Immunological and virological changes during treatment for hepatitis C virus infections

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Immunological and virological changes during treatment for hepatitis C virus infections

Immunologische en virologische veranderingen tijdens de behandeling van hepatitis C virus infecties

(met een samenvatting in het Nederlands)

Proefschrift

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“when a man is growing older, he realizes he begins to look like his (grand)father.”

vrij naar Gabriel Garcia Marquez

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CHAPTER 1

General introduction

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Hepatitis C virus and human immunodeficiency virus coinfection: where do we stand?

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Introduction

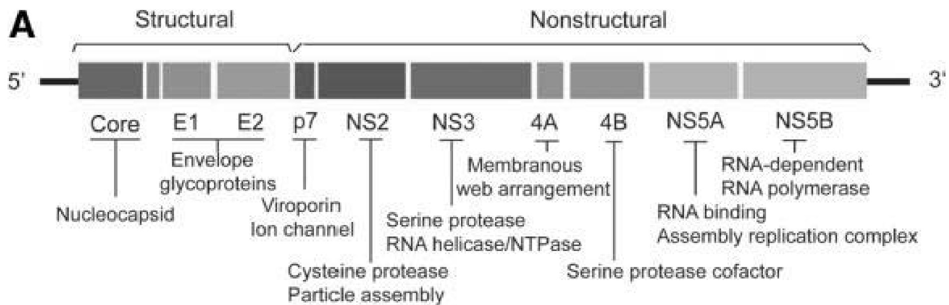
Hepatitis C virus (HCV) infections are a global health problem with an estimated 170 million people (3% of the total population) infected with this virus worldwide [1,2]. In the United States nearly 4 million and in Europe more than 5 million people are infected with HCV [3,4]. In the Netherlands approximately 0.1 to 0.4% of the population is chronically infected (15,000 to 60,000 individuals) [5]. HCV is a single-stranded RNA-virus belonging to the Flaviviridae family to which for example also yellow fever, dengue and tick-borne encephalitis viruses belong [6]. After the discovery of its genomic structure in 1989, screening assays became rapidly available leading to screening of blood donors from 1992 onwards [7]. HCV has 6 known genotypes, denoted 1 to 6, differing from each other in up to 30% of the total sequences. Within these genotypes over 80 subtypes are recognized again differing 15-20% in their nucleotide sequences [8]. Patients infected with a HCV infection are currently treated with weekly injections of pegylated interferon-alfa (pegIFN-alfa) in combination with daily ribavirin tablets [9]. Currently, we are at the brink of a new era with respect to the introduction of revolutionary novel therapeutic anti-HCV agents into clinical practice. However, a lot of questions remain regarding the interplay between the virus and the host defense mechanism.

HCV virology

Hepatitis C virus

HCV has a positive-strand RNA genome of 9.6 Kb pairs consisting of a 5′-non-coding region (NCR), including an internal ribosome entry site (IRES), an open reading frame encoding for the viral proteins and a 3′-NCR (figure 1). After entry into the hepatocytes, IRES-mediated translation results in the formation of a large poly-protein that is subsequently cleaved by both viral and cellular proteases into structural proteins and non-structural (NS) proteins [10]. The structural proteins, consisting of the core protein and the envelope glycoproteins E1 and E2, form the HCV nucleocapsid. The NS-proteins (NS2, NS3, NS4A/B, NS5A and NS5B) are important in coordinating the intracellular processes of viral replication [11]. The most-studied viral proteases and currently targets of newly developed anti-HCV therapies are NS3, NS5A and NS5B proteins. NS3/4A is a multifunctional protein harboring both a helicase and protease domain. The NS4A protein serves a cofactor for the NS3 protease forming an integrated protein [12] that

Figure 1: Genomic structure of HCV divided in structural and non-structural proteins.



The functions of the different proteins are briefly explained (reproduction with permission from the auteur)

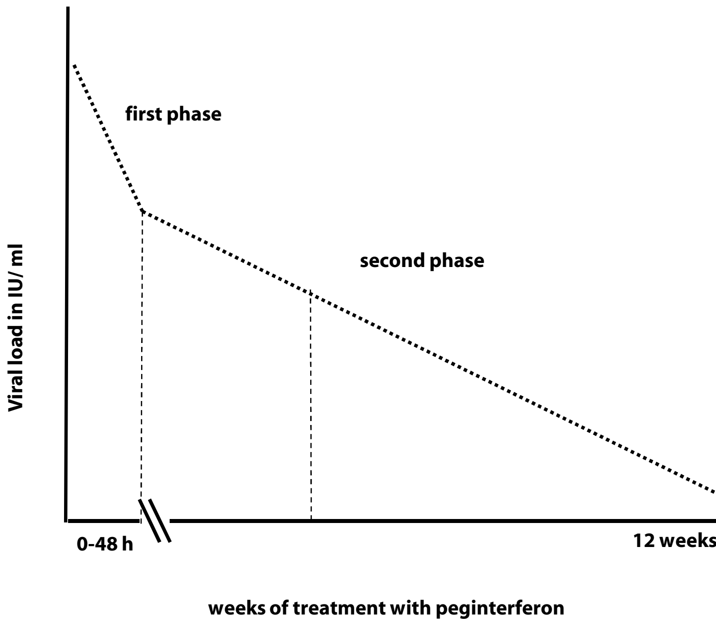
has recently been shown to be able to inactivate intra-cellular signaling molecules Triff [13] and Cardif [14]. The NS3 helicase domein is important in RNA unwinding [15]. The phosphorylated NS5A protein plays a role in the efficacy of HCV-RNA replication [16]. Lastly, NS5B is the RNA-depended RNA polymerase (RdRp), responsible for the formation of genomic positive-strand RNA from its negative-strand RNA template, the last step in the viral replication [17].

Viral Kinetics

HCV has a high replication rate of 1 x 10^{12} virions/ day and a half-life of only three hours [18,19]. Viral load levels of HCV remain relatively stable over time but are higher in HIV-infected patients [20,21]. The decline in HCV-RNA after initiation of interferon-alfa (IFN-alfa) and ribavirin treatment on both mono-infected and coinfected patients shows a biphasic pattern (figure 2) [22,23]. The first phase is rapid and occurs within 24 to 48 hours after the start of treatment. At that time the viral production and release of HCV is blocked. This reflects the sensitivity of the virus to interferon [22]. The second phase is slower and more variable in time, which is thought to reflect the rate of immune-mediated clearance of HCV infected cells [23]. Previous studies in HCV mono-infected patients have shown that the steepness of the slope of the second phase is a good predictor of achieving a sustained viral response (SVR, i.e. undetectable HCV-RNA 24 weeks after treatment discontinuation) [19,24-27]. The existence of a biphasic pattern in HIV/ HCV coinfected patients is unclear [28-30].

To date, it was generally accepted that there was minimal variance in the first phase viral load decrease. Two retrospective studies in HCV mono-infected patients demonstrated that the first phase viral load decrease may predict SVR in patients treated with

Figure 2: Viral kinetics during anti-HCV therapy



A schematic picture of a biphasic model (first and second phase) of HCV-infected patients with on the x-axis the duration of pegIFN-alfa treatment and on the y-axis the viral load in IU/ ml.

IFN-alfa monotherapy [31,32]. If a decline in viral load at day two (48 hours) after the start of pegIFN-alfa/ ribavirin therapy could serve as a decision moment, unnecessary treatment of many HCV-infected patients might be prevented. However, this remains to be confirmed in future trials.

HCV immunology

The human defense against invading pathogens consists of an innate and an adaptive immune system. The main function of the innate immune system is 2-fold, namely to contain the infection via production of different proteins (complement and CRP) and cytokines (type I IFN's, i.e. IFN-α and IFN-β). Second, the adaptive immune system is triggered for clearance and immunological memory.

Innate immunity

After invasion of a pathogen, the innate immune system is activated by triggering of the so-called pathogen-associated molecular patterns (PAMPs) recognizing small molecular motifs conserved in the pathogen. After entrance of HCV into the hepatocyte, through receptor-mediated endocytosis [33], viral RNA is sensed by the helicase RIG-I, forming a complex that inhibits activation of signaling molecules [34]. Moreover, HCV-RNA in plasma is detected by the Toll-like receptor-3 (TLR-3) on the hepatocyte surface also capable of inducing intra-cellular signaling pathways (via IFN regulatory factor-3 (IRF-3)) leading. Together with the RIG-I pathway this leads to up-regulation of Interferon Stimulating Genes (ISG) resulting in production of IFN-β. HCV proteins Core and NS3/4A have been shown to block these intracellular signaling pathways and thereby can inhibit an effective initiation of an anti-HCV immune response [35]. Other cell types like natural killer cells (NK-cells) and dendritic cells (DCs) are also thought to play a role in viral clearance or persistence of HCV although their functional role is still largely unknown [36].

Adaptive immunity

Neutralizing antibodies, produced by B-cells, directed first against core and NS3 and later against the hypervariable region of the E1 and E2 proteins, are detectable several weeks after the initial HV-infection. In contrast to studies in chimpanzees [37], the importance of HCV-neutralizing antibodies in both viral clearance and sterilizing immunity in humans is still controversial [38].

Much better characterized is the role of T-cells in viral clearance. Successful clearance of acute HCV-infection is associated with a strong and vigorous HCV-specific CD4+ and CD8+ (i.e. cytotoxic T lymphocytes or CTLs) T-cell response targeting multiple HCV epitopes [39-42]. In contrast, in chronic HCV-infection both HCV-specific CD4+ and CD8+ T-cells exert diminished proliferative properties and low interferon-gamma (IFN-γ) secretion [43,44]. Furthermore, the breadth of the HCV-specific T-cell response in chronic HCV infection is narrow, targeting only a very small number of HCV antigens [45-47]. Some studies have shown that successful outcome (SVR) of pegIFN-alfa/ ribavirin therapy is associated with higher baseline CD4+ and/ or CD8+ specific T-cell responses [48-51] while others did not observe such a relationship [52-56]. Similarly, contradictory studies have been reported on the role of HCV-specific T-cells during pegIFN-alfa/ ribavirin therapy showing either augmentation [54,57,58] or decline [48,49,52] of T-cells. Therefore, there is no conclusive evidence whether HCV-specific immunity

contributes to therapy induced viral clearance for several reasons. Good animal models are unavailable [59], frequencies of circulating HCV-specific T-cells are very low [41] and therefore hard to detect, and a consensus on the optimal *ex vivo* experimental cell culture protocols is lacking [60]. A robust and sensitive assay able to detect low frequencies of HCV-specific T-cell responses is needed to resolve the above mentioned controversies.
Recently, a genome-wide association study in chronic HCV genotype 1- infected patients treated with pegIFN-alfa/ ribavirin has revealed a single nucleotide polymorphism (SNP) in the interleukin-28B (IL-28B) gene [61]. Thereafter, several other IL-28B SNPs have been identified to be associated with spontaneous viral clearance of acute HCV [62] and successful outcome [61,63] or non-response [64,65] to therapy in chronic HCV. The IL-28B gene encodes for a protein called interferon-lambda (IFN-λ3) known to induce the JAK-STAT pathway by binding to the IFN-receptor after which ISG are upregulated aiding in suppression of the viral infection [66]. However, its exact contribution to viral clearance remains to be elucidated.

