their HCV transmission. At baseline, week 4 during treatment and 12 weeks after treatment, blood was drawn for additional analysis. All patients that initiated treatment with a viral load above 1000 IU/ml (CAP/CTM, V2, Roche diagnostics or Abbott Realtime M2000) were selected for WGS. Patients with a poor response to therapy at week 4 (above 1000 IU/ml) and patients who relapsed after treatment were analyzed for acquired resistance. All patients signed a written informed consent. This study was registered under NCT01912495.

Double stranded cDNA synthesis

Nucleic acid was extracted from EDTA plasma using the DSP Virus/Pathogen kit on the QiaSymphony with the complex200 protocol and nucleic acid was eluted in 60ul. Approximately 55 µl of the RNA extractions were concentrated down to ~11 µl and the complete 11 µl were used for first strand cDNA synthesis using the Superscript III Reverse Transcriptase kit (Life Technologies, 18080093) following the manufacturer's instructions, which required Random Primers (Life Technologies, 48190011), 10 mM dNTP Mix (Life Technologies, 18427013) and RNaseOUT (Life Technologies, 10777019). For second strand cDNA synthesis 20 µl from the first stand synthesis were processed with the Second Strand cDNA synthesis kit (NEB, E6111) following the manufacturer's instructions. All of the ds cDNA was purified using the Genomic DNA clean and concentrator TM -10 kit (Zymo Research, D4011) following the manufacturer's instruc-

tions and using 5x binding buffer and eluting in 30 µl of ultrapure nuclease free H_2O (Life Technologies, 4387936).

SureSelectXT Target Enrichment: RNA baits design

The 120-mer RNA baits spanning the length of 953 GenBank HCV partial and complete reference genomes were designed using an in-house PERL script developed by the PATHSEEK consortium. The specificity of the baits was verified by BLASTn searches against the Human Genomic plus the Transcript database. The custom designed HCV bait library was uploaded to SureDesign and synthesised by Agilent Technologies.

SureSelectXT Target Enrichment: Library preparation, hybridisation and enrichment

The purified ds cDNA were quantified using the Qubit dsDNA HS assay kit (Life Technologies, Q32854) and between 200-500 ng of ds cDNA was sheared for 150 seconds, using a Covaris E220 focused ultra-sonication system (PIP 175, Duty factor 5, Cycles per Burst 200). End-repair, non-templated addition of 3' poly A, adapter ligation, hybridisation, PCR (12 cycles pre-capture and 18 or 22 cycles post capture) and all post- reaction clean-up steps were performed according to the SureSelect^{XT} Automated Target Enrichment for Illumina Paired-End Multiplexed Sequencing 200 ng protocol (version F.2) on the Bravo platform WorkStation B from Agilent Technologies. All recommended quality control steps were performed on the 2200 TapeStation from Agilent Technologies.

Illumina sequencing

The samples were sequenced in multiple runs on an Illumina MiSeq sequencing platform with 500 bp v2 reagent sets. Base calling, adapter trimming and sample de-multiplexing were generated as standard producing paired FASTQ files for each sample.

Sequence data analysis

Trimming and quality control

Genome mapping, assembly and finishing was performed using CLC Genomics Workbench (version 7.5/7.5.1) including the CLC Microbial Genome Finishing Module (version 1.4) from Qiagen. For each data set, all read-pairs were subject to quality

control and reads were quality trimmed based on a cut-off of an average Phred score of 30 and the presence of ambiguous nucleotides.

Whole genome consensus sequencing

First an unbiased sequencing approach was taken using *de novo* assembly. For *de novo* assembly all trimmed reads from each sample were pre-filtered against a GenBank reference list containing 953 partial and complete HCV genomes. From the trimmed, paired HCV reads a subset of 50,000-100,000 reads were subsampled and used for *de novo* assembly. The contig(s) generated from each sample were aligned against 19 HCV reference genomes including different HCV genotypes (1a-6k) and if required, contig(s) were manually assembled and reversed complemented based on the best matching reference. All trimmed reads were mapped back to the generated contig(s) and duplicated mapped reads were removed. This was followed by local realignment, indels and structural variants were identified based on unaligned ends and locally realigned a second time. *De novo* consensus sequences were extracted using a threshold of 6x coverage and insertion of 'N' ambiguity symbols where coverage was low.

Some samples failed to generate contig(s) using *de novo* assembly due to a low number of HCV sequence reads (samples with low viral loads). For samples with a high number of HCV reads which did not generate contigs using *de novo* assembly, reference based mapping was performed. All trimmed reads were mapped against a GenBank reference list containing 953 HCV genomes to identify the best matching HCV reference. The best matching reference was identified by choosing the reference sequence with highest percentage of total sequence coverage (>30% as minimum) together with highest number of mapped reads. Each sample was mapped to the best matching reference using high stringency affinity gap cost parameters (mismatch cost 5, insertion cost 3, deletion cost 3, insertion open cost 6, deletion open cost 6, deletion extend cost 1, length fraction 0.8 and similarity fraction 0.9). The trimmed mapped reads were locally realigned, indels and structural variants were identified based on unaligned ends and locally realigned a second time. Consensus sequences were extracted using a threshold of 6x coverage and insertions of 'N' ambiguity symbols where coverage was low. To investigate whether dual infection was the reason for *de novo* assembly failure, unmapped reads were used to find the second best matching reference out of the 953 HCV genomes. Minimal requirements for reference coverage were set at >30% of reference coverage and number of reads

mapping to the second best matching reference needed to be more than 5% of the number of reads mapping to the best matching reference. Using the second best matching reference a consensus sequence was generated using the same parameters as described above.

Phylogenetic analysis

Consensus sequences from the samples were aligned to full genome consensus genotype references obtained from the Los Alamos HCV sequence database (2014 reference set) using Bioedit version 7.2.5(190). Sequences were cut to the same length which resulted in a 7632 nucleotide length alignment. Best fitting evolutionary substitution model was determined using BIC model testing in the CLC workbench version 8.0. Phylogenetic trees were made using Mega version 6 using the best fitting model (General Time Reversible with invariant sites and a gamma distribution of among site rate heterogeneity (GTR+I+G) with bootstrap value determined by 100 bootstrap resampling of the data(191). In addition pairwise distances were calculated using the Maximum Composite Likelihood model with a 100 bootstrap resampling of the data. The rate variation among sites was modelled with a gamma distribution (shape parameter = 5).

Directly Acting Antivirals (DAAs) resistance analysis

Positions of interest for DAA resistance (see tables S1-S3 in supplementary material) were identified in the GenBank reference HCV strain H77 polyprotein gene, complete codon (accession no. AF011751). All trimmed reads from each sample were mapped against the genes of interest (NS3/NS4a, NS5a and NS5b) using the default affine gap cost parameters described above followed by removal of duplicated mapped reads. The trimmed mapped reads were locally realigned, indels and structural variants were identified based on unaligned ends and locally realignment was performed a second time using the identified indels and structural variants as guidance track. Low frequency variant detection was called with a min. coverage filter of 20x and a min. count of 5 independent reads. Variant frequencies were called down to 1% if the coverage allowed for it (see table S4 in supplementary material). A relative read direction filter was used and variants with a read direction distribution significantly different from the expected were removed.

RESULTS

Sample selection

DAHHS baseline samples were available from 50 of the 65 patients (table 1). Fifteen patients were excluded because of samples being not available (n=12), viral load <1000 IU/ml (n=3). Two patients with a poor response to therapy at week 4 (above 1000 IU/ml) and three patients who relapsed after treatment were analyzed for acquired resistance. The total number of samples analyzed was 55.

Table 1. Baseline characteristics

		n=50	IQR
male		100%	
age	years	40	(33-47)
genotype 1	a	98%	
	b	2%	
HCV viral load	IU/ml	439.500	(40.450-2.362.500)
Cd4	E6/mL	650	(450-833)
Il 28B genotype			
CC		38%	
non-CC		54%	
unknown		8%	

Characteristics of patients analysed at initiation of treatment. All results are presented as median with interquartile range (IQR) or as percentage.

Result of whole genome sequencing

In this study we performed WGS directly on clinical specimens from 55 HCV positive samples. Using *de novo* assembly, we generated complete genomes (>90-100% recovery of the HCV genome) from 51 samples, partial genomes (>80-90% recovery of the HCV genome) from 3 samples and 1 low titre sample (3070 IU/ml) failed to generate a HCV genome (<50% recovery of the HCV genome) (table S5 in supplementary material). Overall the sequence data showed a strong correlation between the number of HCV copies in the diagnostic sample and the mean read depth across the genome (figure 1). From this data-set the lower limit of detection for WGS of HCV directly from clinical samples was estimated to a diagnostic value of ~3500 IU/ml.

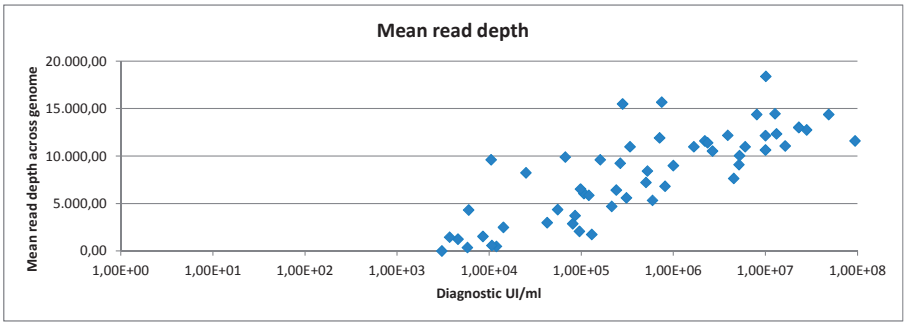

Figure 1: Figure 1 shows the relationship between the HCV copy number identified in the diagnostic sample (IU/ml x 2.7 = copies/ml) and the mean read depth obtained across the whole HCV genome.

Four samples (Na01, Ad04, Ef06, Ol12) failed *de novo* assembly despite having >90% on-target reads (reads mapping to HCV). Reference based mapping showed, from the highly variable region of the HCV *E2* gene (H77 numbering 872-1968), an inter-subtype (GT1A plus GT1A) dual infection in patient Ol12 (supplemental figure 1). For the samples from patient Na01 and Ad04, the second best mapping reference did not generate sufficient reference coverage i.e. to generate a second consensus sequence indicating that these patients did not have a dual infection. The first and second best reference mapping consensus sequence from patients Ef06 rooted very close to each other in the phylogenetic tree, suggesting strain variation rather than dual infection within the patient. Samples from patients Oc03 and Ob02 were used as controls and the reference based mapping approach, using the best and second best matching reference, generated two identical consensus sequences from each patient, both of which were identical to the *de novo* generated sequences (data not shown).

Phylogenetic analysis

Using the consensus sequences obtained by *de novo* assembly and the four best sequences obtained by reference based mapping a phylogenetic tree was generated. Consensus reference sequences from the Los Alamos 2014 HCV full genome reference set were added (figure 2). The figure shows several clusters suggesting various networks in which sexual transmission of HCV occurred.

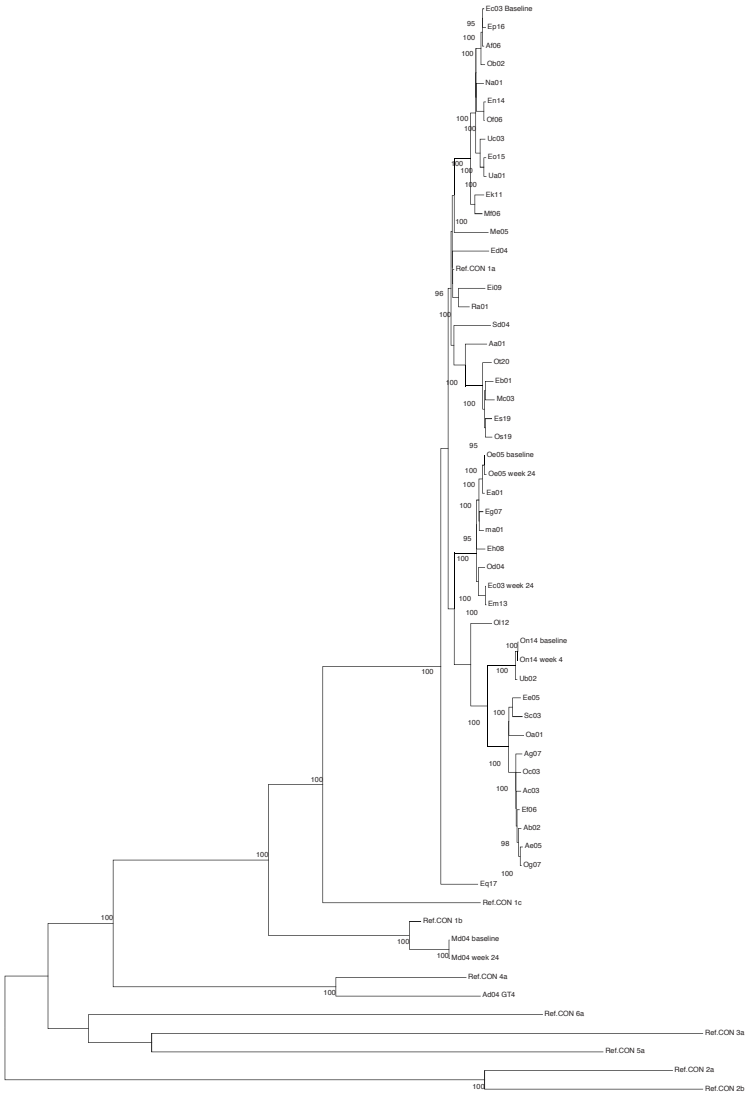

Figure 2. Maximum Likelihood tree based on the General Time Reversible model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.8480)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.0000% sites). There were a total of 7632 positions in the final dataset. All samples are baseline samples, except for samples with 'week 24' or 'week 4'added. These samples are taken at week 4 during or 12 weeks after the end of a 12-week treatment with boceprevir, peginterferon and ribavirin.

Variant detection

After exclusion of dual infection, four samples showed high variation between the viral quasispecies: two at baseline (Ef06, Na01) and a baseline together with week 4 (Ad04). Low frequency variant detection showed a high number of heterozygous single nucleotide variant (SNV) sites throughout the genome in patient Ef06 and Na01 compared to a homogenous sample from patient Oe03 (supplementary material Figure S2a-c). The variant data indicated an approximate 50:50% mix in patient Ef06 and a 40:60% mix in patient Na01. Low frequency variant detection was performed on the two samples from patient Ad04 and all shared heterogeneous biallelic SNV sites were identified to assess the ratio of the variants. The variant data from this patient indicated an approximate 50:50% mix of the two genotype 4 variants at baseline and after four weeks of treatment the ratio changed to approximate 10:90% mix (figure 3).

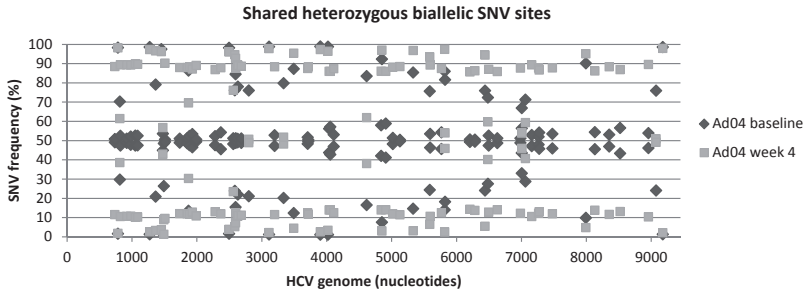

Figure 3: Figure 3 illustrates the shared heterozygous biallelic single nucleotides variant sites in patient Ad04 at baseline (dark grey) and after week 4 of treatment (light grey). The horizontal axis shows the HCV genome in nucleotides and the vertical axis shows the biallelic variant frequencies in percentage.

Directly Acting Antivirals (DAAs) resistance analysis

Detailed information on the target genes NS3/4a, NS5a and NS5b was extracted from the WGS data and mutations known to be associated with DAA resistance could be excluded or were identified in all baseline samples down to a 1% variant frequency level. In the acute HCV cohort DAA Resistant associated substitutions (RASs) were identified in 31 of 50 baseline samples. In 18 of the samples no RASs were identified and in one sample coverage was too low for accurate resistance analysis. In 38.7% (12/31) of the samples a single DAA RASs was identified and in 61.3% (19/31) multiple RASs were detected. The most prominent RAS in NS3 was Q80K in 40% of the samples. In the NS5a genes both M28V (22%) as well as H58P (26%) were found as

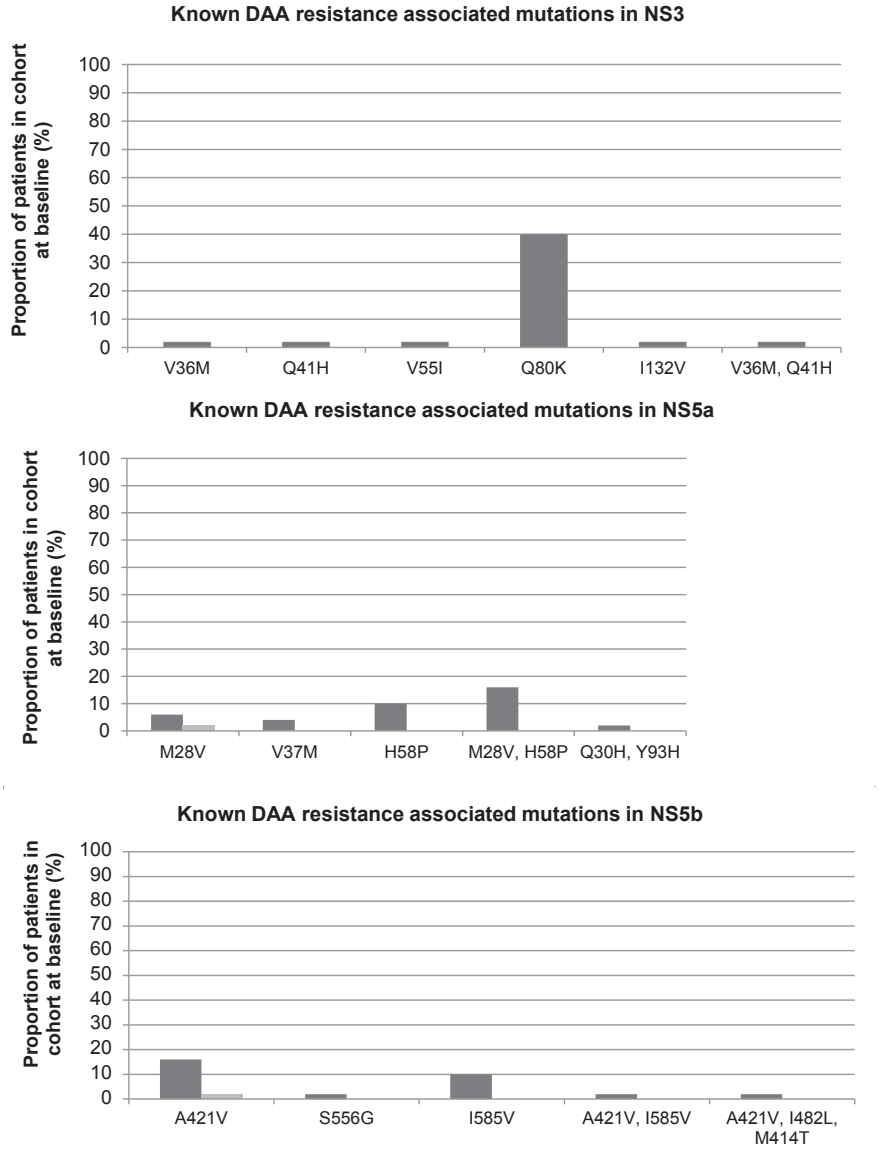

Figure 4: Figure 4 illustrates the known DAA resistance associated substitutions identified at baseline (n=50) in the acute HCV cohort. The vertical axis shows the proportion of patients (in percentage) in which the different mutations were identified. a) Known DAA resistance associated mutations in NS3/NS4a, b) Known DAA resistance associated mutations in NS5a and c) Known DAA resistance associated mutations in NS5b. Dark gray: variant frequency >50%, light gray: variant frequency <50%.

dominant substitutions with in 8 of 49 being combined. The A421V (18%) and I585V (12%) were seen as most occurring RASs in the NS5b gene (Figures 4 a/b/c). Remarkably, in patient Me05, an uncommon combination of Q30H and Y93H occurring at high frequency (both 99%) in NS5a was seen. This combination is associated with 93.136 fold resistance in EC50 against daclatasvir (192).

Development of RASs during therapy

Insufficient response at week 4 of therapy

Patient Ad04 was diagnosed with genotype 1a and had a baseline HCV viral load of $16.9x10^6$ IU/ml. At week 4 the patient presented with a viral load of $23.1x10^6$ IU/ml and reported that he had discontinued therapy one week before. BOC resistance associated mutations were not identified in patient Ad04 at the two sampling points (Supplementary table 6). Surprisingly, based on the sequence results this patient turned out to have a genotype 4 infection at both time points instead of the genotype 1.

Patient On14 was diagnosed with genotype 1a with a baseline viral load of $16.4x10^6$ IU/ml. At week four of treatment he had a viral load of 5790 IU/ml and reported to be treatment adherent. No baseline DAA RASs were detected, but after four weeks of therapy 2 BOC associated mutations (T54A and R155K) were identified (Supplementary table 6) at 53.3% and 14% variant frequencies, that result in a 10-fold decrease in BOC susceptibility (Tong et al, Biochemistry).

Relapse 12 weeks after treatment

Patient Ec03 was treated for 12 weeks and had an undetectable HCV RNA at week 4, but became detectable at week 12 after treatment. The baseline and week 12, post-treatment samples showed two different HCV genotype 1a strains (figure 2, Ec03 Baseline and Ec03 week 24). Second best reference approach showed that the genotype present at failure was not detected at baseline (data not shown). Therefore, WGS proved that this patient did not have a relapse but a reinfection.

Patient Md04 (genotype 1b) and Oe05 (genotype 1a) were successfully treated for 12 weeks. Both had a detectable viral load at week 4 of treatment but became negative at week 8. Both patients became detectable at week 12 after therapy and strains in both cases were identical to the baseline strains. At the time of relapse, patient

Oe05 showed a R155K dominant (92%) mutation, which is associated with major BOC resistance. In contrast, Md04 had no known acquired BOC mutations at relapse (supplementary figure S3a/b).

DISCUSSION

We have previously shown that targeted-enrichment is a useful approach to recover complete viral or bacterial genomes directly from clinical specimens without any prior amplification or *in vitro* culture (188, 189, 193, 194). Here we use this novel methodology to recover complete HCV genomes directly from clinical HCV positive plasma samples. The combination of whole-genome enrichment and deep sequencing allowed us to recover close to complete genomes for 98% of the samples, to perform a comprehensive DAA resistance and phylogenetic analysis and to assess the population structure in four samples with high variant numbers.

In this study, we hypothesized that patients with an acute HCV infection in 2013 or 2014 could theoretically have been infected with a resistant variant. Because failure to the first generation protease inhibitors was common, treatment experienced viremic patients could have transmitted their acquired resistant variant to their sexual partners. It is reassuring that the presence of typical boceprevir or telaprevir RASs in these treatment-naïve patients was limited and potentially observed in only three cases (6%). This involved the V36M substitution, which is associated with low level resistance (7 fold change in EC50) and V55I which is also associated with low level resistance (1.4 fold increase in EC50) (195, 196). However, these substitutions can occur also as natural variants so transmission from DAA failing patients cannot be proven (197).

As soon as the first HIV drugs became available, transmission of drug resistant HIV was observed among MSM (198, 199). This has led to the recommendation to test for transmitted drug resistance before the start of antiviral therapy in international HIV treatment guidelines. Recently, the first case of transmitted HCV DAA resistance was described by Franco and colleagues(181). As far as we know, this study is the largest of its kind that used whole genome deep-sequencing to look at the prevalence of transmitted DAA resistance in patients that became HCV infected in the DAA era.

Additionally, we observed a 40% prevalence of the Q80K substitution in the NS3/4a region. This substitution is found almost exclusively in genotype 1a(180). Our findings correlate with proportions found in AHCV cohorts in Germany (56%) but is higher than was seen in England (16%)(200, 201). However, in a recent Australian study, only 4% of the acutely infected patients had a Q80K substitution(202). The Q80K substitution results in a median 11-fold increase in the EC50 of simeprevir (IQR 7.4-13) against HCV genotype 1a. This has been associated with increased treatment failure with simeprevir, pegIFN and RBV and therefore resistance testing is advised(203, 204). In contrast, recent studies have shown that there is no impact on the overall response when simeprevir is given for 12 weeks in combination with sofosbuvir for chronic HCV (205, 206). However, when acute infections are treated with very short regimens (i.e. 8 week a simeprevir/sofosbuvir) and other negative predictors like high baseline viral loads are present, the Q80K substitution could have an impact on the sustained viral response rate(145).

At the time the patients in the current study became HCV infected, NS5a or NS5b inhibitors were not yet in clinical use. Therefore, the RASs that we detected in NS5a and NS5b were not selected by drug exposure but occurred as natural variants. The NS5a M28V substitution was found in 22% of the patients. This substitution could influence the effectivity of ombitasvir and elbasvir modestly(180). The reduced treatment success that was observed in patients with a M28V substitution that were treated with grazoprevir and elbasvir resulted in the first drug label that recommends resistance testing for treatment naïve genotype 1a patients(207). In a single other patient, the combination of the Q30H and Y93H substitutions in NS5a was documented, which results in high level resistance to NS5a inhibitors(180). Within the NS5b gene we found the clinical relevant M414T (2%) and the S556G (2%) substitutions. These substitutions can occur as a naturally occurring polymorphism but are also associated with virological failure in response to dasabuvir(180, 208).

Currently, there is ongoing debate as to whether resistance testing in treatment naïve patients is indicated. The presence of well-defined clusters of HCV transmission together with ongoing sexual transmission and the presence of a highly resistant HCV strain in one of our patients, clearly shows the potential of the transmission of a NS5a resistant virus. If patient Me05, with the Q30H and Y93H substitutions in NS5a, had been founder of a cluster (figure 2), this could have resulted in loss of susceptibility to all NS5a inhibitors in the entire cluster. With a median of 10 sexual partners per

6 months found in a recent study, this resistant variant could rapidly spread among MSM(209). Therefore, in particular if unexplained treatment failure is observed, baseline resistance testing may be indicated and certainly if treatment failure would be observed in >1 patient from a potential transmission cluster.

In conclusion, this study showed that a WGS technique that used target enrichment can be successfully applied to patients with an acute HCV. It was able to detect mixed infections and resistance associated mutations that were present at baseline as well as at the time of treatment failure. Fortunately, at the moment no convincing evidence of transmitted drug resistance was found. However, the presence of naturally occurring resistant variants could result in low treatment response rates when certain regimens are used.

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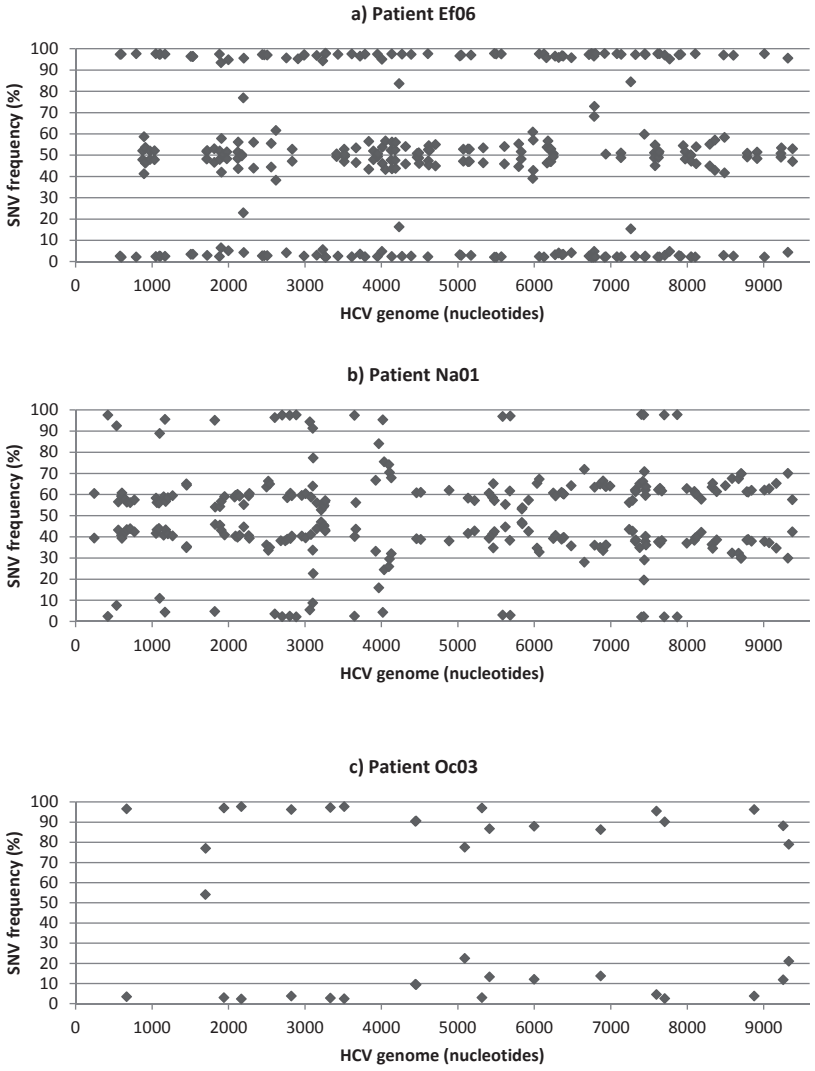
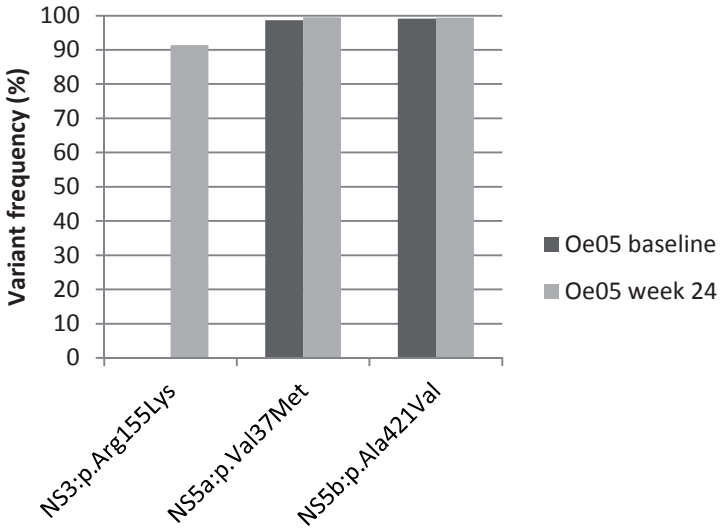
Suzan D. Pas, Gertine W. van Oord, Andre Boonstra. We acknowledge all partners within the PATHSEEK consortium (University College London, Erasmus MC, QIAGEN AAR, and Oxford Gene Technology). Collaborators in the DAHH-Study: Mark A.A. Claassen, Guido E.L. van den Berk, Jan T.M. van der Meer, Dirk Posthouwer, Fanny N. Lauw, Eliane M.S. Leyten, Peter P. Koopmans, Clemens Richter, Arne van Eeden, Wouter F.W. Bierman, Joop E. Arends. We acknowledge the infrastructure support of the MRC Centre for Medical Molecular Virology

SUPPLEMENTS

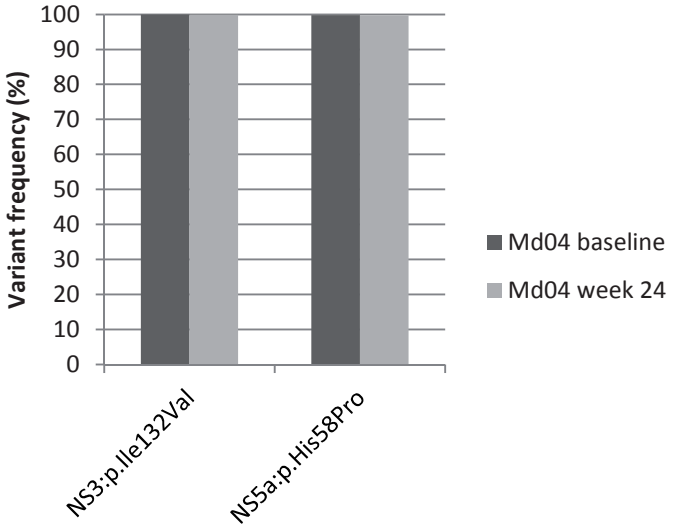
Supplementary figure 1: Maximum likelihood phylogenetic tree of full length HCV sequences with incorporation of 4 samples analyzed with reference based mapping



Supplementary figure S2a/b/c: All heterozygous single nucleotide variant sites found throughout the HCV genome in patient Ef06 (a), Na01 (b) and Oc03 (c) plotted against frequency. Patient Ef06 showed potential evidence of a 50:50% mix and patient Na01 a 40:60% mix. Patient Oc03 had only a few heterozygous variant sites and showed no evidence of an intermediate mix



a



b

Supplementary figure S3a/b shows the DAA resistance associated mutations identified in patient Oe05(a) and Md04 (b). The patients were sampled at baseline (dark grey) and at week 24 after treatment (light grey). The vertical axis shows the resistance variant frequency in percentage.