Acute hepatitis C

Transmission of HCV has traditionally been thought to occur mainly via blood-blood contact putting intravenous drug users and patients receiving blood transfusions at high risk of contracting acute HCV. Risk of sexual transmission in HCV discordant couples is assumed to be around 1% per year [67]. After infection, only 20-40% of the patients develop symptoms of acute HCV like jaundice, nausea, abdominal pain or general unwellness [68]. The severity of symptoms and/ or laboratory abnormalities has been positively associated with the chances of spontaneous viral clearance [68-71]. Due to the lack of severe symptoms in the majority of patients, the diagnosis of acute HCV infection is difficult.
In recent years, several studies have shown a marked increase in the incidence of acute HCV among human immunodeficiency virus (HIV)-infected man who have sex with men (MSM) [72-77]. Phylogenetic analysis revealed clustering of specific HCV-strains among large international HIV-positive MSM networks making sexual (permucosal) exposure the most likely transmission route [78,79]. Compared to acute HCV mono-infections, overt clinical symptoms rarely occur leading to much lower spontaneous viral clearance rates of 10-15% in HIV-infected patients with acute HCV [73,80-82]. It is important to realize that failure to diagnose HCV in the acute phase of the infection compromises the chances of

cure as higher SVR rates are observed upon early therapy when compared to treatment of the chronic disease [83].
Treatment of acute HCV mono-infected patient is undisputed since studies with pegIFN-alfa monotherapy have shown high SVR rates between 72-94% [84-88]. Addition of ribavirin has not been shown to further increase the SVR rates [89]. However, in HIV-infected patients with an acute HCV-infection the optimal treatment strategy has been debated [83,90]. Several retrospective studies evaluating the efficacy of pegIFN-alfa monotherapy have reported varying SVR rates from 0% [91],8% [76], 62% [92] and 100% [93,94]. Two prospective non-randomized studies compared pegIFN-alfa/ ribavirin combination therapy to pegIFN-alfa monotherapy showing either superiority of the latter [95] or favoring combination therapy over monotherapy [96]. Studies using pegIFN-alfa/ ribavirin combination therapy reported SVR rates between 50% and 80% [73,80-82,97-99]. All studies differ with respect to design, patient characteristics and number of included patients making comparison between studies difficult.

Chronic HCV

Currently, the standard treatment for chronic HCV infection is pegIFN-alfa in combination with ribavirin. PegIFN-alfa is a chemical modification of interferon-alfa by the covalent attachment of a polyethylene glycol (PEG) molecule that resulted in a changed pharmacokinetic profile. PegIFN-alfa is thought to exert both anti-viral and immunomodulatory properties [100-102]. With this regimen, a successful response is achieved in HCV genotypes 2 and 3 of around 80-90% in contrast to much lower SVR rates of 40%-50% for genotypes 1 and 4 [103-105]. Importantly, the standard duration of treatment for the latter group of patients is 48 weeks compared to the 24 weeks for genotypes 2 and 3.
PegIFN-alfa's main side-effects are flu-like symptoms, weight loss, depression and fatigue. It's mechanism of action is thought to up-regulate ISG leading to interference with viral replication, activation of the innate immune response and apoptosis [106]. Ribavirin is a synthetic guanosine nucleoside analogue with a moderate anti-viral effect (-0.5 to 1.6 log10 reduction) in the first 2 to 3 days after ingestion [107]. Despite the many existing hypothesis, the exact mechanism of action of ribavirin is still unknown. Well known are ribavirin's effects on erythrocytes inducing anemia.
Several patient characteristics serve as predictors of a successful outcome of therapy such as the presence of cirrhosis [108], HCV genotype [104] and baseline HCV viral load [109]. Very recently, several SNPs in the IL-28B gene have been identified as strong

predictors for treatment success [61-65]. Moreover, an on-treatment predictor for the successful outcome of therapy, is the achievement of a rapid virological response (RVR, i.e. HCV-RNA <50 IU/ml at week 4 of therapy). Studies have shown that in HCV genotype 1-infected patients achieving a RVR, shortening of therapy from 48 to 24 weeks is feasible [109].

In HIV-infected patients, chronic HCV is treated similarly to HCV mono-infected patients with pegIFN-alfa in combination with ribavirin. Different from the variable treatment durations based on HCV genotype in chronic HCV, HIV/ HCV coinfected patients are treated for 48 weeks regardless of the HCV genotype [110-112]. However, evidence is accumulating that also in HIV/ HCV coinfected patients shortening of treatment duration is possible in patients with a low viral load at baseline and achievement of a RVR [113-115]. In contrast to these so-called "easier-to-treat" patients, another group of patients exists with genotype 1 or 4 and a partial response in HCV-RNA at week 12 of therapy (i.e. partial Early Virological Response (pEVR)). In those patients prolonging therapy to 72 weeks might be beneficial [116].

The combination of severe side-effects of pegIFN-alfa/ ribavirin leading to discontinuation of therapy in quite a number of patients on the one hand and the low SVR rates in HCV genotype 1 infected patients on the other hand, has led to the development of new classes of anti-HCV drugs. As mentioned before, the viral proteins NS3 (protease-inhibitors), NS5A and NS5B (polymerase-inhibitors) are targets to which these STAT-C (specifically targeted anti-viral therapy for HCV) drugs have been developed. Currently, the protease-inhibitors telaprevir (VX-950) and boceprevir (SCH503034) are at the end of their phase III trials and final results and subsequent licensing (expected in 2011) are eagerly awaited. Treatment with telaprevir in chronic HCV-infected patients resulted in high SVR rates of around 70% for those naive for anti-HCV therapy and 50% for previous non-responders to anti-HCV therapy [117,118]. Revolutionary, was the INFORM-1 trial [119] evaluating the efficacy of a 14-day combination regimen of a protease and a polymerase inhibitor without the addition of pegIFN-alfa/ ribavirin. Interim-analysis showed a very pronounced viral load decrease with 7 of 8 patients being HCV-RNA undetectable (<40 IU/ml) at the end of the 14 day treatment period [119]. Today more than 20 STAT-C compounds are in development leading to a new era in the battle against HCV.

There is a group of mainly HIV/ HCV coinfected patients that have been unsuccessfully treated with pegIFN-alfa/ ribavirin in the past and currently progress to moderate to severe liver fibrosis [120,121]. For these patients there is no current form of therapy and the new protease and polymerase inhibitors are still a few years away. Therefore, they are at increased risk for development of cirrhosis and subsequently endstage liver

disease or development of a hepatocellular carcinoma (HCC). Immunohistochemical studies in patients with chronic HCV have shown both an increased presence of apoptotic hepatocytes as well as a correlation between hepatocyte apoptosis rates (percentages of caspase-3 positive) and the amount of liver fibrosis [122-125]. This has led to the development of a novel class of antagonists of the apoptosis pathway called caspase-inhibitors [126].

HIV and HCV coinfection

Due to a similar mode of transmission (blood-blood contact and sexual), 30% of the HIV-infected patients are also coinfected with HCV [127]. This increases to around 80% in intravenous drug users [128,129]. In chronic HCV, progression of liver fibrosis to cirrhosis and HCC is known to be slow, taking decades to develop. This varies according to individual variables such as duration of infection, age at time of infection, male sex, amount of alcohol consumption and HIV coinfection [130-133]. Multiple studies have examined the effects of HIV infection on the natural history of chronic hepatitis C infection, showing an increased fibrosis progression rate leading to more rapid development of liver cirrhosis leading to HCC and/ or liver failure [134,135]. A possible explanation could be the immune compromised state of the HIV-patients showing an inverse correlation between the CD4 cell count and the rate of fibrosis progression [136,137]. Moreover, no protective effect of combination anti-retroviral therapy (cART) on slowing liver fibrosis progression has been demonstrated [121,138,139].
Conflicting results have been reported about the effect of chronic hepatitis C on the natural history of an HIV infection with some showing a faster progression to AIDS and death [140-142] while more recent studies did not detect differences in progression to an AIDS-defining illness, progression to a CD4 cell count below 200/ mm^3 or survival between HIV/ HCV coinfected or HCV-negative HIV-infected patients [143-145]. It is generally accepted that although there is no effect of HCV on HIV, HIV negatively influences the natural history of HCV.

Outline of this thesis

The studies in this thesis describe the immunological and virological changes during anti-HCV therapy. It focused on the "difficult-to-treat" patients infected with HCV genotypes 1 or 4 with or without HIV. Three different studies have been designed in order

to investigate the HCV-specific T-cell response and viral kinetics (PRIVICOP-study), the optimal treatment regimen for HIV-infected patients with acute HCV (UMCU/ UMCG cohort study) and the rates of apoptotic T-cells (caspase-study).

In **chapter 2** the discrepant therapeutic regimens for treatment of acute HCV mono-infection and acute HCV in HIV-coinfection are discussed together with hypothesizing that pegIFN-alfa monotherapy for acute HCV in HIV-infected patients could be sufficient. In **chapter 3** the cohort-study is described, evaluating the efficacy of pegIFN-alfa monotherapy for the treatment of acute HCV in HIV-infected patients. The results of this study together with a growing need for evidence based recommendations for the treatment of acute HCV in HIV-infected patients led to the establishment of the first Dutch guidelines described in **chapter 4**.

Chapter 5 evaluates the role of the HCV viral load decrease in the first 48 hours after treatment initiation as predictor for successful outcome of pegIFN-alfa/ ribavirin therapy.

The following 2 chapters describe the immunological studies performed in chronic HCV-infected patients. **Chapter 6** evaluates the HCV-specific T-cell response both at baseline and during pegIFN-alfa/ ribavirin therapy in chronic HCV-infected patients. In **chapter 7** similar analyses into the role of HCV-specific T-cells during pegIFN-alfa/ ribavirin therapy are described only now in HIV-infected patients coinfected with chronic HCV.

A novel agent in the field of HCV is described in **chapter 8**. The effect of a caspase-inhibitor on peripheral T-cells is investigated.

In **chapter 9**, the main findings of this thesis are discussed, new hypotheses are generated and put into perspective. Finally, ideas for future research are presented.

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Chapter 2

Peginterferon-alfa monotherapy for the treatment of acute hepatitis C in HIV-coinfected patients

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Introduction

In recent years, the incidence of acute hepatitis C virus (HCV) infections among human immunodeficiency virus (HIV)-infected men who have sex with men (MSM) in Western Europe is increasing [1-3]. To date, only a small number of clinical trials have been performed and no evidence based guidelines are available to guide the treatment of acute hepatitis C in HIV- coinfected patients. With much interest we read the publication by Soriano *et al.* [4] on the updated recommendations from the HCV-HIV International Panel, published recently in this journal. After a short overview of acute HCV in patients with chronic HIV-infection, the authors recommended that these patients should be treated early with both pegylated interferon and ribavirin. They state that "addition of ribavirin ensures maximal clearance of HCV". Since this recommendation in merely based on an expert opinion, we would like to argue this and advocate that these patients could be treated with pegylated interferon-alfa solely, for reasons to be mentioned below.

Treatment of acute HCV mono-infections

First, the most available data about the treatment of acute hepatitis C come from trials performed in HCV mono-infected patients. The first therapeutic trials in acute HCV mono-infection, with varying interferon regimes and small numbers of included patients, showed a beneficial effect of treatment with responses varying between 25% and 91%. The first landmark trial by Jaeckel *et al.* [5], treating 44 patients with interferon alfa-2b for a total of 24 weeks, resulted in a sustained viral response rate (SVR) of 98%. More recently, a few trials [6-9] have shown that high response rates, varying between 72 % and 94 % can also be reached with pegIFN-alfa monotherapy. Up to now, there is no convincing evidence that addition of ribavirin to pegIFN-alfa will add any benefit [10]. Therefore, current guidelines for the treatment of acute hepatitis C monoinfection recommend peginterferon monotherapy instituted 12 weeks after seroconversion.