Table S1. Mutations known to be associated with resistance in NS3/NS4a

Amino acid position (per gene)	Sensitive aa	Amino acid in ref. H77 1a AF011751	3-letter code in H77	Resistant amino acid
36	V	V	Val	M/L
41	Q	Q	Gln	R/N/L/K/H
43	F	F	Phe	V/S/L/I
54	T	T	Thr	S/A
55	V	V	Val	I/A
56	Y	Y	Tyr	H
80	Q	Q	Gln	R/N/L/K/H
95	T	T	Thr	S
109	R	R	Arg	K
122	S	S	Ser	S/R/N/G/A
132	I	I	Ile	V
155	R	R	Arg	W/T/S/Q/M/K/G
156	A	A	Ala	V/T/S/N/G/F
168	D	D	Asp	Y/N/I/H/G/E/A
170	V	I	Ile	A

Table S1 lists the known direct acting antiviral resistance associated mutations in NS3/NS4a which was assessed in this study.

Table S2. Mutations known to be associated with resistance inNS5a

Amino acid position (per gene)	Sensitive aa	Amino acid in ref. H77 1a AF011751	3-letter code in H77	Resistant amino acid
28	M/L	M	Met	V/T
30	R/Q	Q	Gln	E/H/Q/R
31	L	L	Leu	F/M/V
37	V	V	Val	M
58	H	H	His	P
93	Y	Y	Tyr	C/H/N

Table S2 lists the known direct acting antiviral resistance associated mutations in NS5a which was assessed in this study.

Table S3. Mutations known to be associated with resistance in NS5b

Amino acid position (per gene)	Sensitive aa	Amino acid in ref. H77 1a AF011751	3-letter code in H77	Resistant amino acid
282	S	S	Ser	T
316	C	C	Cys	Y/N
365	S	S	Ser	?
368	S	S	Ser	T
395	A	A	Ala	G
411	N	N	Asn	S
414	M	M	Met	T
419	L	L	Leu	S
421	A	A	Ala	V
422	R	R	Arg	K
423	M	M	Met	V/T
444	N	N	Asn	K
445	C	C	Cys	F/N/G
448	Y	Y	Tyr	H/C
451	C	C	Cys	S/R
482	I	I	Ile	L
486	A	A	Ala	V
494	V	V	Val	?
495	P	P	Pro	S/L
496	P	P	Pro	?
499	V	A	Ala	?
553	A	A	Ala	V
556	S	S	Ser	F/N/G
559	D	D	Asp	G
565	S	S	Ser	F
585	I	I	Ile	V

Table S3 lists the known DAA resistance associated mutations in NS5b which was assessed in this study.

Table S4. Variant detection cut-off

Depth	Frequency
50x	10%
100x	5%
200x	2.5%
400x	2%
>1000x	1%

Sequence read depth cut-off used for variant detection with a minimum count of five independent reads.

Table S5. Overview of sequence results

Patient ID	Diagnostic value IU/ml	Note	Total no. of reads	On-target reads (%)	Coverage of ref. (%)	Mean read depth	Consensus Sequence (bp) length	Out-come
Eb02	9.55E+04	baseline	642,798	69.24	89.02	2,060.07	8546	partial
Ec03	5.24E+05	baseline	1,480,434	95.46	97.93	8,426.85	9401	complete
Ed04	1.67E+06	baseline	1,237,426	95.33	97.75	10,976.69	9384	complete
Ee05	4.87E+07	baseline	1,227,084	97.34	98.43	14,376.27	9449	complete
Ef06	1.91E+07	baseline	1,078,260	96.63	96.85	19,075.34	9298	complete
Eh08	1.00E+07	baseline	1,305,908	96.89	97.63	12,147.69	9372	complete
Ei09	8.13E+05	baseline	1,249,624	89.5	97.72	6,812.59	9381	complete
ma01	5.95E+05	baseline	892,498	72.53	96.82	5,331.41	9295	complete
Na01	6.85E+05	baseline	1,035,510	96.06	97.09	20,855.58	9321	complete
Oa01	4.27E+04	baseline	634,808	92.45	92.97	2,987.47	8587	complete
Ra01	1.20E+04	baseline	285,792	47.59	92.69	484.47	8898	complete
Oc03	1.05E+04	baseline	1,157,600	90.71	98.20	9,595	9427	complete
Of06	1.43E+04	baseline	821,494	42.25	98.10	2,485	9418	complete
Od04	3.38E+05	baseline	1,239,850	92.93	98.30	10,985	9437	complete
Ob02	8.06E+06	baseline	1,343,942	70.57	98.33	14,385	9440	complete
Og07	8.08E+04	baseline	928,300	39.4	91.39	2,863	8773	complete
Oe05	1.27E+07	baseline	1,033,120	94.9	86.10	14,460	8266	partial
Aa01	2.81E+05	baseline	1,459,442	93.39	98.17	15,485	9424	complete
Ab02	6.03E+06	baseline	918,110	93.33	98.28	10,985	9435	complete
Ek11	1.07E+05	baseline	904,798	67.98	98.02	6,056	9410	complete
Em13	1.61E+05	baseline	1,104,180	83.41	99.15	9,612	9518	complete
Eo15	2.31E+07	baseline	1,036,060	92.87	97.60	13,013	9370	complete
Ep16	2.80E+07	baseline	1,084,814	94.28	98.00	12,755	9408	complete
Eq17	2.65E+05	baseline	1,305,270	81.85	98.02	9,250	9410	complete

Table S5. Overview of sequence results (continued)

Patient ID	Diagnostic value IU/ml	Note	Total no. of reads	On-target reads (%)	Coverage of ref. (%)	Mean read depth	Consensus Sequence (bp) length	Out-come
Es19	5.23E+06	baseline	896,744	87.2	97.73	10,045	9382	complete
Ea01	7.49E+05	baseline	1,577,244	94.03	95.11	15,671	9131	complete
Ac03	9.81E+04	baseline	947,992	85.39	97.74	6,508	9383	complete
Ad04	1.69E+07	baseline	870,658	89.66	96.86	12,099	9299	complete
Ae05	3.10E+05	baseline	829,314	87.15	98.53	5,587	9459	complete
Af06	6.71E+04	baseline	1,079,900	91.65	96.00	9,890	677, 4366, 4174	complete
Ag07	5.55E+04	baseline	1,037,550	81.82	97.79	4,345	9388	complete
Mc03	9.40E+07	baseline	934,552	95.53	98.19	11,596	9426	complete
Md04	1.21E+05	baseline	911,260	89.46	98.04	5,864	9412	complete
Me05	4.52E+06	baseline	1,002,154	91.98	97.79	7,637	9388	complete
Mf06	2.51E+04	baseline	1,019,344	94.08	98.15	8,227	9422	complete
On14	1.64E+07	baseline	898,712	96.92	96.42	11,054	7643, 1613	complete
Op16	1.00E+07	baseline	789,490	94.68	90.65	10,643	599, 2702, 2309, 3093,	complete
Oq17	1.07E+04	baseline	594,852	20.25	88.75	572	7595, 925	partial
Os19	2.36E+06	baseline	1,014,772	92.6	98.59	11,406	9465	complete
Ot20	2.66E+06	baseline	875,084	95.74	97.99	10,521	9407	complete
Ec03	5.04E+05	week 24	926,832	91.63	94.00	7,208	9024	complete
Oe05	3.90E+06	week 24	1,250,586	97.19	97.97	12,173	9405	complete
Sc03	8.59E+04	baseline	716,228	96.14	97.68	3,716	9377	complete
Sd04	2.40E+05	baseline	1,022,048	92.17	97.92	6,415	9400	complete
Ua01	1.32E+07	baseline	1,006,068	97.52	98.02	12,330	9410	complete
Ub02	1.01E+07	baseline	2,075,844	92.81	98.13	18,395	9420	complete
Uc03	7.10E+05	baseline	1,530,604	94.73	97.85	11,909	9394	complete

Table S5. Overview of sequence results (continued)

Patient ID	Diagnostic value IU/ml	Note	Total no. of reads	On-target reads (%)	Coverage of ref. (%)	Mean read depth	Consensus Sequence (bp) length	Out-come
Ad04	2.30E+07	week 4	979,162	96.94	96.86	19,789.72	9299	complete
Md04	2.14E+05	week 24	956,070	94.4	93.70	4,688	9383	complete
On14	5.79E+03	week 4	638,858	15.77	93.70	364	9205	complete
Ol12	4.58E+03	baseline	2,410,436	30.97	95.97	16,081	9214	complete
En14	3.73E+03	baseline	3,607,426	6.58	95.86	1,448.10	9203	complete
Ou21	6.00E+03	baseline	2,135,530	28.53	96.36	4,308.14	9251	complete
Eg07	8.59E+03	baseline	1,942,860	10	91.20	1,520.65	8755	complete
El12	3.07E+03	baseline	1,799,658	0.78	31.00	0.92	-	fail

The column on-target reads (%) refers to the proportion of the total number of reads from each sample mapping to HCV. Coverage of ref. (%) is the fraction of the HCV genome recovered from the whole genome sequencing and is estimated from the consensus sequence (bp) length divided by 9600 (bp), which is an approximated size of the HCV genome. The mean read depth is the average sequence coverage across the genome. Sequence outcome is defined as complete when >90-100% of the HCV genome is recovered (coverage of ref.), partial when >80-90% of the HCV genome is recovered and fail when <50% of the HCV genome is recovered.

Table S6. DAA resistance associated mutations found in patients with poor treatment outcome

Sample ID	Gene	Amino acid change	Frequency (%)
Ad04 baseline	NS3/NS4a	none	
	NS5a	Met28Val	85.0
	NS5b	Ala421Val	96.3
	NS5b	Ile482Leu	98.6
	NS5b	Met414Thr	97.2
Ad04 week 4	NS3/NS4a	none	
	NS5a		
	NS5b	Ala421Val	99.8
	NS5b	Il482Leu	99.8
	NS5b	Met414Thr	99.5
	NS5b	Cys445Phe	97.0
	NS5b	Ala553Val	99.8
On14 baseline	NS3/NS4a	none	
	NS5a		
	NS5b		
On14 week 4	NS3/NS4a	Thr54Ala	53.6
	NS3/NS4a	Arg155Lys	14.0
	NS5a	none	
	NS5b		

Patient Ad04 and On14 were sampled at baseline and after four weeks of treatment. Known direct acting antiviral resistance associated substitutions were assessed at both sampling points and the variant frequencies were identified from the sequence data.

Chapter 8

Frequencies of circulating MAIT cells are diminished in chronic HCV, HIV and HCV/HIV co-infection and do not recover during therapy

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ABSTRACT

Introduction

Mucosal-associated invariant T (MAIT) cells comprise a subpopulation of T cells that can be activated by bacterial products and cytokines to produce IFN-γ. Since little is known on MAIT cells during HCV infection, we compared their phenotype and function in comparison to HIV and HCV/HIV co-infected patients, and determined the effect of IFN-α-based and direct-acting antiviral therapy on MAIT cells of HCV patients.

Methods

Blood samples from patients with chronic HCV (CHCV), virologically suppressed HIV, acute HCV/HIV co-infection (AHCV/HIV) and healthy individuals were examined by flowcytometry for phenotype and function of MAIT and NK cells.

Results

Compared to healthy individuals, the frequency of CD161+Vα7.2+ MAIT cells was significantly decreased in patients with CHCV, HIV and AHCV/HIV co-infection. CD38 expression on MAIT cells was increased in AHCV/HIV patients. MAIT cells were responsive to IFN-α *in vitro* as evidenced by enhanced frequencies of IFN-γ producing cells. IFN-α-based therapy for CHCV decreased the frequency of IFN-γ+ MAIT cells, which was still observed 24 weeks after successful therapy. Importantly, even after successful IFN-α-based as well as IFN-α-free therapy for CHCV, decreased frequencies of MAIT cells persisted. We show that the frequencies of MAIT cells are reduced in blood of patients with CHCV, HIV and in AHCV/HIV co-infection compared to healthy individuals. Successful therapy for CHCV did not normalize MAIT cell frequencies at 24 weeks follow up.

Conclusion

The impact of HIV and HCV infection on the numbers and function of MAIT cells warrant further studies on the impact of viral infections and the antimicrobial function of MAIT cells.

INTRODUCTION

Following infection with hepatitis C virus (HCV), hepatocytes are triggered to produce type I and III interferons (IFN), which induce the expression of hundreds of IFN stimulating genes (ISG) with anti-viral activity (210-212). However, despite the induction of ISG, viral titers increase during acute HCV infection, and in the majority of infected individuals the virus is able to establish a chronic infection of the liver, which indicates that the immune response is ineffective (213, 214). Besides the induction of ISG, IFN also activates natural killer (NK) cells, T cells and dendritic cells (DCs), and are therefore important immunomodulators (211, 215-218). Similar as in HCV, type I IFN are produced in large amounts after infection with human immunodeficiency virus (HIV), causing induction of antiviral responses that target every step of the HIV life cycle (218).

In recent years, our understanding of Mucosal-Associated Invariant T (MAIT) cells in chronic HIV infection has increased substantially. Most MAIT cells are CD8+ or double negative for CD4 and CD8, and characterized by the expression of CD161 and the invariant T cell receptor (TCR) Vα7.2 that recognizes vitamin metabolites presented by MR1, a MHC class I related protein, on the surface of antigen-presenting cells (219, 220). MAIT cells are also activated by IL-12 and IL-18 in an MR1-independent manner (221). MAIT cells are abundant in human blood (1-10% of CD8+ T cells) and are known for their antimicrobial activity to bacteria and yeast in the gut and lungs (222, 223) via release of cytokines and cytotoxic enzymes (219). Interestingly, MAIT cells are reduced in peripheral blood and lymph nodes of patients with chronic HIV infection, and their cytokine production and cytolytic functions are severely affected which has been suggested to be the result of exhaustion. Importantly, the loss and dysfunction of MAIT cells are not recovered after successful combination antiretroviral therapy (cART) therapy (224-231). It has been suggested that the functional impairment and numerical decline of MAIT cells contributes to the high incidence of bacterial infections observed in HIV patients (227). At the moment it is unclear what causes the depletion of MAIT cells in HIV infection. Similar findings were reported recently in patients chronically infected with HCV where the MAIT cell numbers in blood were severely reduced during persistent infection (232). Also in chronic HCV, successful HCV clearance by IFN-free therapy does not result in normalization of MAIT cell numbers.