Arguments in favour of peginterferon-alfa monotherapy

What about the evidence in HIV/HCV-coinfected patients? In recent years, 3 prospective trials have been published on the treatment of acute HCV in HIV-coinfected patients [11-13]. Both Dominguez *et al.* and Gilleece *et al.* have treated their patients

with the combination of pegIFN-alfa and ribavirin whereas Vogel *et al.* compared pegIFN-alfa monotherapy with pegIFN-alfa/ ribavirin combination therapy. The SVR rates reached in these trials were comparable (59% to 71%). Moreover, no difference in SVR rate was seen between patients treated with pegIFN-alfa/ ribavirin combination therapy versus pegIFN-alfa monotherapy [13]. Therefore, in our opinion, there is no firm evidence at the moment to support the addition of ribavirin to pegIFN-alfa in the treatment of acute HCV infection in HIV-coinfected patients.

Another reason to withhold ribavirin is that it can be added to the treatment regimen in the later stage once chronic HCV is established, i.e. in case of a non-response to early treatment or a relapse. Overall SVR rates in coinfected patients are substantially lower than in mono-infected patients (60% versus 90%). Therefore, a high percentage of patients will fail treatment and will become chronic HCV carriers requiring re-treatment. Several trials in both HCV mono- and coinfected patients have shown that re-treatment with pegIFN-alfa/ ribavirin combination therapy in (peg)interferon experienced patients results in much lower SVR rates than in (peg)interferon naïve patients [14-17]. Furthermore, it has been shown that a less potent regime in the naïve setting gives a higher chance of an acceptable SVR once re-treatment is necessary. The addition of ribavirin in treating interferon non-responders resulted in a higher SVR than re-treatment with interferon alone [18-20].

Moreover, HCV mono-infected patients who have a relapse after combination therapy need to be re-treated with higher doses of ribavirin and with prolonged courses of therapy [21,22]. Therefore, withholding ribavirin can be advantageous in order to optimise the chances of achieving a SVR once the pegIFN-alfa monotherapy failed and the patient becomes a non-responder or a relapser.

Lastly, treatment of acute HCV infection in HIV coinfected patients with only pegIFN-alfa will result in less side-effects (anaemia and thrombocytopenia), less interactions with antiretroviral agents and a lower pill burden resulting in a better compliance and a higher chance to complete this therapy.

Conclusion

Based on these arguments, we believe that at the moment there is not enough evidence to support combination therapy with pegIFN-alfa/ ribavirin for the treatment of acute HCV infection in HIV-positive patients. Withholding ribavirin in the acute treatment phase can maximize the chances of an acceptable SVR in case the patient is a non-responder or has a HCV relapse and needs re-treatment.

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Chapter 3

Peginterferon-alfa monotherapy leads to low response rates in HIV-infected patients with acute hepatitis C

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Submitted.

Abstract

Background: Despite a rising incidence of acute hepatitis C virus infections (HCV) in patients infected with human immunodeficiency virus (HIV), the optimal therapeutic strategy (peginterferon-alfa monotherapy or in combination with ribavirin) is still under debate.

Methods: Twenty-three HIV-infected patients were prospectively diagnosed with acute HCV and treated with peginterferon alfa-2a monotherapy (pegIFN-alfa-2a; 180 µgr /weekly) for 24 or 48 weeks. Add-on ribavirin was allowed from week 4 of therapy onwards. Three patients were not included for different reasons. Blood samples were routinely drawn for viral load measurement and IL-28B polymorphism analysis.

Results: Spontaneous viral clearance occurred in 1 patient (4%). Nineteen patients (13 genotype 1 and 6 genotype 4) received treatment with pegIFN-alfa monotherapy (3 with add-on RBV) resulting in a rapid virological response (RVR, HCV-RNA <50 IU/ml at week 4) in 7 patients (37%). A sustained virological response (SVR) was reached in 7 patients (37%) whereas 9 patients (47%) were null-responders to treatment (non-EVR, i.e. $<2Log_{10}$ drop in HCV-RNA at week 12 of therapy). The unfavorable G-allele of the IL-28B polymorphism rs8099917 was detected in 66% of the non-responders. In case of re-emergence of HCV viremia after treatment discontinuation, sequence analysis of quasispecies confirmed a HCV relapse in 3 patients while 2 patients were re-infected by their previously non-responding partner.

Conclusion: PegIFN-alfa monotherapy resulted in a low SVR rate and a high percentage of null-response whereas non-SVR was associated with a polymorphism in the IL-28B gene (rs8099917).

Introduction

In recent years, the incidence of acute hepatitis C virus (HCV) infections among men having sex with men (MSM) infected with the human immunodeficiency virus (HIV) has markedly increased [1-3]. Recent phylogenetic analysis revealed clustering of specific HCV-strains among large international HIV-positive MSM networks with permucosal exposure as most likely transmission route [1,4]. Since acute HCV in HIV-infected patients rarely presents with overt clinical symptoms, yearly testing for the presence of HCV antibodies and routine laboratory assessment of transaminases is the preferred method of screening [5]. Failure to diagnose HCV in the acute phase of the infection compromises the chances of cure as higher rates of sustained virological response (SVR) are seen upon early therapy when compared to treatment of chronic HCV [6].

In contrast to HCV mono-infected patients, where SVR rates between 72%-94% are reached with (peg)interferon-alfa monotherapy [7-9], the optimal treatment strategy for acute HCV in HIV-infected patients is debated [10,11]. In HIV-infected patients with acute HCV fairly consistent SVR rates of around 51%-80% have been reported with combination therapy of peginterferon-alfa (pegIFN-alfa) and ribavirin [12-18]. However, treatment with pegIFN-alfa monotherapy has resulted in a wide variance of SVR rates from 0% to 100% [19-23]. Two prospective non-randomized trials have compared pegIFN-alfa monotherapy with pegIFN-alfa/ ribavirin combination therapy with 1 study [24] showing superiority of pegIFN-alfa monotherapy over combination therapy while the other study [25] reported the opposite result. Comparing these trials is difficult because of differences in study design and patient characteristics.

This study describes the low efficacy of pegIFN-alfa monotherapy in the largest cohort so far of HIV-infected patients with acute HCV. Since recent publications highlight the importance of IL-28B gene single nucleotide polymorphism (SNP) in spontaneous clearance in acute HCV [26] and either a favorable outcome [27,28] or non-response [29,30] to pegIFN-alfa/ ribavirin therapy in chronic HCV genotype1 infections, this study evaluated the role of IL-28B SNP in treatment outcome of pegIFN-alfa monotherapy in HIV-infected patients with acute HCV.

Patients and Methods

Patient cohort

All HIV-patients in follow up in two academic hospitals (University Medical Center Utrecht and University Medical Center Groningen) consecutively being diagnosed with acute HCV infection between January 2006 and January 2009, were included in this study. Routine monitoring of liver enzymes every 3 to 4 months and yearly screening for HCV-antibodies was performed in all patients. Diagnosis of acute HCV was based on the following criteria; i) detectable plasma HCV-RNA (COBAS taqman 2.0, Roche Molecular Diagnostics, Almere, the Netherlands or real-time PCR, Abbott Diagnostics, Hoofddorp, The Netherlands); ii) anti-HCV seroconversion (AxSYM automated immunoassay instrument system or Abbott Architect CMIA 3.0, Abbott Diagnostics, Hoofddorp, The Netherlands) following any elevation of liver enzymes above the upper limit of normal (ULN is 35 U/L) compared to previous measurements; iii) negative HCV-serology in the last stored plasma samples within the previous 6 (n=18) to 12 months (n=5) before diagnosis. HCV genotype was determined by second generation InnoLIPA assay (Versant HCV genotype 2.0 assay, Siemens Healthcare Diagnostics, Breda, the Netherlands).

Treatment

Patients were treated and followed up by their own Infectious Diseases physicians in accordance with international guidelines [6,31]. After the diagnosis of acute HCV, spontaneous viral clearance was awaited for 12 weeks after which anti-HCV treatment was initiated with pegIFN-alfa-2a 180 ugr weekly (Pegasys®; Roche, Basel, Switzerland). When no rapid virological response (RVR, i.e. HCV-RNA <50 IU/ml) but more than $2\log_{10}$ decrease in HCV-RNA was observed at week 4 of therapy, addition of weight based ribavirin (<75 Kg: 1000 mg in two daily doses; ≥ 75 Kg: 1200 mg in two daily doses; Copegus®; Roche, Basel, Switzerland) was allowed. Patients were treated 24 or 48 weeks at the discretion of the treating physician. A 12-week on-treatment analysis showing high rates of non-response [32], resulted in modification of our treatment practice and thereby ending the inclusion of patients into this cohort.

Viral load and definitions

Plasma HCV-RNA was routinely measured pre-treatment and at weeks 4, 12, 24, 48 and 24 weeks after discontinuation of therapy using the qualitative Roche Amplicor® (lower limit of detection <50 IU/ml; Roche Molecular Systems, Pleasanton, California), the standard PCR-assay performed in our clinic. A null-response was defined as $<2\log_{10}$ drop in HCV viral load at week 12 of therapy after which pegIFN-alfa monotherapy was discontinued in all but 2 patients who were treated for 24 weeks. An early viral response (EVR) was defined as $>2\log_{10}$ drop in HCV-RNA at week 12 of therapy.

Viral sequencing

Total RNA was isolated from plasma using TRIzol reagent (Invitrogen, Breda, the Netherlands), dissolved with RNAse inhibitor (Applied Biosystems, Foster City, USA) after which 10 μl of RNA template was used as input for the reverse transcription using iScript cDNA Synthesis Kit (BioRad, Veenendaal, the Netherlands) according to the manufacturer's protocol. The used mix of primers for the NS5B amplification and sequencing was as follows: outer primers (sense: CTGACRACTAGCTGYGGTA-AYAC (position 8454-8476) and anti-sense: CCTGGAGAGTAACTRTGGAGTG (position 9040-9019)) and inner primers (sense: GCTCCRGGACTGCACSATGCTCGTG (position 8522-8546) and anti-sense: AATGCGCTRAGRCCATGGAGTC (position 9016-8995)) for genotype 1 and outer primers (sense: ACACTGACGTGCTAYCTHAARGC (position 8475-8497) and anti-sense: TTCGTGTGGAGAGTATCCRTGCA (position 9044-9022)) and inner primers (sense: CTGAGAGACTGCACSATGYTGGT (position 8523-8545) and anti-sense: AATGCGCTRAGRCCATGGAGTC (position 9016-8995)) for genotype 4. PCR products were ligated into the pGEM-T vector and 10-20 clones were sequenced using the M13 primers (M13 sense: 5'-TTTTCCCAGTCACGAC-3' and M13 antisense: 5'-CAGGAAACAGCTATGAC-3'). Sequences were aligned and analyzed using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Quasispecies were aligned to their reference strain: H77 for genotype 1a, D90208 for genotype 1b, Y11604 for genotype 4a and D86638 for genotype 4d. Phylogenetic trees were constructed by the rooted neighbor-joining method and visualized using MAFFT software (Version 6, http://mafft.cbrc.jp/alignment/server/). The robustness of the phylogeny was assessed by bootstrapping with 100 rounds of replications (values >70 were considered robust clusters).