Because little information is available on the role of MAIT cells in HCV infection, we examine in this study the impact of HCV infection on MAIT cells. In addition, we investigate the consequence of IFN-α exposure on NK cells and MAIT cells during IFN-α based therapy for CHCV and acute-HCV/HIV co-infection.

METHODS

Patients and healthy subjects

Heparinized blood was collected from 33 patients with chronic HCV (CHCV) infection, 9 acute HCV patients with cART-suppressed HIV (AHCV/HIV), 10 patients with cART-suppressed HIV mono-infection and 12 healthy subjects. The patient characteristics are listed in table 1. 33 CHCV patients were treated in 4 different historical treatment regimens, and blood was collected at multiple time-points. In cohort 1, 11 patients were treated with pegylated-IFN-alpha-2a (PegIFN-α) and ribavirin for 24 or 48 weeks, according to HCV genotype (NCT00422838, (233)). Patients in cohort 2 (n=11) were treated with telaprevir, PegIFN-α and ribavirin for 24 or 48 weeks, according to their fibrosis level and previous treatment response to PegIFN-α and ribavirin. Consistent with international guidelines (48), patients were treated with telaprevir, PegIFN-α and ribavirin for the first 12 weeks, and continued treatment consisting of PegIFN-α and ribavirin only (NCT01641094). In this treatment cohort, naïve patients, patients with a partial response (>2log drop in viral load) to previous IFN-based therapy, and patients without cirrhosis were treated for 24 weeks when HCV RNA was undetectable (<15 IU/l) at week 4 and 12 during therapy. In cohort 3, 5 patients were treated with daily daclatasvir and twice daily asunaprevir for 24 weeks (NCT02282709, (234)). In cohort 4, 6 patients were treated with sofosbuvir and daclatasvir with or without ribavirin for 12 or 24 weeks according to the international guidelines (AASLD/IDSA Recommendations for testing, managing, and treating hepatitis C 2015 (hcvguidelines.org) and EASL: recommendations on treatment of hepatitis C 2015). The selection of the treatment regimen was solely made by the treating physician. For patients with CHCV, blood was collected at baseline, week 4, week 12 during therapy and 24 weeks after end of therapy in all 4 cohorts. Patients with acute HCV and cART-suppressed HIV were treated in cohort 5 (n=9). Patients were treated within 26 weeks after HCV infection with 12-weeks boceprevir, PegIFN-α and ribavirin (NCT01912495). Blood was collected at baseline and at week 4 during therapy. Patients with cART-suppressed HIV mono-infection (n=10) and 22 healthy subjects (67% male, average age: 54

Table 1: Patient characteristics

	All CHCV	CHCV	CHCV	CHCV	CHCV	AHCV/HIV	HIV
		cohort 1	cohort 2	cohort 3	cohort 4	cohort 5	
Treatment strategy		PegIFN/riba	PegIFN/riba telaprevir	asunaprevir daclatasvir	sofosbuvir daclatasvir	PegIFN/riba boceprevir cART	cART
Number	33	11	11	5	6	9	10
Gender (% male)		73	82	80	67	100	100
Age (mean, yrs)		45 (27-60)	50 (25-61)	52 (43-66)	59 (36-70)	40 (23-58)	49 (27-65)
HCV RNA (mean, IU/ml)		$4.6x10^6$ ($3.7x10^2$-$2.7x10^7$)	$2.90x10^6$ ($3.5x10^4$-$8.2x10^6$)	$1.2\ x10^6$ ($1.6x10^5$-$2.3x10^6$)	$2.7\ x10^6$ ($8.3x10^4$-$5.3x10^6$)	$0.3\ x10^6$ ($2.0x10^2$-$1.9x10^6$)	
ALT (mean, U/l)		79 (34-164)	70 (29-140)	188 (94-269)	126 (24-196)	324 (33-1070)	
Fibrosis (%) 0-1/2/3/4/n.d.		30/50/20/0/0	27/18/36/18/0	80/0/0/20/0	0/0/33/67		
SVR (%)		82	82	100	100	100	
HCV genotype (%) 1/2/3/4		36/18/46/0	100/0/0/0	100/0/0/0	83/17/0/0	100/0/0/0	
HIV Load <20 geq/ml (%)						100	80
CD4 (mean, $x10^9$/ml)						0.87 (0.64-1.37)	0.67 (0.47-0.96)

CHCV: chronic hepatitis C virus; AHCV: acute hepatitis C virus; cART: combination antiretroviral therapy; ALT: alanine-aminotransferase; SVR: sustained virological response; PegIFN: peginterferon; riba: ribavirin; n.d.: not-defined

(range 42-70)) were included as controls. The institutional ethical review board of the Erasmus Medical Center approved the protocols, and written informed consent was obtained from all individuals.

Analysis of cell surface molecule expression by flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by (Ficoll-PaqueTM plus, Amersham) and frozen at -150°C. PBMC were thawed and washed with RPMI 1640 (Lonza) with 10% FCS (Lonza). For flow cytometry, 500,000 PBMC were used for each staining. Cells were stained with anti-CD38-PerCp-eFluor710 (HB7), anti-CD3-PE-Cy7 (UCHT1), anti-CD161-Pacific Blue (HP-3G10, all eBiosciences), anti-CD4-APC-H7 (SK3, BD Biosciences), anti-TCR Vα7.2-PE (3C10, Biolegend), anti-CD56-APC (N901, Beckman) and live/dead marker (Aqua, Life technologies) for 20 minutes at 4°C in the dark. Positivity for CD38 was determined by setting the gates using internal negative cells. Cells were washed and marker expression was detected by flow cytometry (Canto-II, BD). MAIT cells were defined as CD3+CD161+Vα7.2+ cells within the lymphocyte gate and NK cells were defined as CD3-CD56+ cells within the lymphocyte gate. Data was analyzed using FlowJo version 10.1 (Tree Star Inc).

Analysis of intracellular cytokines by flow cytometry.

The percentage of cells producing IFN-γ was measured by flowcytometry using various stimuli. For each condition, 500,000 cells were stimulated in a 24 wells plate with IL-12 (0.25 ng/ml, Miltenyi), IL-18 (50 ng/ml, MBL) and IFN-α (2500 IU/ml, Intron-A, Merck). For all conditions, cells were stimulated for 16 hours at 37°C at 5% CO_2. Brefeldin A (10 µg/ml, Sigma) was added and the cells were incubated for another 3 hours. Cells were stained with anti-CD3-PerCp-Cy5.5 (UCHT1), anti-CD4-APC-H7 (SK3, both BD biosciences), anti-CD69-PE-Cy7 (TPI.55.3), anti-CD161-PB (HP-3G10, both eBiosciences), anti-TCR Vα7.2-PE (3C10, Biolegend), anti-CD56-APC (N901, Beckman) and Live/ dead marker (Aqua, Life technologies). Cells were fixed, permeabilized and stained with anti-IFN-γ-FITC (25723.11, BD Biosciences). Cytokine-producing cells were detected by flow cytometry (Canto-II, BD). Quadrants were set on low or absent expression on lineage negative cells. Data was analyzed using FlowJo version 10.1 (Tree Star Inc).

Statistics

Statistical comparison was tested using the Kruskal-Wallis and Mann-Whitney test for unpaired non-parametric analyses and the Paired student t-test for paired ob-

servations. A *p* value ≤ 0.05 was considered significant. All data was analysed using GraphPad Prism (GraphPad Software).

RESULTS

MAIT cells are decreased in patients with chronic HCV, HIV and AHCV/HIV co-infection compared to healthy controls.

We investigated whether MAIT cells are affected during chronic viral infections and compared them to NK cells. For this purpose, the frequencies of CD3+CD161+Vα7.2+ MAIT cells and CD3-CD56+ NK cells were determined in peripheral blood of patients with CHCV, HIV, AHCV/HIV and healthy individuals by flow cytometry (figure 1A and 1B). A significant decrease was observed in the frequencies of MAIT cells in CHCV, HIV and AHCV/HIV patients as compared to healthy controls (p=0.004, p=0.04 and p=0.01, respectively, figure 1B). No differences were observed in frequencies of NK cells in patients with AHCV/HIV co-infection compared to healthy controls (235-238), whereas NK cells in CHCV and HIV mono-infected patients were decreased compared to healthy controls (p=0.009 and p=0.003, respectively) (figure 1B). Next, we determined the expression of the activation marker CD38 on MAIT cells and observed that patients with AHCV/HIV co-infection had increased frequencies of activated MAIT cells in peripheral blood compared to HIV, CHCV and healthy individuals (p=0.01, p<0.001, p=0.002 respectively, figure 1B). No association between the frequency of MAIT cells and ALT levels (r-value: -0.185, p=0.42) or viral load (r-value: 0.019, p=0.93) was observed (figure 1C). Also, stratification of patients with CHCV of their fibrosis stage showed similar frequencies of MAIT cells (figure 1C).

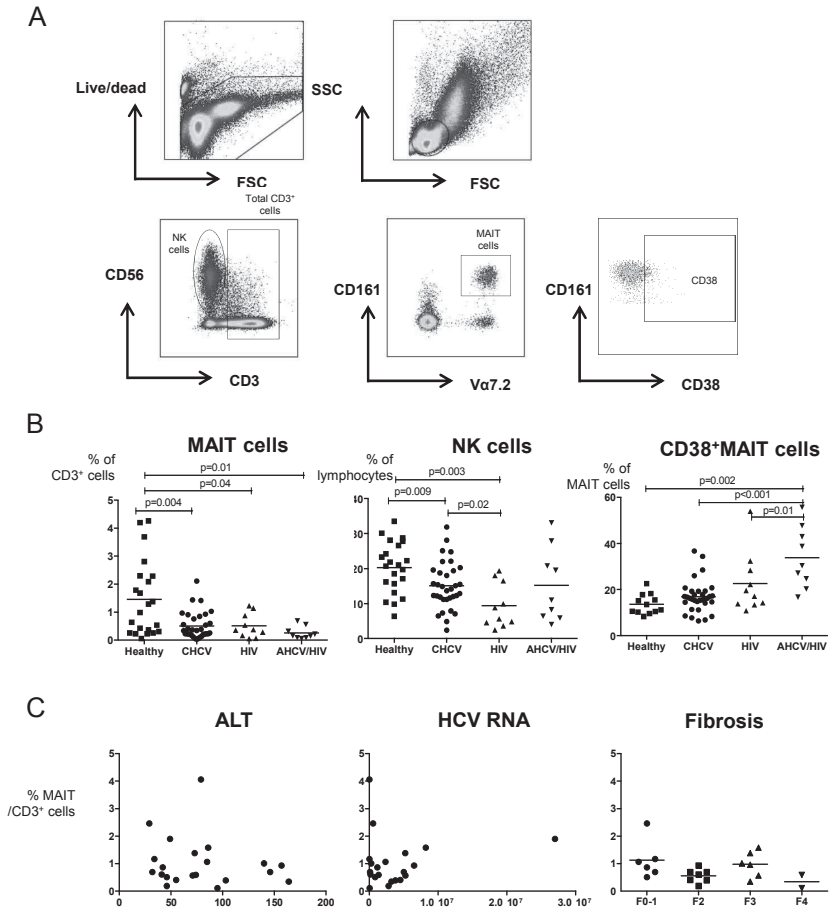


Figure 1: Frequencies of MAIT cells are decreased in patients with CHCV, HIV and AHCV/HIV co-infections compared to healthy individuals. (A) Representative dot plots and gating strategy of CD3+CD161+ Vα7.2+ MAIT cells and CD3-CD56+ NK cells. (B) The percentage of MAIT cells within the CD3+ T cell population, NK cells within the lymphocyte population, and CD38+MAIT cells within the total MAIT cell population of healthy individuals (n=22 for frequencies of MAIT and NK cells; n=12 for frequencies of CD38+ MAIT cells), chronic HCV (CHCV) patients (n=33), cART-suppressed HIV (HIV) patients (n=10) and patients with acute HCV/HIV co-infection (AHCV/HIV) (n=9). (C) Frequency of MAIT cells within the CD3+ T cell population in CHCV patients do not correlate with ALT, HCV RNA or fibrosis score levels. No association between the frequency of MAIT cells and ALT levels (r-value: -0.185, p=0.42) or viral load (r-value: 0.019, p=0.93) was observed. Statistical comparison was tested using Mann-Whitney test for unpaired analyses. Spearman's correlation test was used for the first two panels of figure 1C.

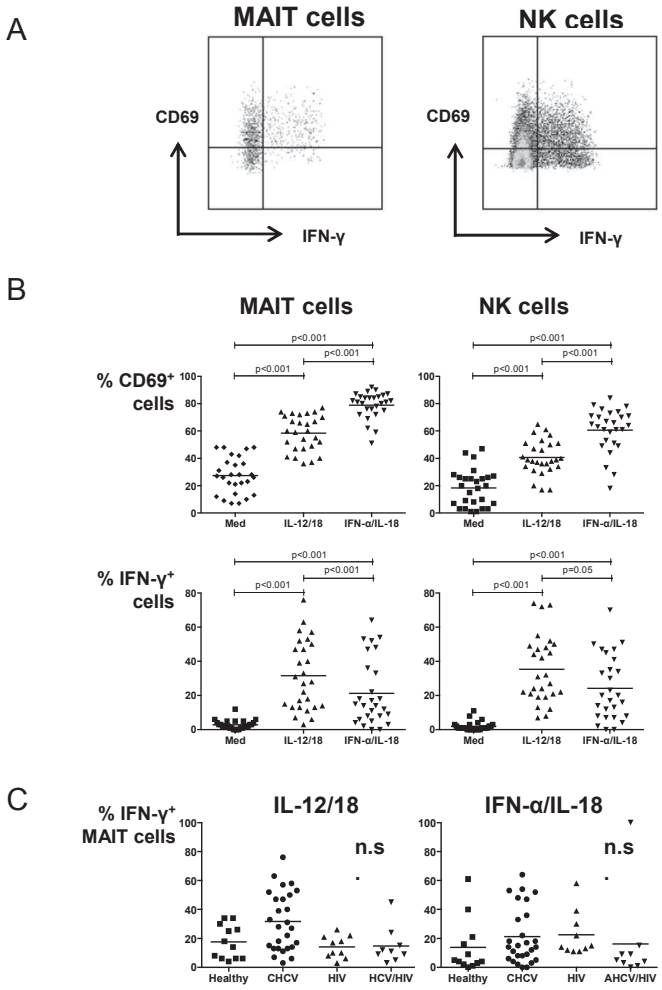


Figure 2: MAIT cells function does not differ between patients with chronic HCV, HIV and AHCV/HIV co-infection. (A) Representative dot plots of CD69 expression and IFN-γ production by MAIT and NK cells in CHCV after stimulation with IL-12/IL-18. (B) Frequency of CD69 expression and IFN-γ producing MAIT cells and NK cells in CHCV patients after 19 hours stimulation with medium, IL-12/IL-18 and IFN-α/IL-18. (C) Frequency of IFN-γ producing MAIT cells after various stimuli between healthy individuals, patients with CHCV, HIV and AHCV/HIV co-infection. Statistical comparison was tested using paired T test (2B) and Mann-Whitney test (2C).

Frequency of IL12/IL-18 induced IFN-γ producing MAIT cells does not differ between patients with CHCV, HIV, AHCV/HIV co-infection or healthy individuals.

Next, we determined the ability of MAIT cells from the various patient groups to respond to IL-12/18 as well as to IFN-α/IL-18 and evaluated CD69 surface expression and production of IFN-γ. A representative dot plot and gating strategy after stimulation of CHCV peripheral blood with IL-12/18 is shown in figure 2A. As shown in figure 2B, CD69 expression on both MAIT cells and NK cells was upregulated upon stimulation with IL-12/18 and IFN-α/IL-18, as compared to medium control conditions. The frequency of IFN-γ producing MAIT cells was significantly increased upon stimulation with IL-12/18. Importantly, IFN-α was also found to be a potent activator of MAIT cells (figure 2B). Next, we determined the function of MAIT cells in different viral infections and observed that MAIT cells are equally capable to become activated and produce IFN-γ upon stimulation with IL-12/18 and IFN-α/IL-18 in CHCV, HIV, AHCV/HIV co-infection and healthy individuals (figure 2C).