IL-28B analysis

PBMC were isolated from sodium heparin tubes using a Ficoll-Hypaque density gradient centrifugation or with sodium citrate tubes for cell separation (CPT™, BD Biosciences, San José, CA, USA). One million PBMC were lysed using L6 lysis buffer after which DNA was isolated using standardized isopropanol/ ethanol. SNP genotyping was performed using Allelic Discrimination assays (Applied Biosystems, Foster City, USA) for the SNP rs8099917 and rs12979860 following the instructions of the manufacturer. The primers for the two IL-28B SNP were as follows: rs8099917 (TTTTGTTTTCCTTTCTGTGAGCAAT[G/T]TCACCCAAATTGGAACCATGCTGTA) and rs12979860 (GGGAGCTCCCCGAAGGCG[C/T]GAACCAGGGTTGAA). Before the PCR reactions, DNA was added with Allelic Discrimination Assay Mix and TaqMan Universal PCR Master Mix to MicroAmp Optical 96-Well Reaction Plates (Applied Biosystems). Real-Time PCR reactions were performed using the ABI Prism 7900HT system (Applied Biosystems). Data were analyzed using the ABI PRISM SDS software v.1.7 (Applied Biosystems).
All patients provided written informed consent and institutional ethical review boards at participating centers approved the protocol.

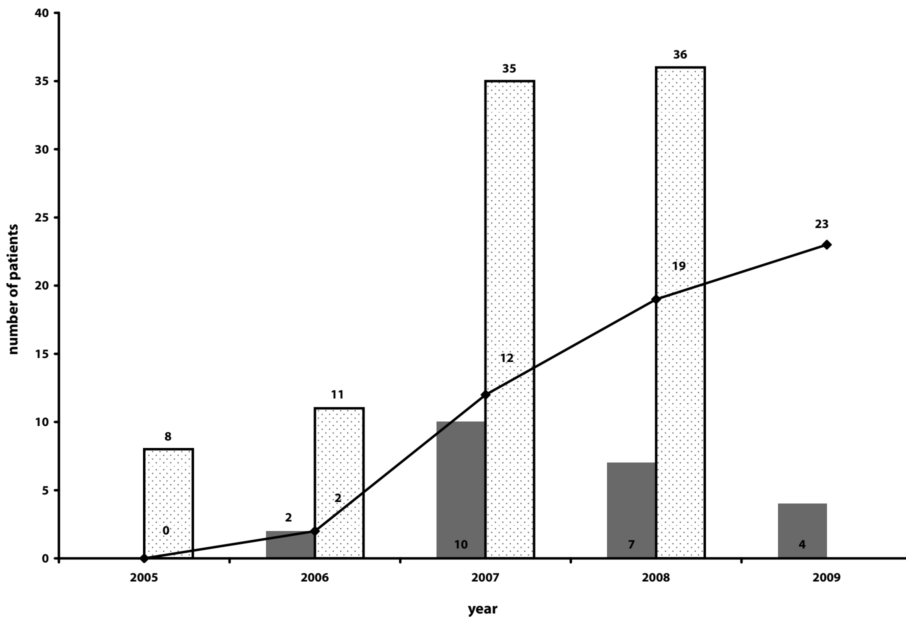
Statistical analysis

Data were analyzed non-parametrically using a Fisher's exact test. Statistical significance was reached with a p-value of ≤0.05 and all tests used were two-sided. All data were analyzed using GraphPad Prism (version 4.0 for Windows, GraphPad Software, San Diego, California, USA).

Results

Twenty-three HIV-infected patients were diagnosed with an acute HCV-infection (figure 1). One patient (4%) spontaneously cleared the infection while another patient was treated with combination treatment pegIFN-alfa/ ribavirin from the beginning. Two patients (one psychologically unfit and one retrospectively diagnosed) were not treated leaving 19 patients for treatment with pegIFN-alfa monotherapy, of which 3 received add-on ribavirin from week 4 onwards. Patient characteristics of those treated are shown in table 1. All patients were male and infected with either HCV genotype 1 (68%) or genotype 4 (32%). The HCV viral load was high with a median of

Figure 1: numbers of HIV-infected patients with acute hepatitis C



Distribution of the number of diagnosed acute HCV infections in HIV+ patients (y-axis) between January 2006 and January 2009 (x-axis) in our cohort (black bars). The black solid line (–) shows the cumulative number of patients during this period. The white speckled bars show the annual number of patients with acute HCV-infection in the Netherlands collected by the RIVM (data of 2009 are still lacking; RIVM-report 210261005).

6.49 Log_{10} IU/ml (IQR 5.77–6.70). The time from HCV seroconversion to start of therapy was 12 weeks (IQR 7-16). Nearly half of the patients (47%) were treated for 24 weeks. Patients had a relatively high CD4 cell count (median of 500 /mm^3, IQR 300 – 693) and 8 patients (42%) received combination antiretroviral treatment (cART) for their HIV-infection.

Overall, pegIFN-alfa monotherapy was well tolerated with no dose modifications necessary. Both leukocytopenia (white blood cell counts below $3.5x10^9$/L) and thrombocytopenia (platelet counts below $150x10^9$/L) were generally mild and seen in 53% of patients without the need to administer growth factors. Anemia occurred in 2 of 3 patients treated with add-on ribavirin, but hemoglobin concentration did not drop below 10 g/dl (6.2 mmol/L). CD4 cell counts dropped below 200/mm^3 in 4 patients (21%) but no opportunistic infections occurred. All patients finished their anti-HCV treatment as intended and according to laboratory data and patients self-reporting, compliance to pegIFN-alfa monotherapy during the course of treatment was considered to be good.

Table 1: Patient characteristics of treated patients

Patient characteristics*	n = 19
age (years)	42 (39 – 48)
genotype 1 / 4	13 (68) / 6 (32)
sex M / F±	19 / 0
HCV viral load (log10 IU/ml)	6.49 (5.77 – 6.70)
maximal ALT (U/L)	541 (233 – 849)
time from seroconversion to start therapy (weeks)	12 (7 - 16)
CD4+ cell count (mm^3)	500 (300 – 693)
on anti-retroviral therapy (HAART) ¶	8 (42)
PI-based	6
NNRTI-based	2
add-on ribavirin at week 4	3 (16)
duration of treatment ¥	
24 weeks	9 (47)
48 weeks	3 (16)

* for continuous data median values are given with (interquartile range) while categorical data are presented with (%).

¶ all 8 patients receiving HAART had a undetectable HIV viral load (<50 copies/ml).

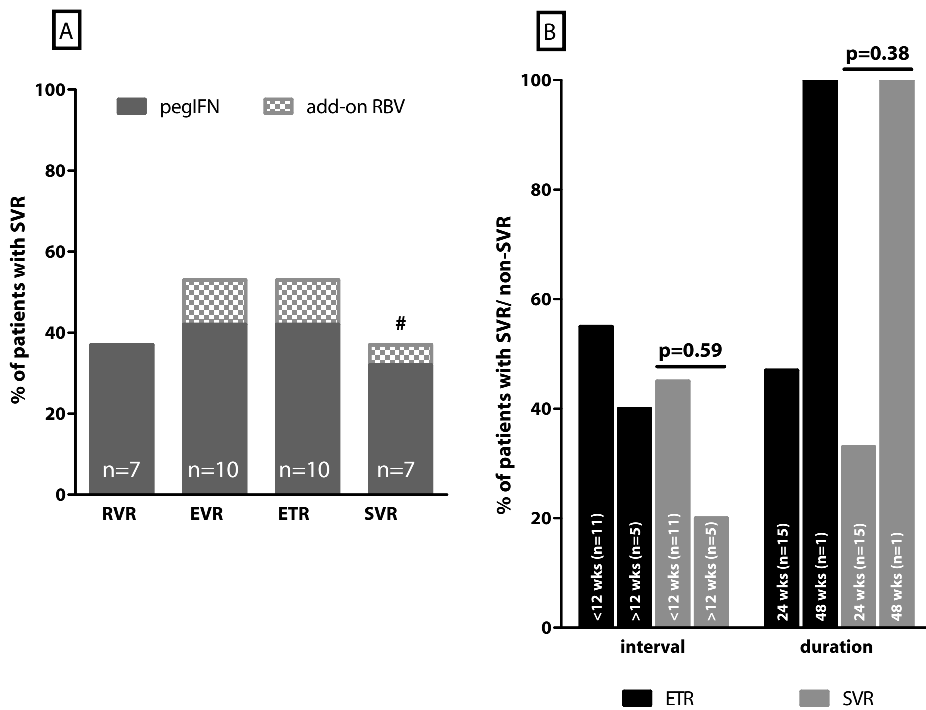
¥ 9 patients were null-responders (<2Log_{10} drop in HCV-RNA at week 12 of therapy) of which 7 were treated for 12 weeks and 2 for 24 weeks.

± in all patients the supposed mode of HCV transmission was permucosal (man having sex with men (MSM))

Treatment outcome

Of the 19 treated patients, 7 (37%) reached a RVR (figure 2A). One of the RVR patients subsequently had a viral rebound with high plasma HCV-RNA levels at week 12 and week 24 effectively being a null-responder and thus therapy was discontinued. After 12 weeks of treatment an EVR was reached by 10 patients (53%), of which two used add-on ribavirin from week 4 onwards. All these patients achieved an end-of-treatment (ETR) response. A total of 9 patients (47%) treated with pegIFN-alfa monotherapy, with 1 patient concurrently receiving add-on ribavirin, failed to respond to therapy and were considered null-responders. A SVR was achieved by 7 patients (37%) with five of these 7 patients (71%) achieving a RVR and 1 patient using add-on ribavirin.
Subsequently, a subgroup analysis was performed of patients only being treated with pegIFN-alfa monotherapy (n=16), excluding the patients with add-on ribavirin. Compared to the overall group of patients, clinical outcome in this subgroup was similar with respect to RVR, EVR, ETR and SVR (44%, 50%, 50% and 38% respectively; data not shown). Next, a stratified analysis was perform based on the interval between diagnosis and start of therapy (within 12 weeks or thereafter) and the duration of therapy (24 or 48 weeks; figure 2B). This resulted in small numbers of patients per group. Although