IFN-based therapy for chronic HCV reduces the frequency of IFN-γ producing MAIT cells upon IL-12/IL-18 stimulation.

Figure 2B shows that IFN-α is a potent stimulator of MAIT cells *in vitro*. Since IFN-α has potent antiviral activity against HCV, we determined whether IFN-α-based therapy for CHCV activates MAIT cells and affects MAIT cell frequencies in CHCV and AHCV/HIV co-infection. Twenty-two CHCV patients were treated with IFN-based therapy with (n=11) or without (n=11) telaprevir. All patients were HCV RNA negative at week 12 during therapy. We observed that IFN-α-based therapy did not alter MAIT cell frequencies, but did increase CD38 expression on MAIT cells ($p<0.001$, figure 3A, upper panels). No difference was observed between treatment with or without addition of telaprevir (supplementary figure 1). The NK cell frequency was reduced early during IFN-based therapy ($p<0.001$), but this was not sustained at week 12 (figure 3A, upper panels). Recently, IFN-free therapy became available for clinical use and has substituted IFN-α-based therapy because of higher SVR rates and reduced side-effects. Eleven CHCV patients were treated with an IFN-free regimen and we determined if the observed effects on MAIT cells were due to a direct effect of IFN-α or to viral load decline. In all patients, HCV RNA titers were undetectable after 4 weeks of treatment; however, no effect was observed on MAIT cell frequency or activation (figure 3A, middle row). In addition, no effect was observed on NK cell frequencies (figure 3A, middle panels). These data suggest that the increased frequencies of CD38-expressing MAIT cells

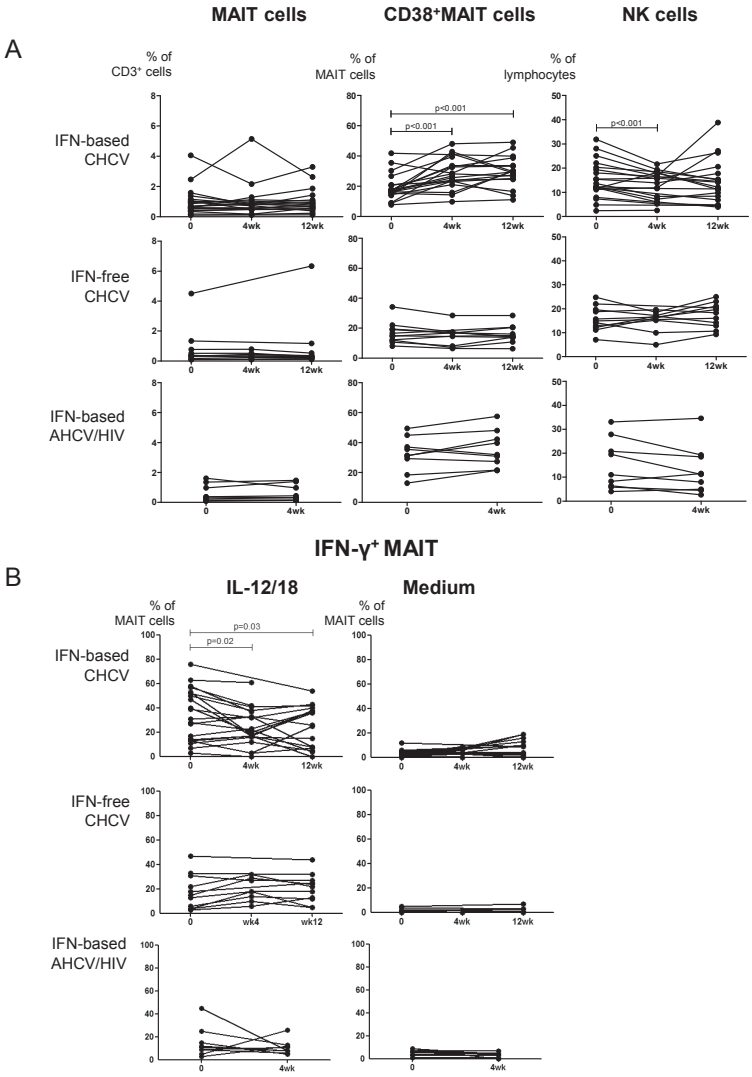


Figure 3: IFN-based therapy for chronic HCV increases expression of CD38 on MAIT cell but reduces MAIT cell function. (A) Frequency of MAIT, CD38+MAIT cells and NK cells during IFN-based therapy for CHCV (cohorts 1 and 2, n=22), IFN-free therapy for CHCV (cohort 3 and 4, n=11) and IFN-based therapy for AHCV/HIV co-infection (cohort 5, n=9). At week 12 during therapy for CHCV and at week 4 during therapy for AHCV/HIV, all patients were HCV RNA negative. (B) Frequency of IFN-γ producing MAIT cells after stimulation with IL-12/18, and medium during IFN-based and IFN-free therapy for CHCV infection (baseline, 4wk, 12wk), and during IFN-based therapy for AHCV/HIV (baseline, 4wk). The baseline frequencies of CD38+ MAIT cells and IFN-γ+ MAIT cells of the patient groups receiving IFN-based and IFN-free therapy were similar (Mann Whitney test). All other statistical comparisons were tested using Wilcoxon matched pairs T test.

in CHCV patients is the consequence of exposure to IFN-α, rather than of viral load decline.

Patients with AHCV/HIV co-infection were treated with a combination of boceprevir, PegIFN-α and ribavirin; all patients were HCV RNA negative at week 4 during therapy. IFN-α-based therapy did not alter MAIT cell or NK cell frequencies in this patient population (figure 3A, lower panels). The enhanced expression of CD38 on MAIT cells of patients with AHCV/HIV co-infection (figure 1) was not further increased during therapy (figure 3A, lower panels).

To investigate whether MAIT cell function was altered during therapy for HCV, cells were stimulated with IL-12/18. Surprisingly, although IFN-α-based therapy for CHCV caused increased expression of activation marker CD38 on MAIT cells as shown in figure 3A, a decrease in the frequency of IFN-γ producing MAIT cells was observed upon stimulation with IL-12/18 during therapy compared to medium ($p=0.02$ at week 4, $p=0.03$ at weeks 12; figure 3B, upper panels). Neither in CHCV patients treated with IFN-free therapy, nor in patients with AHCV/HIV co-infection, therapy altered the frequencies of IFN-γ producing MAIT cells (figure 3B, middle and lower panels).

Successful therapy for chronic HCV does not restore MAIT cell frequency 24 weeks after therapy.

Besides evaluation of the effect of therapy on phenotype and function of MAIT cells, it is highly relevant to determine the effect of viral clearance. Blood was collected 24 weeks after end of therapy. No samples from patients with AHCV/HIV co-infection were available after therapy. Figure 4 shows that 24 weeks after cessation of IFN-based therapy as well as IFN-free therapy, the MAIT cell frequencies were not restored in CHCV patients and remained low (figure 4, upper panels). The increased expression of CD38 on MAIT cells during IFN-based therapy for CHCV (figure 3A) was not maintained 24 weeks after therapy and returned to pre-treatment levels (figure 4). Interestingly, the suppressive effect of IFN-based therapy on IFN-γ producing MAIT cells as shown in figure 3, was still observed 24 weeks after cessation of therapy upon IL-12/IL-18 stimulation ($p=0.002$, figure 4). Therefore, it appears that IFN-based therapy for CHCV affects IFN-γ producing MAIT cells at least up to 24 weeks after therapy. No differences in frequencies of MAIT cells, CD38+ MAIT cells or IFN-γ producing MAIT cells were observed before and 24 weeks after IFN-free therapy for CHCV (figure 4, right panels).

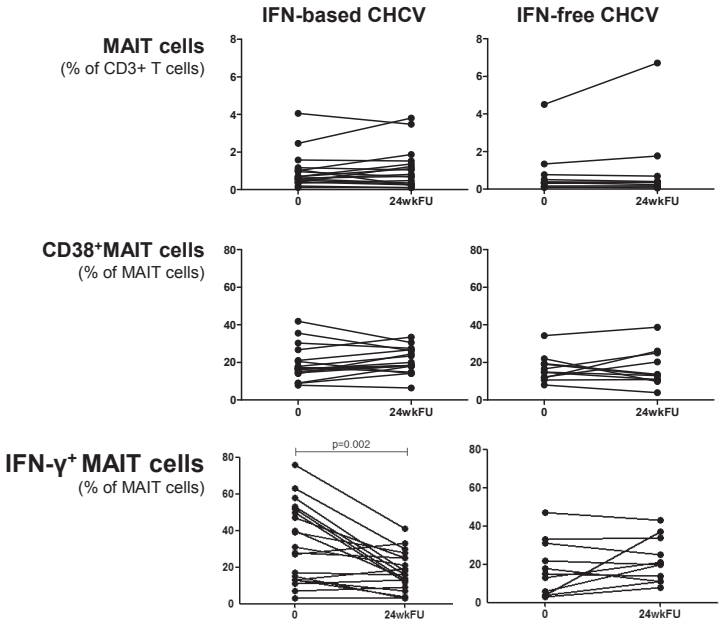


Fig. 4. Frequency of MAIT cells in peripheral blood of chronic HCV patients does not recover after successful therapy. Frequencies of MAIT, CD38+MAIT and IFN-γ+ MAIT cells before and 24 weeks after successful IFN-based (left, n=22) and IFN-free (right, n=9) therapy for CHCV. Frequencies of IFN-γ+ MAIT cells were measured after 19 hours stimulation with IL-12/18. Statistical comparison was tested using Wilcoxon matched pairs T test.

DISCUSSION

In this study we performed a detailed analysis on MAIT cells in CHCV, HIV and AHCV/HIV infections. We observed that the frequency of MAIT cells is decreased in all three groups of infected patients compared to healthy individuals, and that no normalization was observed following successful anti-HCV therapy at a follow-up period of 24 weeks. Moreover, the frequency of IFN-γ producing MAIT cells upon IL-12/IL-18 stimulation was reduced in blood from HCV patients receiving IFN-α-based therapy, but not in blood from patients receiving IFN-free therapy.

Our findings on reduced MAIT cell frequencies in HIV and HCV are in line with others (224-227, 239). We confirmed the recent study by Hengst et al. (23) by showing reduced frequencies of CD161+Vα7.2+MAIT cells in peripheral blood of CHCV patients, long after viral clearance. From our studies, we cannot conclude whether the reduced fre-

quencies of MAIT cells are due to depletion of cells via apoptosis, migration of MAIT cells from blood to peripheral organs or skin, or due to down-regulation of characteristic markers, such as CD161. However, others have demonstrated that it is unlikely that CD161 down-regulation is responsible for the observed MAIT cell numbers in HIV infection (19). In addition, it has been shown that MAIT cells are systemically depleted in simian immunodeficiency virus infected rhesus macaques, a model often used to investigate HIV (240). This suggests that migration of MAIT cells to peripheral organs in HIV is less likely. More research is needed to clarify the cause of the depletion in HIV and HCV. Importantly, virus eradication by IFN-based therapy as well as by IFN-free therapy did not lead to normalization of the reduced MAIT cell frequencies at 24 weeks after cessation of therapy. These findings are similar to the observations by Hengst et al. who showed nonreversible MAIT dysfunction 48 weeks after end of treatment with sofosbuvir and ribavirin (23). The long-term complications of low MAIT cell frequencies may be an increased susceptibility of bacterial infections after viral clearance. These observations are reminiscent of the findings reported in HIV where long-term suppression of viral replication by cART does not result in normalization of MAIT numbers in blood (224, 226, 227). It is well-known that the immune system in cART-controlled HIV patients remains at a higher activation status as compared to control healthy individuals, but it is unknown whether the enhanced immune activation mediates MAIT cell depletion. Besides a lower frequency of MAIT cells, we also observed a lower frequency of NK cells in HIV patients compared to CHCV and healthy individuals ($p=0.02$ and $p=0.02$). Decreased NK cell frequencies and function in HIV patients has been described before to be associated with a more rapid progression to AIDS in untreated patients (241).

Besides their numbers, the function of MAIT cells is also an important determinant of their contribution to the overall response to HIV or HCV. As shown in this manuscript and reported by others, MAIT cells are highly responsive to IL-12/IL-18 (221, 225). We now show that MAIT cells are also responsive to the combination of IFN-α and IL-18, leading to the production of IFN-γ. The ability of MAIT cells to respond to IFN-α is shared with NK cells.

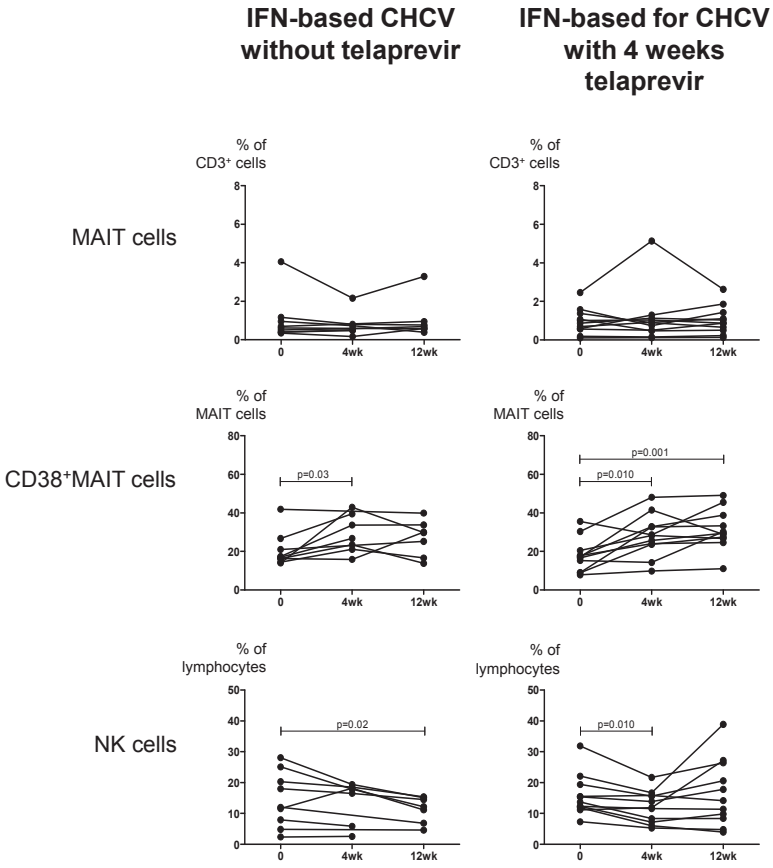
The function of MAIT cells, as reflected by the frequency of IFN-γ producing MAIT cells upon IL-12/IL-18 stimulation was similar in patients with CHCV, HIV or AHCV/HIV as compared to healthy individuals. Recently, dysfunctional MAIT cells from chronic HCV patients with respect to their ability to produce IFN-γ were observed upon MR1-

dependent antigen stimulation, but -similar to our findings- not upon IL-12/IL-18 stimulation (23). However, since MAIT cells are decreased in these patients groups, the total amount of IFN-γ by these MAIT cells is likely to be decreased. Also the activation state of MAIT cells from CHCV and HIV patients were not affected. In contrast, MAIT cells from AHCV/HIV patients exhibited higher frequencies of CD38-expressing MAIT cells, which may be the result of exposure to pro-inflammatory serum cytokines that are known to be present at relatively high levels during acute HCV infection (242).

Viral load reduction by DAA-therapy in CHCV patients did not affect MAIT cell activation or IFN-γ+ frequencies, whereas IFN-based therapy strongly affected both parameters. We observed that IFN-based therapy enhanced the expression of the activation marker CD38 on MAIT cells, but decreased the frequencies of IFN-γ producing MAIT cells. Since the activation state of MAIT cells during IFN-based therapy is augmented, it is unlikely that the effect on IFN-γ production is the result of an overall inhibitory effect on the MAIT cells, but it may be the consequence of functional paralysis due to prolonged exposure to IFN-α. Although highly speculative, this may also explain the opposing effects of IFN-α on the frequencies of IFN-γ producing MAIT cells upon short-term exposure *in vitro* and long-term exposure *in vivo*. The observation that IFN-based therapy, but not DAA-therapy, suppresses the functionality of MAIT cells, may indicate that a disadvantage of IFN-based-therapy over DAA therapy is that it affects the anti-microbial function of MAIT cells and that the reduced functionality of MAIT cells may render individuals more susceptible to pathogens.

In conclusion, we show that MAIT cells are decreased in patients with chronic HCV, HIV and AHCV/HIV co-infection compared to healthy controls. We show that IFN-α modulates MAIT cells *in vitro*, and *in vivo* during IFN-based therapy for HCV but importantly, a sustained viral response for HCV does not rescue MAIT cell frequency.

SUPPLEMENTS



Supplementary Figure 1: No clear differences are observed between CHCV patients treated with IFN/based therapy with or without telaprevir. Patients with CHCV were treated with peginterferon and ribavirin alone (n=9) or in combination with telaprevir for 12 weeks (n=11). All patients were responsive to either treatment and were HCV RNA negative at week 12 of therapy (<15 U/ml). Frequencies of MAIT, CD38+MAIT and NK cells before and 12 weeks during therapy are shown.

Chapter 9

Decreased pro-inflammatory immune responses during recurrent acute HCV infections in HIV co-infected patients

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Under review

Chapter 10

Summarizing discussion

The first phase of a hepatitis C virus (HCV) infection, in this thesis named acute HCV, has a certain intangibility. This phase clearly starts with the transmission of the virus. The exact time-point however, is rarely known because of multiple potential exposures in risk groups. Thereby, the end of an acute infection remains undefined as well. At the moment, there is no clear marker that identifies the end of the acute and beginning of the chronic stage of infection. This results in different perspectives on acute HCV from epidemiological, virological, immunological and clinical points of view and complicates the research field. These perspectives need to be kept in mind when reading the following discussion and give some guidance through the summary of the different chapters.

Generally, it is believed that the use of HCV RNA amplification with retrospective negative testing is the most accurate way of diagnosing an acute HCV infection. In the Netherlands, samples of patients in HIV-care are systematically stored, so retrospective testing is easily performed. This policy makes it possible to diagnose a HCV infection in the acute stage with very high certainty. In **chapter 2**, we describe the results of a system based on voluntary reporting of acute HCV infections by HIV treating physicians. In 2014, a total number of 99 newly diagnosed HCV infections were reported in 19 HIV treatment centers. This resulted in a mean incidence rate of 11/1000 person years of follow-up in HIV positive men having sex with men (MSM). Most of them (78%) were diagnosed through HCV RNA amplification with retrospective negative testing. This study, based on voluntarily reporting, was successful because the concurrent Dutch Acute Hepatitis C in HIV Study (DAHHS) created awareness among physicians, nurses and patients on the importance of diagnosing HCV in the acute stage. When diagnosed with an acute HCV infection, participation in the DAHH-Study allowed for a 50% shorter treatment duration. The study in **chapter 2** provides some additional interesting insights on the characteristics of the Dutch HCV epidemic. First, the incidence rate we found was very comparable to the observed incidence in other national and international studies (34, 156). Remarkably, despite the fact that acute HCV is one of the infectious diseases that physicians are obliged to report to the National Institute for Public Health and the Environment (RIVM), the RIVM reported only 30 infections in 2014 (24). This indicates that the current method of surveillance on the incidence of HCV infections is inadequate and therefore the magnitude of the epidemic underestimated. Second, before the results of this study became available, acute HCV infections were considered to be rather limited to 'big cities' like Amsterdam and Rotterdam. In **chapter 2**, we show that in more than 50%

of the 19 participating Dutch hospitals the incidence of HCV was above 8.7/1000 patient years of follow up. So, it is fair to conclude that transmission of HCV among HIV positive MSM is a nationwide problem. Also in 2015 the epidemic continued, which was nicely illustrated by a reported outbreak in a city in a rural area named Enschede (254). Last but not least, we confirmed that the number of HCV reinfections was very high, as 25% of the patients with an acute HCV infection in 2014 had been cured from a previous infection. The high incidence rates of reinfection have been previously reported and our study confirms these findings (37, 38). Therefore, the alarming rate of reinfections should be of special concern to physicians. Patients with their first HCV infection should be counseled thoroughly about their, potentially, ongoing high risk behavior.

When an acute HCV infection is studied from a virological point of view, a dynamic pattern of HCV plasma viral loads is the hallmark of a recently acquired infection. After intravenous transmission, HCV is detectable in peripheral blood within 2 weeks(255). When an acute HCV infection is diagnosed, three patterns of HCV kinetics can be distinguished: spontaneous clearance, plateau viremia or fluctuating viremia (53, 58). These patterns, which are discussed in **chapter 1**, can be detected with HCV RNA measurements in plasma. HCV RNA amplification by polymerase chain reaction (PCR) is, however, a precise but expensive method to observe these patterns (256). Therefore, equally reliable and cheaper tests are required to diagnose and monitor acute HCV infections in the acute stage of the disease. In **chapter 3**, we study the clinical use of HCV antigen (Ag) testing for diagnosing acute HCV infections and to evaluate the response in patients treated in the DAHH-Study. HCV Ag proved to be a very accurate method to diagnose chronic HCV infections (131, 257). Also during the acute stage of disease, we could conclude that HCV Ag has a good correlation with HCV RNA. However, in 5 out of 44 patients with very low HCV RNA loads (<2200 IU/ml), HCV Ag was not detectable. Compared to HCV RNA as the golden standard, the sensitivity and specificity of HCV Ag testing were 89% and 100%. These results were similar to another study performed in a comparable British patient population(129). Therefore HCV Ag seems to be a reasonable alternative for HCV RNA testing to diagnose an acute HCV infection. Unfortunately, we were unable to study the kinetics of HCV Ag in patients with different HCV RNA kinetics during the acute stage of infection because the DAHH-Study was not designed to study the natural course of an acute HCV infection.

Apart from the detectability of HCV RNA and HCV Ag, IgG seroconversion is another hallmark of an acute HCV infection. Unfortunately, IgM testing, which is often used to diagnose infections in the acute stage, is not an adequate marker for acute HCV infections (258). IgG seroconversion can be diagnosed in the acute stage if there are preceding negative test results. If no previous test result is available, the combination of a negative IgG antibody with HCV RNA positivity resembles the incomplete immune response which is characteristic for an acute infection. A study by Thomson and colleagues among HIV infected patients with acute HCV showed that IgG seroconversion occurs mostly within 6 months up to one year after acute HCV diagnosis(49). These results suggest that an acute HCV infection, especially in HIV infected patients, will often be missed when relying on seroconversion. Therefore, as PCR is available, seroconversion is not frequently used as the only marker to diagnose acute HCV infections in the Netherlands. Remarkably, as shown in **chapter 3**, 91% of the patients included in the DAHHS compared to 63% of the patients described by Thomson and colleagues seroconverted within 4 months after calculated transmission. These discordant results could be explained by slight differences in anti-HCV testing methods and CD4+ T-cell counts. Altogether, these findings suggest that HCV IgGseroconversion could be, besides HCV RNA and HCV Ag, an adequate marker for diagnosing an acute infection. This is especially relevant when prices and practicability of these diagnostic tests are compared: HCV antibody testing is easier and significantly cheaper than HCV RNA amplification.

From a clinical perspective, the definition of an acute infection is maybe debated the most. Because the natural presentation of an acute HCV infection is associated with few clinical symptoms, physicians have to rely on laboratory testing(133, 259). Besides the differences in the nomenclature of acute HCV infections (recently acquired HCV infection, new HCV infection or early HCV infection) there is also a broad spectrum of definitions used in studies on acute HCV. In a systematic review by Hajarizadeh and colleagues, 169 studies were compared on the definition of acute HCV infections(47). These studies covered epidemiological, biological and clinical definitions. In 32,5% of the studies, HCV seroconversion was the only criterion for the definition acute HCV. Other frequently used definitions were: seroconversion combined with HCV RNA positivity and alanine aminotransferase elevation (14,8%) or HCV RNA detectability with being antibody negative (13,6%). The major point stated by the authors is therefore that more standardized case definitions are necessary.

The DAHH-Study, presented in **chapter 4**, used a very strict definition of acute HCV: treatment within 26 weeks after the calculated transmission date. We defined 'calculated transmission date' as the exact midpoint between the last negative and the first positive sign of hepatitis in blood. In the DAHH-Study, patients were treated with boceprevir, pegIFN and ribavirin during 12 weeks. Boceprevir is a first generation NS3a/4 protease inhibitor and was, together with telaprevir, the first direct acting antiviral (DAA) registered for the treatment of chronic HCV genotype 1 in the Netherlands in 2012. In the primary endpoint analysis we showed a 100% sustained virological response (SVR) rate in patients with a rapid virological response, which is defined as an undetectable HCV RNA at week 4 of therapy. This so-called rapid virological response is a well-defined predictor of treatment success in acute and in chronic HCV infected patients receiving pegIFN-based therapy (98, 133, 137, 260). We proved that also during boceprevir, pegIFN and ribavirin therapy for acute HCV, a rapid virological response is a very reliable predictor of treatment success. Additionally, the SVR rate in the intention-to-treat group was 86%. In comparison with a historical Dutch cohort of patients that received 24 weeks of pegIFN and ribavirin, the SVR rate was very comparable (86% and 84% respectively).

If we compare the cure rates in the DAHH-Study to previously published studies in which patients were treated with pegIFN with or without ribavirin for 24 weeks, the cure rates in the DAHH-Study were substantially higher. In a review by Rockstroh on behalf of the European AIDS Treatment Network, a composite cure rate of 65% was found within HIV positive patients with an acute HCV infection (95). However, we consider the comparison with the Dutch historical cohort the more appropriate one as these controls were treated in the same centers as in the DAHH-Study and had very comparable baseline characteristics. The high cure rates in the DAHH-Study in comparison with the literature may be partially explained by selection bias, because the patients that opted-in for study participation could have been the most motivated ones. Another reason could lie in the definition of acute HCV infection, which was very strict. This made the accidental inclusion of patients with a previously undiagnosed chronic HCV infection and therefore a much lower chance of treatment success, impossible.

Prior to the DAHH-Study, the efficacy of shortened treatment with a first generation DAA in combination with pegIFN and ribavirin had only been studied once in a small pilot study by Fierer and colleagues (115). They treated 19 acute HCV HIV-coinfected

patients with telaprevir, pegIFN and ribavirin for 12 weeks and cured 16 patients. So, in comparison with the DAHH-Study, this cure rate was very similar. Soon after completion of the DAHH-Study, several small pilot studies were presented with newer DAA combinations for the treatment of acute HCV. Recently, we described a case series of six acutely HCV genotype 4 infected patients treated with pegIFN, ribavirin and simeprevir. In this study we showed that all patients were treated successfully with 12-16 weeks of triple therapy. Also other groups have presented pilot studies with combinations of the NS5b inhibitor sofosbuvir with ribavirin or sofosbuvir combined with a NS3a/4 or NS5a inhibitor. Remarkably, the cure rates varied substantially from 32% to 100%. In two studies, the combination of sofosbuvir and ribavirin when given for 12 weeks was tested and resulted in cure rates of 59% (10/17) and 92% (11/12) respectively (143, 261). When given for 6 weeks, the combination of sofosbuvir and ribavirin was even less effective with a cure rate of 32% (6/19)(144). Combinations of sofosbuvir with ledipasvir (4 weeks) or simeprevir (8 weeks) in patients with acute HCV mono-infections seem to be much more promising with response rates of 93% (13/14) and 93% (13/15) respectively (145). However, in HIV coinfected patients the cure rate of 6 weeks sofosbuvir and ledipasvir was 85% (22/26) or even 77% (20/26) when two patients who were lost to follow-up are considered failures(262). In conclusion, also in acute HCV, the combination of DAAs seems to be very effective as well. However, the preferred combination and duration remains to be defined.

Following the success of the DAHH-Study in the Netherlands, we initiated a new study: the DAHHS-2. In this study we test the efficacy and tolerability of a combination of the NS3a inhibitor grazoprevir and NS5a inhibitor elbasvir in 80 acute HCV HIV-coinfected patients (NCT02600325). This combination was shown to be effective when given for 12 weeks to patients with chronic HCV genotype 1 and 4. Therefore both genotypes will be treated in the DAHHS-2. This also means that we are able to treat almost all acute HCV infected patients, because, as described in **chapter 2,** genotype 1 and 4 are by far the most dominant genotypes in the Dutch population. The choice for the treatment duration of 8 weeks is based on the assumption that acute HCV can still be treated with shorter duration regimens, even when pegIFN-free regimens are used. However in the light of the results of the previous pilot studies presented above, 8 weeks of treatment could be the minimum duration in HIV coinfected patients with an acute HCV infection. Another difference between the DAHHS 1 and 2 is that also patients from Belgium can be treated in a single center in Antwerp. Because the reimbursement of DAAs in Belgium is more stringent than the

Dutch reimbursement policy, we expect that a substantial part of the patients will be included in Belgium to prevent their acute HCV infection from becoming chronic. Another difference with DAHHS-1 is that also acute HCV mono-infected patients can be treated in the DAHHS-2. Recently, pre-exposure prophylaxis (PREP) studies have reported HCV infections in HIV-negative participants (263). This raised the concern that HCV is transmitted also between HIV negative MSM. Because sexual transmission of HCV between HIV negative MSM is probably rare, part of the transmissions may be the result of the intravenous use of chemicals ("party drugs") like crystal meth, ketamine or mephedrone. This so-called 'slamming' is a recent trend entering the gay scene and has been associated with a rise in HIV and HCV infections (264).

During combination antiretroviral therapy (cART), special attention should be given to potential drug-drug interactions with concomitantly prescribed or non-prescribed medication. We studied the interaction between rilpivirine, a component of certain cART regimens given to patients with HIV, and boceprevir in **chapter 5**. Because boceprevir is a CYP3a inhibitor and rilpivirine a CYP3a substrate, this could result in increased plasma rilpivirine levels. During dose-finding studies with rilpivirine, it was found that increased plasma rilpivirine levels resulted in QT interval prolongation on the electrocardiogram, which are associated with cardiac arrhythmias(265). In this DAHHS sub-study we found a significant increase in rilpivirine plasma concentrations in patients treated with boceprevir, without significant QT-prolongation on the electrocardiogram. The increase in rilpivirine $AUC_{0\text{-}24h}$ (71%) found in this study was higher than described in healthy volunteers (39%)(146). Therefore, we conclude that healthy volunteer data should be translated cautiously to HIV infected patients.

Maybe the most important question that arose after the DAHH-Study was completed is whether there is still a role for pegIFN based treatment options in patients with an acute HCV infection. Although high cure rates can be obtained with pegIFN based therapy in the acute stage of the disease, it is associated with significant side effects. Flu like symptoms, anemia and depressive symptoms are common pegIFN induced side effects. They result in a decrease in the quality of life and productivity during and sometimes also persist after the end of therapy (**chapter 4**). Therefore, also acute HCV infected patients could benefit from pegIFN free treatment if the same cure rates are obtained during acute HCV therapy as seen in chronically HCV infected patients. Unfortunately, the availability and reimbursement of sofosbuvir-based therapies remains limited in parts of the resource rich as well as resource limited

countries. In the USA, Medicaid programs in some states denied nearly half of all claims (46%) for treatment with DAAs in the chronic stage. Denials were significantly more common in patients who did not have signs of cirrhosis, which per-definition is the case in patients with acute HCV (266). In conclusion, as long as reimbursement issues continue, a short-course of a DAA with pegIFN and ribavirin may be a valid option for patients with an acute HCV infection.