Figure 2: outcome of pegIFN-alfa therapy



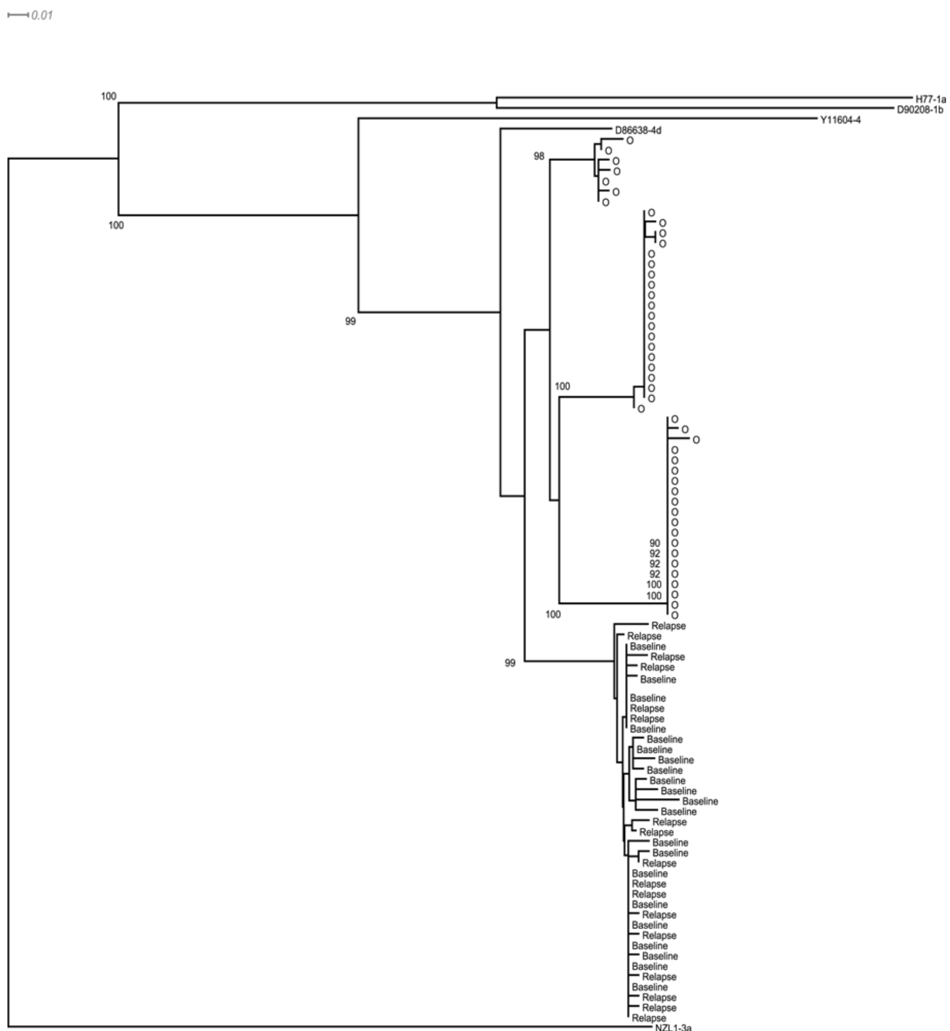
Panel A: treatment response of all 19 patients treated with pegIFN-alfa monotherapy (grey bars) and add-on ribavirin from week 4 onwards (grey speckled). # 2 patients were re-infected by their partner after discontinuation of treatment.
Panel B: outcome by ETR (black bars) and SVR (grey bars) is shown of 16 patients only treated with pegIFN-alfa monotherapy. Virological response is stratified by the interval between diagnosis and start of treatment (interval) and 24 or 48 weeks of treatment (duration). On the y-axis the percentage of patients with a SVR/ ETR is shown. RVR, rapid virological response; EVR, early virological response; ETR, end-of-treatment response; SVR, sustained virological response.

not significant, 5 of 11 patients (45%) starting treatment within 12 weeks after the diagnosis achieved a SVR in contrast to 1 of 5 patients (20%) starting therapy thereafter, suggesting a benefit of early treatment initiation. Finally, anti-HIV treatment with cART did not influence acute HCV treatment outcome (data not shown).
In 5 patients HCV-RNA re-emerged after discontinuation of pegIFN-alfa monotherapy. To differentiate between relapse and re-infection of HCV, NS5B sequence analysis of the quasispecies of these patients was performed. A rooted neighbor-joining phylogenetic tree analysis clearly demonstrated a relapse in 3 out of 5 patients (figure 3A). Compared to the overall patient cohort, in these relapse patients, no distinctive parameters were found, known to negatively influence the outcome of therapy, like time from seroconversion to start of treatment, baseline viral load, HIV-status or

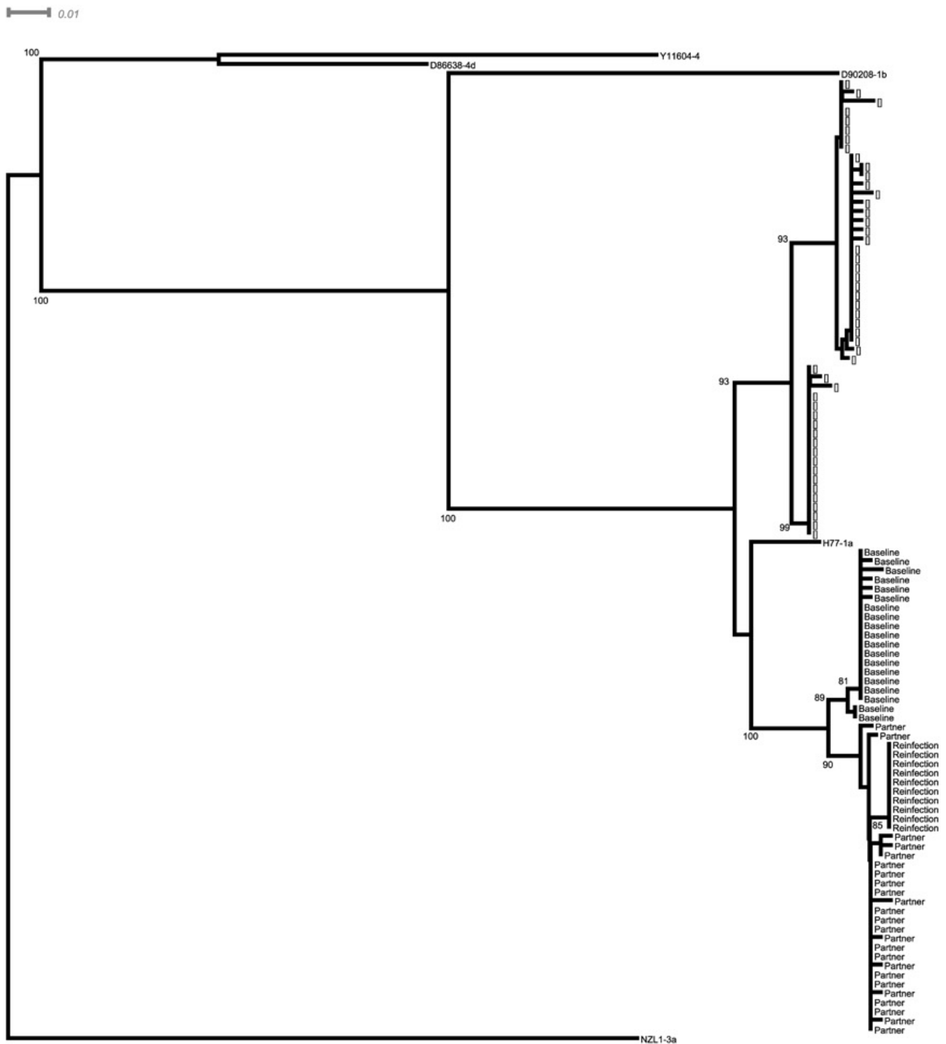
genotype. The other 2 patients (1 achieving a RVR and 1 with a HCV-RNA of 80 IU/ml at week 4 of therapy) were re-infected by their partner since the HCV sequences of the re-infection strains in the patients clustered with the partners' HCV strains. These partners were both null-responders to acute anti-HCV treatment (i.e. null responders in the study cohort; figure 3B). Therefore, these two re-infected patients were regarded as sustained responders for the original pegIFN-alfa monotherapy treatment resulting in a total of 7 SVR-patients (37%).

Figure 3: phylogenetic analysis of patients with a HCV relapse and a HCV reinfection

A



B



Rooted neighbor-joining phylogenetic tree analysis of a patient with a HCV relapse (baseline and relapse strains; panel A) and one with a HCV re-infection from his partner (baseline, re-infection and partner strains; panel B) are shown. The reference strains H77-1a and D90208-1b are shown together with multiple strains of 3 chronic HCV patients of the same genotype (o and □). Bootstrap values of >70 are considered robust and are therefore depicted.

Distribution of IL-28B polymorphisms according to outcome of therapy

Given the importance of the recently published IL-28B gene SNPs [27], an evaluation of 2 of these SNPs (rs8099917 G-allele for non-response and rs12979860 C-allele for response) was performed to possibly explain the outcome of pegIFN-alfa monotherapy. The rs8099917 G-allele is the minority allele in the general population and is therefore modeled dominantly, by combining the minor homozygote G/G with the heterozygote G/T leading to 2 categories for analysis (G/G+G/T versus T/T). Though the rs12979860 C-allele is the minor variant worldwide, in the Caucasian population C/C is the dominant genotype and was therefore modeled recessive, combining the minor homozygote T/T with the heterozygote C/T (i.e. C/C versus C/T+T/T). Of the 12 non-SVR patients, 8 patients (67%) were carrier of the rs8099917 G-allele whereas 4 patients (33%) were homozygous for the T/T-genotype (table 2). In patients achieving a SVR, 3 and 4 patients were carrier of the G-allele and T-allele, respectively (43% and 57%). The rs12979860 C/C-genotype was present in 2 patients (29%) achieving a SVR compared to 5 SVR-patients (71%) carrying the T-allele (table 2). In patient without a SVR, the majority of 9 patients (75%) were carrier of the T/T-genotype compared to only 3 patients (25%) who were carrier of the C/C-allele.

Table 2: Distribution of IL-28B polymorphisms according to outcome of acute HCV treatment

SNP	SVR	non-SVR	p-value
rs8099917			
G/G+G/T	3 (43)	8 (67)	0.38
T/T	4 (57)	4 (33)	
rs12979860			
C/C	2 (29)	3 (25)	1.00
C/T+T/T	5 (71)	9 (75)	

categorical data are presented with (%)

Discussion

This prospective study analyzed the efficacy of pegIFN-alfa monotherapy in the largest cohort reported so far of HIV-infected patients with acute HCV. The results of this study are twofold. First, a low SVR rate with pegIFNA-alfa monotherapy was observed due to a high percentage of non-response with detailed quasispecies analysis showing both HCV re-infections as well as relapses. Second, the majority of the patients not achieving a SVR were carriers of the unfavorable G-allele of the IL-28B SNP rs8099917.

In acute HCV mono-infected patients, high SVR rates varying between 72-98%, were reached with pegIFN-alfa monotherapy making addition of ribavirin unnecessary [8,9,33]. As the optimal therapeutic regimen for HIV-infected patients with acute HCV is currently debated [10,11] the current cohort was established demonstrating a low SVR rate of 37% in HIV-infected patients with acute HCV. Retrospective studies and case-series into the role of pegIFN-alfa monotherapy, have shown contradictory results with a large variety in SVR rates from 0% [17,22] to 100% [21,23]. A small cohort study treating 7 patients with pegIFN-alfa monotherapy for 12 weeks showed a SVR rate of 67% [20]. Two small non-randomized trials comparing pegIFN-alfa monotherapy with pegIFN-alfa/ ribavirin therapy reporting either higher SVR rates for the later (40% versus 57%) [25] or the opposite with superior efficacy of pegIFN-alfa monotherapy over combination therapy (80% versus 48%) [24]. This wide variance in SVR rates might be explained by the included HCV genotypes being genotype 1 and 4 (in majority or exclusively) in studies reporting low SVR rates [19,22,25] and all HCV genotype (including 2 and 3) in studies with higher SVR rates [20,21,23,24]. Alternatively, clustering of all patient HCV strains into one group, suggesting a common source [22], was not observed in this study by phylogenetic analysis. In contrast, most studies investigating the efficacy of combination therapy with pegIFN-alfa with ribavirin reported more consistent SVR rates ranging between 59-80% [12-18]. Moreover, different from the varying rates of null-response reported in these studies (5-30%), in our study as much as 47% of patients proved to be non-responsive to pegIFN-alfa monotherapy. Therefore, this study contributes considerably to the growing body of evidence that pegIFN-alfa/ ribavirin combination therapy is the preferred treatment regimen for acute HCV in HIV-coinfected individuals in order to maximize SVR rates. The large number of non-responders (63%, i.e. null-response and relapse) to pegIFN-alfa monotherapy in this study was surprising. The reason for this is most probably multi-factorial with viral, host and treatment related factors all being important [34]. One plausible explanation could be that ribavirin was not part of the treatment regimen. Although the working mechanism of ribavirin is still largely unknown [35], it has been suggested that optimizing concentrations of ribavirin both to body weight and plasma levels, will achieve higher SVR rates thus leading to lower relapse rates [36]. Previous treatment studies in chronic HIV/ HCV-coinfected patients demonstrated higher SVR rates in patients receiving weight based ribavirin compared to those taking standard dose ribavirin [37]. Furthermore, a study in chronic HCV mono-infected patients treated with the new protease inhibitor telaprevir showed that the absence of ribavirin led to a decrease in SVR rates with a high percentage of non-response [38]. Therefore, combination of

pegIFN-alfa and ribavirin seems necessary to treat acute HCV in HIV-coinfected patients.
In contrast to the classical route of HCV transmission (iv drug use or needle stick injuries), the current epidemic of acute HCV in HIV-infected MSM indicates that high risk sexual behavior is also a possible mode of transmitting HCV [4]. Therefore, when patients continue their high risk behavior during or after anti-HCV therapy, in-depth quasispecies analysis should be performed to reliably distinguish between HCV relapse and re-infection. This is clearly shown in two patients in our cohort who denied any unsafe sexual intercourse at the time of recurrence of HCV viremia. NS5B quasispecies sequencing, but not genotype, line blot analysis and population sequencing, did discriminate between re-infection or relapse. As SVR rates of re-treatment of HCV relapse are much lower than for naive infections (i.e. in case of re-infection), discriminating between these entities is important from both a patient and a physicians perspective.
Recent genome wide association studies have revealed several SNPs in the IL-28B region to be strongly associated with either spontaneous clearance of acute HCV [26] and successful outcome [27,28] or non-response [29,30] to therapy in chronic HCV. The C/C-genotype in the rs12979860 IL-28B SNP was shown not to attribute to the SVR rates in HIV-infected patients with acute HCV (73% versus 60%)[39]. Similarly, our study confirmed the observation that IL-28B C/C-phenotype does not seem to play a role in the treatment-induced viral clearance of acute HCV in HIV-infected patients. However, in this study, the SNP rs8099917 G-allele, was found in the majority of non-responders to treatment and could therefore be an explanation for the high rates of non-response to pegIFN-alfa monotherapy in this cohort. However, this observation has to be confirmed in a larger group of HIV-infected patients with acute HCV.

Conclusion

PegIFN-alfa monotherapy is insufficient for the treatment of acute HCV in HIV-coinfected patients. Furthermore, detailed quasispecies analysis is important in this high risk population to distinguish relapse from re-infection. Lastly, a polymorphism in the IL-28B gene was associated with non-response in this population.

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Chapter 4

Treatment of acute hepatitis C virus infection in HIV+ patients – Dutch recommendations

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Submitted.

Abstract

With a rising incidence of acute hepatitis C virus (HCV) infections in patients coinfected with the human immunodeficiency virus (HIV), there is a need for evidence based treatment recommendations. There are no randomized trials available and published studies differ with respect to design, patient characteristics and number of patients included, making a comparison between studies difficult. However, it is critical to standardize treatment for this group of patients in order to optimize the outcome of therapy.

Here, the Dutch society for HIV physicians proposed to write recommendations for the treatment of acute HCV in HIV-coinfected patients. Combination therapy with pegylated interferon-alfa and ribavirin is the preferred regimen initiated preferably within 12 weeks after the diagnosis of acute HCV. A treatment duration of 24 weeks is recommended in case of a favorable virological response (either achievement of a Rapid Virological Response (RVR) or a >2log10 decrease at week 4 plus undetectable HCV-RNA at week 12). In all other patients prolonging the duration of therapy to 48 weeks should be considered.

Quality of studies on which a recommendation is based:

Grade	**Definition**
A1	Meta-analysis of at least 2 independent studies of A2 level
A2	Randomized double-blind placebo controlled study of adequate quality and size
B	Comparative study not fulfilling the characteristics of an A2 level study (including case-control studies and cohort studies)
C	Non-comparative studies
D	Expert opinion

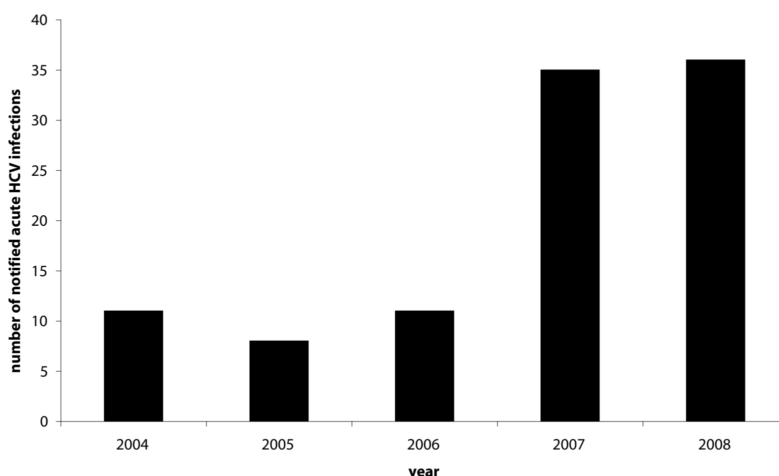
Level of evidence based on the quality of the study on which a recommendation is based:

Level	**definition**
I	Study of level A1 or at least two independent studies of level A2
II	Single level A2 study or at least 2 independent level B studies
III	Single level B or C study
IV	Expert opinion

Introduction

In recent years, the incidence of acute hepatitis C virus (HCV) infections among men having sex with men (MSM) coinfected with the human immunodeficiency virus (HIV) has markedly increased [1-4] (figure 1). HIV-infected patients with an acute HCV-infection hardly ever present with overt clinical symptoms, thereby hampering early detection of acute HCV infection. Frequent routine laboratory assessment of transaminases is the most commonly used method of detection [5,6]. In contrast to treatment of acute HCV mono-infected patients, where SVR rates between 72%-94% are reached with pegIFN-alfa monotherapy [7-11], the optimal treatment strategy for acute HCV in HIV coinfected patients is less clear [12,13]. Therefore, there is a need for evidence based treatment recommendations, guiding clinicians in the field to treat acute HCV in this population. Here, we summarize the available literature, after which recommendations are made regarding the case definition for acute HCV (textbox 1), the preferred treatment regimen, the time to start therapy and the duration of therapy (text box 2).

Figure 1: Number of notified acute HCV infections per year, among HIV-infected MSM in the Netherlands



Source: RIVM-report 210261005 'Sexually Transmitted Infections, including HIV, in the Netherlands in 2008. RIVM-OSIRIS notification data

Literature search

An English-language literature search was conducted using the Pubmed database through April 1th 2010. As search terms "hepatitis C or acute hepatitis C or HCV or acute

HCV" AND "peginterferon-alfa or interferon-alfa or ribavirin or combination therapy or monotherapy or RBV or HCV treatment or HCV therapy" AND "HIV or human immunodeficiency virus or Acquired Immunodeficiency Syndrome" were used. Titles and/ or abstracts were screened to determine the relevance of the studies. Of all relevant studies, full-text publications were reviewed. Furthermore, references of full-text studies were reviewed for missing publications. Lastly, conference abstracts of the annual meetings of the American Association for the Study of Liver Diseases (AASLD), the Conference on Retroviruses and Opportunistic Infections (CROI) and the European Association for the study of the liver (EASL) were reviewed for treatment of acute HCV.

Definition of the diagnosis *acute HCV* in HIV-coinfected patients

To date, there is no uniform definition for the diagnosis acute HCV. Most studies use a raise in transaminases from a previous measurement, during routine lab monitoring of HIV-patients, as criterion for acute HCV [14-21]. However, sometimes a cut-off value is defined above which acute HCV is suspected (for example, >10x above the upper limit of normal (ULN) or a pre-defined value of >300 U/L). Other studies combine a raise in transaminases with clinical symptoms and/ or exposition to HCV as criterion for acute HCV [22-25]. Furthermore, all studies use a documented seroconversion from negative to positive anti-HCV IgG with subsequently detectable HCV-RNA as criterion for acute HCV. The time frame of seroconversion differs per study though; within 6 months [14,16,17,23,25,26], 12 to 24 months [18,19,22,24] or unknown [20,21,27].

HCV seroconversion

In the past, the sensitivity of HCV serology in HIV-infected patients has been questioned. In a large cohort-study conducted in the United States of America, 3.2% of HIV-infected patients coinfected with *chronic* HCV were anti-HCV negative but plasma HCV-RNA positive [28]. Thomson et al. retrospectively tested stored serum samples of HIV-patients on the presence of HCV antibodies. At the time point that HCV-RNA was first detectable, only 25% of the samples were anti-HCV IgG positive. This percentage subsequently increased to 63%, 87% and 90% at month 3, 6 and 9 respectively after the first positive HCV-RNA [29] , suggesting that relying solely on HCV antibody testing induces the risk of missing patients with acute HCV infections. Furthermore, the study showed that at the time of HCV diagnosis, 76% of patients had an elevated ALT value (40 U/L being the upper limit of normal) making this a valuable method for detecting

acute HCV. In the Utrecht cohort study, 18 of the 23 HIV-infected patients with acute HCV experienced a HCV seroconversion within the previous 6 months whereas extending this interval to 12 months resulted in a 100% seroconversion rate [14].
Based on the available literature, we recommend the following case-definition for acute HCV (modified from future NEAT-recommendations; textbox 1):

Textbox 1: Case-definition of acute HCV

Preferred criteria (*Level II)*

1) Positive anti-HCV IgG in the presence of a documented negative anti-HCV IgG in the previous 12 months

or

2) Detectable HCV-RNA in the presence of either a documented negative HCV-RNA or a documented anti-HCV IgG seroconversion within the previous 12 months

Alternative criteria (If historical data/ stored samples are lacking) *(Level III)*

1) Detectable HCV-RNA or positive anti-HCV IgG in association with:

A) an acute rise in ALT with a documented normal ALT and no change in anti-retroviral regimen within the past 6 months.

and

B) anti-HAV IgM negative and anti-HBVcore IgM negative, and exclusion of other causes of an acute hepatitis.