In **chapter 6** we modelled the effects on the epidemic of treating all acute and chronic HCV infected patients with a multiple DAA regimen with 89-100% cure. We compared this 'immediate treatment' to 'delayed' treatment with DAAs. Delayed treatment was defined as 24 weeks of pegIFN based treatment of acute HCV with a cure rate of 52%-84%. If treatment fails, a pegIFN-free DAA regimen is given at METAVIR F2 or F3 fibrosis. This model was based on the reimbursement policies of the Dutch Ministry of Health in 2014/2015. The limited reimbursement had to do with the extremely high therapy costs. In 2014/2015, a 12 week regimen of multiple DAAs was priced somewhere between 40.000 and 80.000 euro(267). The policy to delay treatment is based on the assumption that a chronic HCV infection is asymptomatic and results in limited morbidity until stage of F2 or F3 fibrosis and therefore does not need to be treated immediately. Treatment can therefore be delayed and, theoretically, costs can be saved because a substantial part of the infected population will never progress to F2 or F3 fibrosis(8). However, in models that have been presented or published, the effects of ongoing transmission from untreated infectious patients were not incorporated. In the study described in **chapter 6**, we therefore modelled the effects of different treatment approaches on the incidence and prevalence of HCV in the Netherlands. The main outcome was that immediate treatment of all HIV positive MSM with acute and chronic HCV did not result in elimination of the epidemic. The incidence declined from 12 to 4.2 per 1000 patients years and the prevalence from 5% to 0.6% in 2055. These results were comparable to a study published by Martin and colleagues, who observed an even more substantial decline when behavioral interventions were successfully implemented as well(184). However, at the moment, no intervention strategy has been proven to be effective in this population. Additionally, we calculated the extra costs and benefits of the immediate treatment policy until 2055. Total additional costs were 29 million euro and benefits were 2596 quality adjusted life years. This resulted in an incremental cost- effectiveness ratio (ICER) of 12.000 euro per quality adjusted life year. This ratio is particularly dependent on the price per DAA treatment. Given the fact that different new DAA regimens are upcom-

ing, it can be expected that the price of DAA therapy will decrease substantially and the ICER as well. We conclude that, for the Netherlands, the impact of the immediate treatment policy on the epidemic is substantial, but that total elimination of HCV is not possible. Furthermore, the overall health budget impact is limited and DAA therapy was very cost-effective.

The high effectiveness of DAAs led to statements that HCV can be eliminated on a worldwide scale. Several threats however can hamper global elimination. One of those could be the development of resistance against DAAs. Currently, almost all DAAs target the NS3/4a, NS5a or NS5b regions of the HCV genome. When patients fail DAA therapy, they mostly have mutations in the NS3/4a or NS5a region resulting in resistance to DAAs that target these regions (268). Polymorphisms in these genes can be present in a population as a naturally occurring variant or can be selected when a viral breakthrough or relapse of HCV is observed during or after DAA treatment. To determine the prevalence of resistance associated substitutions within patients treated in the DAHH-Study we performed resistance testing before and after treatment. The results are presented in **chapter 7**. In this study, whole HCV genomes were sequenced in collaboration with the PATHSEEK project. From 2012, boceprevir and telaprevir were used with limited success in chronically HCV infected patients. Therefore, we expected some baseline (pre-treatment) NS3/4a resistance in the DAHHS patient population with proven acquired HCV in 2013/2014. Fortunately, only in five patients a substitution in NS3/4a associated with low-level resistance to boceprevir or telaprevir was found before initiation of treatment. Because these mutations can also be found as a naturally occurring polymorphisms, they do not prove that transmitted drug resistance occurred(269, 270). The Q80K resistance associated variant in NS3/4a, however, was found in 40% of the baseline samples. This variant is not selected by boceprevir/telaprevir therapy but is a naturally occurring variant which results in resistance against simeprevir, another NS3/4a inhibitor. The prevalence of this variant is high in Europe, but low in some other parts of the world (e.g. Australia 4%) (202). Therefore, when no baseline resistance testing is performed, selection of DAA combinations seems to be dependent on the baseline prevalence of resistance associated variants in the geographic region where the patients were infected. Unfortunately, a significant amount of substitutions were found in the NS5a gene (M28V 22% and H58P 26%). These substitutions should be considered as naturally occurring variants as well, because NS5a inhibitors were not used in Europe before 2014 outside clinical trials.

In conclusion, it is unclear what the effect of resistant variants will be on the future HCV treatment. Patients with resistant variants will probably not benefit from short DAA combination therapies in acute and chronic HCV, but the exact role of the presence of resistant variants remains to be defined. As an example: recently, the FDA approved the combination tablet of grazoprevir and elbasvir for the treatment of HCV genotype 1 and 4. For genotype 1, the USA FDA favored the use of pre-treatment NS5a resistance testing. When resistant associated substitutions are detected at amino acid positions 28, 30, 31 or 93, treatment duration has to be increased to 16 weeks and ribavirin added(207). In contrast, the Canadian prescription label does not recommend resistance testing.

If a patient fails on DAA therapy, sequencing of specific parts of the HCV genome may be useful for two reasons. First, the difference has to be made between relapse and reinfection. As the rate of reinfection is high among HCV infected patients **(chapter 2)**, the ability to differentiate between sequential infections is necessary to monitor treatment response. This is illustrated by patient Ec03 in the DAHH-Study who presented 12 weeks after treatment with a new onset viremia. After genotyping by a Line Immuno Probe Assay, which showed a genotype 1a, one could conclude that the virus had relapsed. However, after whole genome sequencing of the virus from baseline and relapse, a genetically different 1a strain was detected and therefore the patient was considered to be re-infected with a new genotype 1a strain. The second reason to perform genome sequencing is to monitor the emergence of resistant variants. This may help in selecting the most appropriate second line treatment for patients. However, in most patients a DAA from a different class may be selected as the second line therapy without the need for genome sequencing. Of course, sequencing of a specific region instead of whole genome sequencing can be done to detect variants in the NS3/4a, NS5a or NS5b gene.

Immunologically, one of the major challenges is to find a marker that clearly differentiates between the acute and chronic stage of HCV and therefore, could be used to pinpoint acute infections in the individual patient. Because the response to IFN-based treatment is significantly different in the acute stage compared to the chronic stage of HCV, it is clinically important to differentiate between these phases when one would consider to treat a patient with pegIFN. The generally strong immune response against multiple epitopes during acute infection can be clearly distinguished from the weak, exhausted narrowly focused response during chronic infection. However,

there are no straightforward, simple and widely available laboratory tests that can differentiate an acute from a chronic infection and could be used in the clinic. This issue becomes even more complex in patients without an intact immune system like patients with a HIV coinfection or when patients were previously infected with HCV.

In **chapter 8**, we studied the impact of a HCV infection on mucosal-associated invariant T (MAIT) cells. These cells comprise a subpopulation of T cells that are important for antimicrobial defense and can be activated by either the cytokines IL-12 and IL-18 or vitamin B metabolites (riboflavins) processed by bacteria. These vitamin B metabolites are presented by MHC class II related protein 1 (MR1) on antigen presenting cells (APC) to the invariant Valpha T cell receptor on the MAIT cell(271). MAIT cells seem to be reduced in peripheral blood of patients with a HIV infection and show signs of exhaustion in these patients (224). In a previous study, an inverse correlation between liver fibrosis and IL-17 dual expressing CD8+ T cells, a proxy for MAIT cells, was found(272). This suggests a role for MAIT cells in chronic liver diseases like HCV. As the role of MAIT cells in HCV infection was incompletely understood, we studied the functionality of these cells in different stages of a HCV infection. We found that MAIT cell frequencies were decreased in chronic HCV, HIV and acute HCV co-infected patients. After successful HCV treatment, no normalization of MAIT frequencies was observed in the chronic HCV infected patients. Additionally, we observed a significant difference between presence of activation markers (CD38) in MAIT cells between chronic and acute HCV infections. However, this state of increased activation was not in observed in interferon gamma production between MAIT cells of acute and chronic HCV infected patients after stimulation.

The high expression of the activation marker CD38 in acute HCV infections could be the result of stimulation by high levels of cytokines and chemokines in the blood. These cytokines and chemokines could be used as biomarkers to detect an acute HCV infection and in the past, specific patterns of biomarker kinetics have been observed for acute HCV infections (242, 273). In **chapter 9**, we studied kinetics of several biomarkers in patients with HIV mono-infection and during their consecutive HCV infections. First, we looked for differences between healthy volunteers, HIV mono-infected and HIV co-infected patients with an acute or chronic HCV infection. We observed, in general, that the biomarker levels were upregulated the most in acute HCV coinfected patients compared to others. This is in line with our expectations that the acute phase of a HCV infection results in the highest activation of the

immune system. Second, in 15 HIV infected patients with two consecutive acute HCV infections we tested biomarker kinetics at several time points. We observed a clear rise in IL-12p40, IP-10, MIG and MIP-1b during the first acute HCV infection. However, at the second acute HCV infection there was no increase in biomarkers compared to the HIV mono-infected state, before the first acute HCV infection nor after the first acute HCV infection had been cured. We hypothesize that the weak pro-inflammatory cytokine responses observed during acute HCV re-infection may on the one hand further compromise the chances of spontaneous clearance and on the other hand may simultaneously prevent damage to the infected organ, the liver(274). One of the explanations for these weakened immune response is negative regulation by IL-10, TGF-β and regulatory T cells (Tregs). It has been shown that these regulatory mechanisms remain active years after therapy-induced eradication of chronic HCV infections(275). Possibly, similar processes occur in repeated acute HCV infections.

CONCLUSION AND FUTURE PERSPECTIVES

The HCV arena is dynamic and changing very fast. Several new DAAs were successfully introduced over the last years and are able to cure the vast majority of patients with a chronic HCV infection. Because acute HCV infections are in general more difficult to diagnose, the role of DAA based therapy for acute HCV remains to be defined. This thesis addresses new treatment and diagnostic opportunities for patients with an acute HCV infection in the DAA era.

In the Netherlands, acute HCV infections are currently diagnosed almost exclusively among HIV infected MSM and are mostly sexually transmitted. This epidemic is ongoing and its future course will depend on several factors. The most important factors discussed in this thesis are: the adequate use of diagnostic tests, the efficacy, safety and availability of DAA treatment, the emergence of viral resistance and the sexual behavior of MSM.

One of the most interesting questions in the pegIFN-free era remains whether acute HCV infections can be treated with shorter duration regimens than chronic HCV infections. We show in this thesis that a shorter treatment regimen is certainly possible when DAAs are added to pegIFN and ribavirin. Studies on the efficacy of DAAs and on how DAAs can be used during an acute HCV infection are urgently needed. As

we speak, at least two adequately powered prospective studies on the treatment of acute HCV are currently enrolling patients, namely the DAHHS-2 (NCT02600325) and REACT (NCT02625909).

Whether transmitted drug resistance to DAAs will become clinically relevant is difficult to predict. The current circulating HCV wild types among acute or chronically HCV infected patients do not seem to harbor a lot of clinically relevant resistance. Therefore, the large majority of people with acute or chronic HCV can now be cured. However, when millions of patients are being treated, a 5% failure rate results in a significant number of patients harboring a virus with resistance to at least 1 class of DAA. If this is followed by ongoing HCV transmission (as in HIV positive MSM or injecting drug users) resistant variants may start to spread. Therefore, despite the very high cure rates that we are currently observing, HIV treating physicians should be aware of this risk and surveillance is needed.

We showed that the national reimbursement policy driven by the price of DAAs has a huge impact on the epidemic. When immediate DAA treatment is provided to all, the most important barrier to elimination of HCV among HIV positive patients will be the continued high-risk sexual activity of a subset of HIV positive MSM. The elimination of HCV in the Netherlands is just a small step in the eradication of HCV. From a global perspective, opiate addiction and inadequate sterilization will remain major risk factors for ongoing transmission and therefore HCV will probably continue to spread for many years.

The first step in HCV elimination starts with the availability of a cheap and reliable test to diagnose a HCV infection. HCV RNA testing remains the gold standard for diagnosing acute and chronic HCV infections. However, this method is relatively expensive. HCV Ag testing seems to be a promising method, especially in the light of frequently testing in high-risk groups. At the moment however, the infrastructure of many Dutch hospitals is not ready to implement this test in routine care.

Despite the fact that we have an effective treatment for HCV, many immunological issues remain unsolved. In this thesis we study the effects of pegIFN and pegIFN-free regimens on MAIT cells. We conclude that MAIT cell frequencies are diminished in viral infections and do not normalize after effective treatment, regardless of the mode of treatment. Additionally, we found differences in biomarker responses between a

first and consecutive acute HCV infection. Both studies suggest long-lasting effects of a HCV infection even after successful therapy-induced viral eradication. Future studies could address this altered state of the immune system and determine to what extend this affects patient's health.

Chapter 11

Nederlandse samenvatting

Infecties met het hepatitis C virus (HCV) komen overal in de wereld in groten getale voor. HCV zorgt voor een ontsteking van de lever wat een bedreiging is voor de gezondheid van de gastheer. In dit proefschrift worden acute HCV infecties vanuit verschillende hoeken belicht. Hierbij staat de diagnostiek en behandeling van acute HCV infecties bij HIV positieve mannen centraal. Het is niet gemakkelijk een exacte definitie van een acute HCV infectie te geven en daardoor is het moeilijk om te beoordelen of een patiënt een acute HCV infectie heeft. Binnen verschillende medische vakgebieden, bijvoorbeeld virologie, immunologie en bij klinisch onderzoek worden wisselende definities gehanteerd. Het is daarom essentieel om goed voor ogen te hebben wat een arts, onderzoeker of patiënt bedoelt wanneer deze spreekt over een acute HCV infectie. In het algemeen wordt een acute HCV infectie gedefinieerd als een nieuwe HCV infectie die binnen de eerste zes maanden na het oplopen daarvan wordt gediagnosticeerd.

De huidige methodes om een acute HCV infectie vast te kunnen stellen hebben beperkingen. Daardoor is er behoefte aan onderzoek naar nieuwe diagnostische technieken. Daarnaast zorgt de recente opkomst van direct aangrijpende antivirale middelen (direct-acting antivirals = DAAs) voor een revolutie in de behandeling van chronische (niet-acute) HCV infecties. De effecten en lange termijn gevolgen van DAAs voor de behandeling van acute HCV infecties zijn tot op heden minimaal bestudeerd en ook daar probeert dit proefschrift een bijdrage aan te leveren.

In hoofdstuk 1 wordt een uitgebreide inleiding op het ziektebeeld acute HCV infectie gegeven en de stand van zaken omtrent acute HCV infecties in de periode tot aan dit proefschrift besproken. De huidige epidemie van HCV in Nederland is anders dan de epidemie die ontstond in de context van heroïne gebruik in de jaren zeventig en tachtig van de vorige eeuw. Momenteel worden de meeste patiënten in Nederland besmet via seksueel contact. Het grootste deel van de besmettingen vindt plaats bij HIV positieve mannen die seks hebben met mannen (MSM). Bij heteroseksuelen en HIV negatieve MSM komt seksuele overdracht van HCV slechts zeer sporadisch voor. Het is belangrijk zich te realiseren dat de overdracht van HCV bij MSM een relatief kleine groep patiënten betreft. Op globaal niveau wordt HCV echter nog steeds op grote schaal verspreid door inadequate sterilisatie van medische instrumenten en injecterend drugsgebruik. Kortom, nieuwe HCV infecties komen overal op de wereld voor waarbij de overdracht voornamelijk plaatsvindt binnen specifieke risicogroepen. De focus van dit proefschrift ligt op HIV positieve MSM met een acute HCV infectie.