Treatment of acute HCV in HIV-coinfected patients

What to treat with

In recent years, several retrospective studies have been published on the efficacy of pegIFN-alfa monotherapy for the treatment of acute HCV in HIV-infected patients. Sustained virological response rates (SVR, i.e. HCV-RNA negative 24 weeks after discontinuation of therapy) varied from 0% [21], 8% [27], 67% [15] to 100% [20,24] (table 1). In 2 prospective non-randomized studies, patients were treated with either pegIFN-alfa monotherapy or pegIFN-alfa/ ribavirin combination therapy [23,25]. Vogel et al. treated 21 patients with combination therapy and 15 patients with monotherapy, resulting in higher SVR rates of 73% (24 weeks) and 100% (48 weeks) for pegIFN-alfa monotherapy compared to 38% (24 weeks) and 80% (48 weeks) for the patients treated with combination therapy. A protocol violation (treatment duration was extended from 24 to 48 weeks in 9 patients) and a small number of patients per treatment arm (ribavirin, length of treatment) make the results from this study difficult to interpret. Morin et al., from 1999 through 2007, registered all patients with acute HCV, of whom 15 were

also coinfected with HIV. The choice of therapy was left to the treating physician, resulting in 5 patients being treated with (peg) IFN-alfa monotherapy and 7 patients with pegIFN-alfa/ ribavirin combination therapy. In this study, combination therapy was more successful than monotherapy (57% versus 40%). Due to the long period of inclusion, some patients were treated with conventional interferon-alfa again leading to very small numbers of patients in both treatment arms. In a recent study by Arends et al. [30], treatment with pegIFN-alfa monotherapy for acute HCV in 19 HIV-infected patients, resulted in a SVR rate of only 37%. Remarkably, a large percentage of 47% of patients were null-responders to pegIFN-alfa monotherapy (defined as <2log10 decline in HCV-RNA at week 12 of treatment).

All these studies differ with respect to design, patient characteristics and numbers of included patients making comparison between studies difficult. However, studies including mostly or exclusively HCV genotype 1 and 4 infected patients, reported lower SVR rate after pegIFN-alfa monotherapy[14,21,23,27] compared to studies that also included HCV genotype 2 and 3 infected patients [15,20,24,25].

Does addition of ribavirin to pegIFN-alfa lead to a higher SVR rate? In most studies performed with pegIFN-alfa/ ribavirin combination therapy, SVR rates varied between 50% and 80% (table 1). For example, in a recent prospective study by Matthews et al. (n=27), a SVR rate of 80% was reported after 24 weeks of therapy [22]. Likewise, combination-therapy studies differ with respect to patient characteristics (percentage genotype 1 infected patients, time between seroconversion and treatment, CD4 cell count and amount of patients treated with anti-retroviral therapy) and the definition of acute HCV. For example, in the prospective study by Dominquez et al. (n=14) [16], with a SVR of 71%, a low dose of ribavirin (800 mg) was used, the median CD4 cell count was low and the median time between diagnosis and treatment was 14 weeks. In contrast, the study by Gilleece et al. (n=27) [17], reporting a SVR of 59%, combination therapy was already initiated after a median of 4 week after the diagnosis, the median CD4 cell count was not mentioned and ribavirin was dosed according to body weight. Despite these differences, the reported SVR rates were very similar.

Besides a probably higher SVR rate, there might be another argument favoring the addition of ribavirin to pegINF-alfa. A recent conference abstract by Matthews et al. demonstrated that the viral kinetics of HCV was better (i.e. steeper decline in HCV viral load during therapy) in patients treated with pegIFN-alfa/ ribavirin combination therapy compared to pegINF-alfa monotherapy [31].

In conclusion, based on the available literature, treatment with pegIFN-alfa/ ribavirin combination therapy is the preferred treatment regimen with achievable success rates above 60%. Since this is considerably higher than SVR rates reached in HIV-infected

patients with chronic HCV, treatment of HCV in the acute phase of the infection should be pursued.

Textbox 2: Recommendations regarding acute HCV treatment

treatment regimen:

- Combination therapy pegylated interferon alfa-2a/2b and ribavirine (weight based 800-1400 mg) (level III)

when to start:

- Preferably within 12 weeks after the diagnosis of acute HCV (level III)

treatment duration:

- 24 weeks in case of either
  1) a Rapid Virological Response (RVR, HCV-RNA <50IU/ml) or
  2) >2log10 drop of HCV-RNA at week 4 and undetectable HCV-RNA at week 12 (level III)
- in all other patients prolonging the duration of treatment to 48 weeks should be considered (level IV)
- treatment should be stopped when <2log10 drop in HCV-RNA at week 12 of therapy (level IV)

When to start

Deciding at which time point one should start treatment in patients with acute HCV is difficult. On the one hand, a chance of spontaneous viral clearance should be awaited while on the other hand deferring treatment to the chronic phase of HCV diminishes the chances of achieving a high SVR.

Studies in patients with an acute HCV mono-infection have shown that the rate of spontaneous viral clearance can be as high as 40% occurring mostly within 12 weeks after the diagnosis [32]. In HIV-infected patients with acute HCV chances of spontaneous viral clearance were lower (around 10-15%), but also highest within the first 12 weeks after the diagnosis [16,17,19,33]. However, in a recent study by Vogel et al., evaluating spontaneous viral clearance rates in 92 HIV-infected patients with acute HCV, an unusually high clearance rate of 40% was reported [34]. An intent-to-treat (ITT) analysis on week 12 after the initial diagnosis showed a positive predictive value of 89% for development of chronic HCV in case of HCV-RNA positivity. Furthermore, patients not experiencing a $2\log_{10}$ drop in HCV-RNA at week 4 after the diagnosis had an 85% chance of becoming chronically infected with HCV. The used PCR for HCV-RNA detection had a relatively high lower limit of detection of 600 IU/ml in contrast to the currently used detection limits of 10 to 50 IU/ml. This is important since it has been

Table 1: Overview of studies evaluating the efficacy of pegIFN-alfa with or without ribavirin in HIV-infected patients with acute HCV

	number of treated patients (total number of patients)	patient characteristics	genotype	Median time between diagnosis and start of therapy	CD4 / HAART	treatment regimen	SVR rate
Vogel et al. [24] 2005 (retrospective)	11 (16)	male : 91% MSM : 91% symptomatic: 82%	1 & 4 : 91% 2 : 9%	2.6 weeks	507 / 73%	pegIFN-alfa + RBV (n=5), pegIFN-alfa (n=4) en IFN-alfa (n=2) duration varied between 24 and 48 weeks	80% (pegIFN-alfa + RBV) and 100% (pegIFN-alfa)
Danta et al. [27] 2005 (retrospective)	23 (39)	male : NM% MSM : NNM% symptomatic: NM%	1 & 4: 85%	0 weeks for pegIFN-alfa monotherapy; 12 weeks for pegIFN-alfa/ RBV	549 / NM	pegIFN-alfa monotherapy (center 1); pegIFN-alfa/ RBV (center 2)	60% (pegIFN-alfa/ RBV) 8% (pegIFN-alfa monotherapy)
Serpaggi et al. [21] 2006 (retrospective)	10 (12)	male : 100% MSM : 100% symptomatic: 20%	1 & 4 : 92 % 3 : 8%	49 days	625 / 90%	IFN (n=7), IFN + RBV (n=2) and pegIFN (n=1)	0%
Luetkemyer et al. [26] 2006 (retrospective)	4 (9)	male : 100% MSM : 67% symptomatic: 44%	1 : 75% 2 : 25%	8 weeks	329 / 89%	pegIFN-alfa + RBV (WB) 1 patient 24 weeks and 3 patients 48 weeks	50% (1 patient only end-of-treatment available)
De Rosa et al. [15] 2009 (retrospective)	6 (7)	male : 57% MSM : NB% symptomatic: 14%	1 & 4 : 71% 2 : 29%	31 days	539 / 43%	pegIFN-alfa monotherapy 12 weeks	67%

	number of treated patients (total number of patients)	patient characteristics	genotype	Median time between diagnosis and start of therapy	CD4 / HAART	treatment regimen	SVR rate
Lambers et al. [18] 2010 (retrospective)	50 (52)	Male : 100% MSM :100 % symptomatic: NM%	1 : 65% 2 and 3: 4% 4 : 19% unknown : 12%	27 weeks	450 / NM	pegIFN-alfa + RBV duation: 24 weeks (n=21) and 48 weeks (n=29)	75% (24 weeks) 86% (48 weeks)
Schulze zur Wiesch et al. [20] 2009 (case-series)	3 (3)	male : 67% MSM : 33% symptomatic: 0%	1 : 33% 3 : 67%	10 weeks	323 / 0%	pegIFN-alfa (n=2) and pegIFN-alfa + RBV (n=1) duration: mono (22 and 28 weeks) and combination 48 weeks	100% for mono and combination therapy
Fierer et al. [33] 2008 (prospective)	10 (31)	male : 100% MSM : 100% symptomatic: NM%	NM	"acute phase"	527 / NM	pegIFN-alfa + RBV for 24 weeks	80%
Gilleece et al. [17] 2005 (prospective)	27 (50)	male : NM% MSM : 100% symptomatic: NM%	1 : 74% 2,3 and 4 : NM	4 week	NB / 56%	pegIFN-alfa + RBV (WB) 24 weeks	59%
Dominquez et al. [16] 2006 (prospective)	14 (25)	male : 100% MSM : 96% symptomatic: 36%	1 & 4 : 50% 3 : 50%	14 weeks	345 / 86%	pegIFN-alfa + RBV (WB) 24 weeks	71%

	number of treated patients (total number of patients)	patient characteristics	genotype	Median time between diagnosis and start of therapy	CD4 / HAART	treatment regimen	SVR rate
Vogel et al. [25] 2006 (prospective)	36 (47)	male : 100% MSM : 81% symptomatic: 47%	1 & 4 : 75% 2 & 3 : 20%	7 weeks	416 / 61%	pegIFN-alfa (n=15) and pegIFN-alfa + RBV (n=21) - duur: 24 weeks (11 pegIFN and 16 RBV) and 48 weeks (4 pegIFN and 5 RBV)	pegIFN 24 weeks:73% pegIFN 48 weeks: 100% pegIFN+RBV 24 weeks: 38% pegIFN+RBV 48 weeks: 80%
Matthews et al. [22] 2009 (prospective)	22 (27)	male : 100% MSM : 49% symptomatic: 46%	1 : 60% 2 & 3 : 33 %	> 30 weeks	614 / 59%	pegIFN-alfa (n=2) pegIFN-alfa + RBV (n=20) 24 weeks	0% for pegIFN-alfa ; 80% for pegIFN-alfa/ RBV
Schnuriger et al. [19] 2009 (prospective)	20 (38)	male : 100% MSM : NM% symptomatic: 30%	1 & 4 : 95% 3 : 5%	4 weeks	509 / 75%	pegIFN-alfa + RBV (800 mg) 24 weeks	75% (2 patients with SVR became re-infected)
Morrin et al. [23] 2010 (prospective)	12 (15)	male : 93% MSM : 67% symptomatic: 27%	1 & 4 : 73% 3 : 13%	less than 12 weeks (n=10) and more than 12 weeks (n=2)	NM	pegIFN-alfa : 5 (42%) pegIFN-alfa + RBV : 7 (58%) 7 patients 24 weeks and 5 patients 48 weeks	mono : 2 (40%) combi : 4 (57%)
Arends et al. [14] 2010 (prospective)	19 (23)	male : 100% MSM : 100% symptomatic: 0%	1 : 68% 4 : 32%	12 weeks	500 / 42%	pegIFN-alfa monotherapy 24 weeks (n=9) and 48 weeks (n=3)	37% (2 patients with SVR became re-infected)

NM = not mentioned

observed in acute HCV-infected patients that HCV-RNA fluctuates, sometimes even shortly becoming undetectable [35].