Vanaf hoofdstuk 2 komen de diverse studies die de kern van dit proefschrift vormen aan bod. In hoofdstuk 2 wordt het vóórkomen van acute HCV infecties in Nederland bestudeerd. In het kader van de Dutch Acute HCV in HIV Study (DAHHS) werd er in 2014 een meting uitgevoerd waarin aan alle HIV-behandelaren gevraagd werd om acute HCV infecties te melden en anonieme karakteristieken van hun patiënten door te geven. Uit deze studie blijkt dat er in één jaar tijd 99 nieuwe HCV infecties werden gediagnosticeerd onder HIV positieve MSM in de 19 centra die deelnamen aan deze incidentiestudie. Het aantal nieuwe infecties wordt daarmee op 11 per 1000 HIV positieve MSM per jaar geschat. Dit aantal is vergelijkbaar met de voorspellingen die zowel nationaal als internationaal zijn gedaan. Verder concluderen we uit deze studie dat een kwart van de patiënten al eerder geïnfecteerd is geweest met HCV en dat er dus een aanzienlijke groep is die hoog risico gedrag blijft vertonen. Deze studie toont dus aan dat acute HCV infecties binnen HIV positieve MSM, ook in 2014, nog steeds frequent voorkomen.

Er zijn verschillende manieren om een acute HCV infectie aan te tonen, maar deze hebben beperkingen. De aanwezigheid van viraal genoom in het bloed (RNA) is een goede manier om de aanwezigheid van HCV aan te tonen. Hiermee wordt echter geen onderscheid gemaakt tussen een langer bestaande en een recente infectie. Om aan te tonen dat een infectie acuut is, moet een negatieve HCV test beschikbaar zijn. Echter, het aantonen van RNA is relatief duur en de tijd tot het detecteerbaar worden van antistoffen (seroconversie) kan tot een jaar duren. Daarom blijft er behoefte aan een nieuwe test die goedkoop is en een acute HCV infectie op een gevoelige manier kan aantonen. In hoofdstuk 3 presenteren we een studie naar de gevoelige en onderscheidende eigenschappen van de HCV antigeen (Ag) test bij het diagnosticeren van een acute HCV infectie. Het blijkt dat deze test een goede gevoeligheid (sensitiviteit 89%) en een goed onderscheidend vermogen heeft (specificiteit 100%) om een acute HCV infectie aan te tonen, dan wel uit te sluiten. Dus een HCV Ag bepaling is een redelijk alternatief is voor de HCV RNA test.

Uit onderzoeken bij patiënten met HCV infecties is gebleken dat HCV Ag een rol kan hebben in het vervolgen van het effect van een behandeling die gebaseerd is op het geven van peginterferon en ribavirine. Deze middelen waren tot 2012 de enige middelen die enigszins effectief waren voor de behandeling van HCV. De kans op genezing met peginterferon en ribavirine is voor chronische HCV matig maar voor een acute HCV hoog. Na de eerste vier weken kan zowel bij acute als chronische HCV

op basis van de hoogte van het HCV RNA een inschatting gemaakt worden over de kans op genezing na beëindiging van de behandeling. Helaas kunnen we in de studie in hoofdstuk 3 niet aantonen dat HCV Ag deze HCV RNA metingen om effect van therapie te vervolgen goed kan vervangen.

In hoofdstuk 4 komt de DAHH-Studie aan bod. In deze studie zijn patiënten met een acute HCV behandeld met peginterferon, ribavirine en boceprevir. Boceprevir is één van de eerste DAAs die in combinatie met peginterferon en ribavirine voor de behandeling van chronische HCV kon worden gebruikt. Omdat er destijds nog geen onderzoek was verricht naar de effectiviteit van boceprevir bij de behandeling van acute HCV, is de DAHH-Studie opgezet. In deze studie wordt het behandelschema van een acute HCV infectie verkort van standaard 24 naar 12 weken. Deze kortere behandeling met drie geneesmiddelen leidt tot een genezingspercentage van 100% binnen de groep patiënten die op week 4 van behandeling al geen aantoonbaar HCV RNA meer heeft. In de totale groep behandelde patiënten is 86% van de patiënten genezen. Binnen de studie vergelijken we deze resultaten met de reguliere behandeling van 24 weken peginterferon met ribavirine. Het genezingspercentage in deze controle groep is 84%. Dus we concluderen dat door het toevoegen van een extra middel de behandelduur van een acute HCV infectie gehalveerd kan worden. Omdat peginterferon en ribavirine een aantal ernstige bijwerkingen hebben is deze verkorting een belangrijke winst.

De combinatie van HIV-remmers en andere medicatie kan voor interacties zorgen die nadelig zijn voor een patiënt. De combinatie van boceprevir en rilpivirine, een hiv remmer, kan tot een extra stijging van de rilpivirine spiegel in het bloed leiden. Omdat hoge rilpivirine spiegels theoretisch kunnen resulteren in hartritmestoornissen, onderzochten wij het effect van boceprevir op de rilpivirine spiegel. In hoofdstuk 5 laten we zien dat boceprevir daadwerkelijk voor hogere rilpivirine spiegels zorgt, maar dat het electrocardiogram, waarop een afwijking een voorbode kan zijn van hartritmestoornissen, normaal blijft.

DAAs zijn nieuwe middelen voor de behandeling van HCV. Het gebruik van deze middelen leidt over het algemeen tot hele hoge genezingspercentages omdat ze de virusproductie op een heel krachtige manier remmen. Deze remming vindt daarbij, in tegenstelling tot peginterferon, heel selectief plaats waardoor er weinig bijwerkingen zijn. Door de populariteit van deze middelen wordt er gezegd dat HCV een ziekte is

die theoretisch volledig geëlimineerd kan worden. In hoofdstuk 6 wordt een modelleringsstudie gepresenteerd waarin effecten worden getoond van verschillende behandelstrategieën van HCV op landelijk niveau. In deze studie vergelijken we het direct behandelen van alle HCV/HIV positieve MSM met deze DAAs met het uitstellen van behandeling totdat er ernstige leverschade wordt vastgesteld. Wachten op het ontstaan van ernstige leverschade is in theorie mogelijk omdat HCV maar bij een beperkt deel van de HIV positieve patiënten (ongeveer 30%) binnen een termijn van 30 jaar ernstige leverschade ontstaat. Dus zolang er geen leverschade ontstaat hebben de meeste patiënten niet direct baat bij een behandeling. Een andere belangrijke reden om te wachten met therapie is de, op dit moment, zeer hoge prijs van DAAs. Wachten met de behandeling kan op populatieniveau echter ook negatieve gevolgen hebben. Zolang een patiënt een HCV infectie heeft, kan hij deze namelijk ook overdragen. Daarom zou het toch zinvol en doelmatig kunnen zijn om alle patiënten met een nieuwe HCV infectie direct te behandelen. In hoofdstuk 6 laten we zien dat het direct behandelen van alle patiënten een flinke daling in het totale aantal patiënten met HCV en ook in het aantal nieuwe geïnfecteerde patiënten geeft. Echter, in de studie concluderen wij ook dat het niet lukt om HCV binnen de HIV positieve patiënten volledig te elimineren. Als behandelingen worden uitgesteld tot gevorderde leverschade, zal de epidemie zelfs in omvang toenemen. Naast het effect op de epidemie zijn er ook effecten op de kosten en baten van deze andere behandelstrategie. Als de kosten op de lange termijn worden afgezet tegen de baten, dan lijkt het direct behandelen van alle patiënten kosteneffectief. Als de prijs van een behandeling met DAAs van 40.000 euro per behandeling naar 20.000 euro per behandeling zou gaan dan zou dat zelfs resulteren in een kostenbesparing. Kortom, het direct behandelen van alle patiënten met HIV en HCV lijkt een zinvolle investering op landelijk niveau.

Ondanks de zeer hoge genezingskans na behandeling met DAAs, kan het HCV wel ongevoelig voor deze middelen worden. Dit wordt resistentie genoemd en berust op het ontstaan van kleine veranderingen in het genoom (mutaties). In hoofdstuk 7 onderzoeken we de aanwezigheid van resistentie in het virale genoom bij de DAHHS patiënten. Omdat de patiënten van de DAHH-Studie geïnfecteerd werden in 2013 en 2014, liepen deze het risico om geïnfecteerd te zijn met een virus dat al resistentie had verworven tegen boceprevir of telaprevir. Beide middelen, die dezelfde werking hebben, werden namelijk vanaf 2012 gebruikt en bij een aanzienlijk deel van de patiënten mislukte deze behandeling. De kans dat het HCV van deze patiënten

bij wie de behandeling mislukte resistent werd voor deze DAAs is groot. Als deze patiënten vervolgens het virus overdragen, kan het resistente virus zich verspreiden. Als dit op grote schaal gebeurt dan kan dat effect hebben op de effectiviteit van DAAs. In de studie in hoofdstuk 7 vinden we op het moment dat de patiënten starten met hun HCV behandeling geen mutaties die met zekerheid door eerdere therapie met boceprevir of telaprevir veroorzaakt is. Wel vinden we bepaalde variaties in het virale genoom in de DAHHS populatie (bijvoorbeeld Q80K in NS3/4a). Deze kunnen van nature voorkomen maar zijn wel in verband gebracht met significante resistentie tegen nieuwere DAAs. Ondanks het feit dat wij geen overgedragen resistentie vinden, blijft het mogelijk dat dit in de toekomst een probleem wordt. Er zijn namelijk nog steeds groepen waarin DAAs niet optimaal werken of niet optimaal gebruikt worden. Als in deze groepen, na falen van de behandeling, resistentie van HCV tegen deze DAAs ontstaat, zou dit overgedragen kunnen worden. Dus behandelaren moeten ook in de toekomst waakzaam zijn voor het ontstaan van resistente varianten.

Als laatst worden er in hoofdstuk 8 en 9 twee studies gepresenteerd die het immunologisch profiel van een acute HCV infectie bestuderen. In het verleden zijn er duidelijke verschillen aangetoond in de immuunrespons tussen acuut en chronisch geïnfecteerde patiënten. Echter, in de kliniek hebben deze bevindingen nog geen diagnostische waarde. Bij patiënten met een chronische HCV infectie wordt een soort uitgeputte immuunrespons geobserveerd, waardoor het immuunsysteem niet meer in staat is krachtig te reageren zoals tijdens de acute fase. In hoofdstuk 8 wordt de mucosal invariant T (MAIT) cel bestudeerd. Dit is een afweer (T) cel die waarschijnlijk een rol speelt bij de bacteriële afweer. Aangezien patiënten met HIV en HCV vaker bacteriële infecties hebben en de mate van aanwezigheid van deze cel in verband is gebracht met leverschade (fibrose) in eerder onderzoek, waren we geïnteresseerd in het functioneren van deze cellen. We observeren in deze studie lagere frequenties van MAIT cellen binnen de totale T cel populatie bij zowel HIV, chronische HCV als acute HCV/HIV co-infecties ten opzichte van gezonde controles. Na succesvolle behandeling van de HCV infectie lijkt er geen herstel te zijn van deze frequenties. Ook lijken er wat betreft MAIT cellen weinig verschillen te zijn tussen acute en chronische HCV infecties. Het enige aantoonbare onderscheid dat we vaststellen tussen acute en chronische HCV is een toegenomen aantal activatiemarkers op MAIT cellen bij acute HCV. Deze verhoogde staat van activatie vertaalt zich in deze studie echter niet in een verhoogde productie van interferon.

In hoofdstuk 9 wordt een studie gepresenteerd waarin het biomarker profiel van acute HCV infecties en HCV herinfecties wordt onderzocht en vergeleken. Biomarkers zijn in deze studie gedefinieerd als stoffen die door cellen worden geproduceerd om een ontstekingsreactie op gang te brengen of juist tegen te gaan. Het doel van deze studie is om te onderzoeken of tijdens een eerste en een daaropvolgende infectie met HCV dezelfde veranderingen van het immuunsysteem optreden. Dit is dus een infectie met een nieuw HCV nadat de voorgaande infectie genezen is. Hiervoor zijn acht biomarkers geselecteerd en op meerdere momenten tijdens opeenvolgende infecties getest. In eerste instantie onderzoeken we of biomarkers veranderen bij HIV positieve patiënten op het moment dat ze een eerste acute HCV infectie krijgen. Vervolgens zijn dezelfde biomarkers opnieuw bepaald gedurende de tweede acute HCV infectie. Van een aantal biomarkers kunnen we aantonen dat deze significant toenemen gedurende de eerste HCV infectie. Echter, bij de tweede HCV infectie zien we deze toename niet duidelijk. Een goede verklaring hebben we hier niet voor kunnen vinden. Mogelijk zijn er na een eerste infectie langdurige verandering in het immuunsysteem opgetreden die een reactie op een nieuwe infectie anders maken.

CONCLUSIE

Een acute HCV infectie is een zeldzaam ziektebeeld dat in Nederland voornamelijk wordt gediagnosticeerd bij HIV positieve MSM. Bij deze mannen wordt het virus seksueel overgedragen. Het is soms lastig te beoordelen of een HCV infectie in de afgelopen zes maanden is opgelopen en daarmee als acuut kan worden gedefinieerd. Het verschil tussen een acute en chronische HCV infectie is zichtbaar wanneer in detail naar de immunologische respons wordt gekeken. Ook is een behandeling met peginterferon veel effectiever tijdens een acute infectie. De beschikbaarheid van DAAs creëert nieuwe behandelopties voor patiënten met acute HCV infecties. In dit proefschrift tonen we aan dat de toevoeging van boceprevir aan peginterferon en ribavirine de behandeling van acute HCV infecties kan verkorten van 24 naar 12 weken zonder verlies van effectiviteit. Echter, de toekomstige behandeling van acute HCV zal waarschijnlijk uit een combinatie van DAAs zonder peginterferon bestaan. Indien alle acute HCV infecties hiermee direct na diagnose behandeld kunnen worden dan zal dit waarschijnlijk leiden tot een belangrijke afname van de epidemie in Nederland. Het ontstaan van resistentie tegen DAAs lijkt op dit moment nog geen

groot probleem maar waakzaamheid is nodig nu de DAA behandeling in Nederland breed wordt toegepast.

Chapter 12

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Chapter 13

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Chapter 14

About the Author

PHD PORTFOLIO SEBASTIAAN HULLEGIE

Courses

Scientific integrity	2015
Biostatistics	2014
Principles of research in medicine	2014
Methods of clinical research	2014
Good Clinical Practice	2014
Emerging infections	2014
Open Clinica	2013

Oral presentations at conferences

Dutch HIV-treating physicians congress	2016
Dutch HIV-treating physicians congress	2015
9th Dutch conference on HIV	2015
EACS conference Barcelona	2015
10th HIV/hepatitis workshop Paris	2014

Poster presentations at conferences

EASL Barcelona	2016
CROI conference Boston	2016
EACS conference Barcelona	2015
EASL Vienna	2015
CROI conference Seattle	2015
9th Dutch conference on HIV	2015
Dutch Hepatitis Day	2015
10th HIV/hepatitis workshop Paris	2014
8th Dutch conference on HIV	2014
1st acute HCV conference Utrecht	2014

Teaching experience

Supervising second year medical students	2014
Tutor bachelor year 1	2014
Tutor bachelor year 1	2015

Awards

Young investigator:
10th HIV/hepatitis workshop Paris
CROI conference Boston
CROI conference Seattle
Late breaker:
EASL Vienna
EACS conference Barcelona
Best poster presentation:
EACS conference Barcelona

Other

Member 2015 PhD committee
Sub-investigator in the following studies:
Gilead 0109
Gilead 0111
Gilead 1216
Merck 1439
DAHHS-1
DAHHS-2

Attended meetings/conferences

PhD day Erasmus MC	2015
Gilead 1216 investigators	2015
Merck 1439a investigators	2015
5th workshop on HCV therapy	2015
CPO Erasmus MC	2014
PhD day Erasmus MC	2014
7th Dutch conference on HIV	2013

CURRICULUM VITAE

Sebastiaan Hullegie was born on February 27th 1988 in Gouda, the Netherlands. In 2006 he graduated from the Marnix College in Ede and started his medical training at the Erasmus MC University Medical Center. After his second year, he was during one year a full time member of the student society board of the Erasmus MC University Medical Center. Afterwards he was chairman of the student faculty counsel and founded the 'Erasmus Journal of Medicine': a journal that provides a forum for young investigators to publish their first research. In 2013, his graduation research focused on hospital acquired infections after surgery. After obtaining his medical degree he started as a PhD candidate under supervision of prof. dr. Verbon and dr. Rijnders at the Erasmus MC University Medical Center.