Although the absence of spontaneous viral clearance might be predicted 4 weeks after the infection, this does not imply that treatment should be started thereafter. To date, no randomized study has compared the outcome of therapy in HIV-infected patients with an acute HCV infection starting treatment very early after the diagnosis compared to those who initiated treatment 12 weeks after the diagnosis. In the study by Matthews et al. [22] a high SVR rate of 80% was reported with treatment being initiated more than 30 weeks after the diagnosis of acute HCV. Furthermore, a recent retrospective cohort study in 50 patients from Amsterdam, evaluated the effect on SVR in patients with initiation of therapy within 6 months (24 weeks) after the diagnosis versus deferral of treatment thereafter [18]. In this study, the time of infection was defined as the midpoint between the last negative and first positive HCV-test (serology or PCR). Although statistically not significant, in both the 24- and 48-week treatment arms a higher SVR rate was reported in patients starting treatment within 6 months after the diagnosis. Furthermore, in most intervention studies, treatment of acute HCV in HIV-infected patients was initiated after a median of 12 weeks after the diagnosis. In a cohort of acute HCV mono-infected Japanese patients a significantly lower SVR rate was noted when the start of therapy was postponed for 1 year compared to initiating therapy within 8 weeks after the diagnosis (40% versus 81%) [36].

Taken together, although spontaneous viral clearance occurs mostly within 12 weeks after an acute HCV-infection and chances of persistence of acute HCV might be predicted already 4 weeks after the infection, evidence for very early initiation of therapy is lacking.

In conclusion, after establishing the diagnosis acute HCV in HIV-coinfected patients, it is recommended to await spontaneous viral clearance for a period of no more than 12 weeks. In case no spontaneous clearance occurs, anti-HCV therapy should be started.

Treatment duration

Most studies in HIV-infected patients with acute HCV achieved SVR rates above 60% after 24 weeks of treatment (table 1). This is supported by the previously mentioned Amsterdam cohort study evaluating the outcome of therapy in 50 HIV-infected patients with acute HCV [18]. No significant difference in SVR rates was demonstrated between 24 and 48 weeks of therapy (75% versus 86% in all patients starting therapy within 6 months after the diagnosis). Contrary, in a German study higher SVR rates were seen in the 48-week arm compared to the 24-week arm (89% versus 52%;

p=0.062) [25]. It must be noted that these patients were erroneously treated for 48 week due to a protocol violation and that both patients treated with monotherapy peg-IFN-alfa and combination therapy with ribavirin were pooled together for this analysis.
A recent re-analysis by Vogel et al. [13] of previously published studies, evaluated the treatment outcome and the role of HCV viral kinetics during therapy in a group of 111 HIV-infected patients treated for acute HCV. Longer treatment duration did not significantly improve SVR rates. Both achievement of a Rapid Virological Response (in this study defined as HCV-RNA <600 IU/ml) and a complete Early Virological Response (cEVR, undetectable HCV-RNA (<600 IU/ml) at week 12) were strong predictors of achieving a SVR. In other words, in case of favorable viral kinetics (fast decline of HCV-RNA until undetectable at week 4 (RVR) or week 12 (cEVR)), 24 weeks of treatment seems sufficient. On the other hand, only 9% of patients without a cEVR reached an SVR.

Conclusion

Twenty-four weeks of therapy is the preferred duration of treatment in HIV-infected patients with acute HCV achieving either a RVR or a >2log10 drop in HCV-RNA at week 4 with an undetectable HCV viral load at week 12 of therapy (cEVR). In all other patients extending treatment duration to 48 weeks should be considered. In patients without a >2log10 drop in HCV-RNA at week 12 of therapy, treatment can be stopped.

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Chapter 5

Plasma HCV-RNA decline in the first 48 hours identifies hepatitis C virus mono-infected but not HIV/HCV coinfected patients with an undetectable HCV viral load at week 4 of peginterferon-alfa-2a/ ribavirine therapy

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Abstract

Background: During peginterferon-alfa-2a/ribavirin therapy, plasma hepatitis C virus (HCV)-RNA decreases with a rapid first phase and a slower second phase. We compared the viral load decrease and slope in the first 48 hours between patients with a rapid viral response (RVR, i.e. HCV-RNA <50 IU/ml at week 4) and patients without achieving a RVR.

Methods: From 23 HCV infected (14 mono-infected and 9 HIV/ HCV coinfected) genotype 1 or 4 positive peginterferon-alfa-2a/ribavirin treated patients, plasma HCV-RNA was determined at baseline, 48 hours, weeks 1, 2, 4, 8, 12, 48 and 72. The HCV viral load decrease ($\Delta_{0\text{-}48}$), the slope (λ_1) and the efficiency factor (ε) were determined in the first 48 hours after the start of therapy.

Results: Five (36%) HCV mono-infected patients and 3 (33%) HIV/HCV coinfected patients achieved a RVR whereas 6 (43%) HCV mono-infected patients and 5 (56%) HIV/HCV coinfected patients reached a sustained viral response (SVR). In contrast to HIV/HCV coinfected patients, 5 HCV mono-infected patients with a RVR showed both a larger $\Delta_{0\text{-}48}$ and steeper λ_1 (-1.77 log_{10} IU/mL±0.66 and -2.04/day±0.76) compared to 9 non-RVR patients (–0.66 log_{10} IU/mL±0.39; p=0.019 and -0.76/day±0.41; p=0.019). When divided by a SVR, a greater $\Delta_{0\text{-}48}$ and steeper λ_1 were also seen in both HCV mono-infected and HIV/HCV coinfected patients.

Conclusion: In the first 48 hours after the start of therapy, HCV mono-infected patients with a RVR have a larger viral load decrease, steeper viral slope and a higher efficiency factor as compared to non-RVR patients.

(ClinicalTrials.gov: NCT00150904)

Introduction

During the course of a chronic hepatitis C virus (HCV) infection, the concentration of plasma HCV-RNA remains relatively constant [1], whereas treatment with (peg)interferon-alfa and ribavirin results in a decrease in plasma HCV-RNA [2]. Modelling studies have shown that this drop in HCV viral load during therapy occurs in a biphasic pattern [3-5]. The first phase, describing the inhibition of viral production, is characterized by a rapid viral decline and occurs in the first 24 to 48 hours (till day 2) after the start of therapy [3,4,6,7]. It is generally accepted that these first 48 hours reflects the sensitivity of the virus to interferon-alfa. The second phase is slower and more variable in time lasting from day 2 to several weeks after treatment initiation and correlates with the elimination of HCV infected hepatocytes [8]. In HCV-infected patients coinfected with the human immunodeficiency virus (HIV) it is less clear if this biphasic kinetic model is applicable [9-11].
Previous studies in HCV mono-infected patients have shown minimal variance in the first phase viral load decrease while the steepness of the slope of the second phase is a good predictor for achieving a sustained virological response (SVR) after treatment [4,12-15]. Two retrospective studies [16,17] showed in 38 and 22 HCV mono-infected patients respectively, that the first phase viral load decrease (measured at 24 hours) may predict SVR in patients treated with interferon-alfa monotherapy. The original modelling studies demonstrating a first phase viral response, have shown a linear relationship between the viral load decrease and the time in the first 48 hours after the start of therapy [4,6]. In later publications there was general consensus about the duration of the first phase being 24-48 hours [8,15,18]. Therefore, the 48 hours cut-off point was chosen for the first phase in this study.
In recent years, several clinical trials have shown that besides an early viral response (EVR = defined as a 2 $\log_{10}$ decrease (partial EVR) or undetectable levels (complete EVR) of HCV-RNA at week 12 of therapy) or none response (null responders, i.e. less than 2log10 decrease from baseline at week 12 of therapy) there is a subgroup of patients achieving a very fast clearance of the virus [19-21]. This rapid virological response (RVR) is defined as an undetectable HCV-RNA at week 4 of treatment in a qualitative PCR assay (<50 UI/mL). A RVR, regardless of genotype, is the most important determinant of clinical success [22].
To our knowledge, no studies either in HCV mono-infected or HIV/ HCV coinfected patients have been published investigating the first phase response in RVR patients and comparing this with non-RVR patients. Therefore, we undertook this study to determine the viral load decrease, and to calculate the slope, in the first and second

phase of patients achieving a RVR using standard peginterferon-alfa-2a and ribavirin therapy in genotype 1 or 4 HCV mono-infected and HIV/ HCV coinfected patients.

Materials and Methods

Study design

The PRIVICOP-study, an acronym for Peginterferon-alfa-2a (Pegasys®) plus Ribavirin on Viral kinetics in hepatitis C mono-infected and HIV/ HCV Co-infected Patients, is a study in HCV positive patients collecting both plasma samples and peripheral blood mononuclear cells (PBMC) in a standardized way to study virological and immunological phenomena. The study was conducted from September 2005 until September 2008. All patients are treated according to current international guidelines [23-26] and received standard treatment for 48 weeks with peginterferon-alfa-2a (40 kD; Pegasys®; Roche, Basel, Switzerland) and weight based ribavirin, i.e <75 Kg than 1000 mg in two daily doses; ≥ 75 Kg than 1200 mg in two daily doses (Copegus®; Roche, Basel, Switzerland). This study has been conducted in three hospitals in the Netherlands (University Medical Center Utrecht, Erasmus Medical Center Rotterdam and Onze Lieve Vrouwe Gasthuis Amsterdam) at both the Infectious Diseases and the Gastroenterology departments. Patients were required to give written informed consent and should be capable of travelling to the hospital. All institutional review boards have approved the protocol and the study is conducted in accordance with the declaration of Helsinki and good clinical practice guidelines.

Eligible patients

Patients were considered eligible if a chronic HCV infection was documented by anti-HCV positive antibodies (AxSYM® automated immunoassay instrument system, Abbott Diagnostics, Hoofddorp, The Netherlands) confirmed by a RIBA immunoblot (Chiron Corporation, Emeryville, Calif., USA). The amount of plasma hepatitis C RNA was measured with a quantitative signal amplification assay (Versant HCV-RNA V3.0, Bayer B.V. Mijdrecht, The Netherlands) with a lower limit of detection of 615 international units per millilitre (IU/mL). Only patients with HCV genotypes 1 or 4, detected by second generation InnoLIPA assay (Versant HCV genotype 2.0 assay, Bayer B.V. Mijdrecht, the Netherlands) were eligible. Part of the patient population is also coinfected with the human immunodeficiency virus (HIV), as detected by a positive anti-HIV antibody test

(AxSYM® automated immunoassay instrument system, Abbott Diagnostics, Hoofddorp, The Netherlands), confirmed by a positive PCR detecting HIV-RNA in plasma using the real-time COBAS Taqman® PCR (Roche Molecular Systems, Pleasanton, California). All procedures were conducted according to the manufacturer's instructions. Decision to start treatment was taken by the treating physician and guided by standard international treatment guidelines, e.g. excluding other causes of liver disease or liver cirrhosis, determining the extent of liver fibrosis and excluding pregnancy.

Blood samples

Within two weeks before the start of therapy baseline blood samples were drawn. After the start of treatment blood samples were taken after 48 hours and at week 1, 2, 4, 8, 12, 48 (end of treatment) and 72 (end of follow-up). Plasma samples were frozen immediately at -80 ^{0}C until use.

Viral kinetic analysis

HCV-RNA assays were performed in batch using COBAS Ampliprep and real-time COBAS Taqman® PCR (Roche Molecular Systems, Pleasanton, California), a qualitative assay with a lower limit of detection of 15 IU/mL. HCV-RNA levels were determined at all these time points. The assay was performed according to the manufacturer's instruction. Confirmation of the COBAS Taqman assay results was done by performing a Versant V3.0 bDNA analysis from the same patient plasma sample. When undetectable in the bDNA assay, a qualitative Roche Amplicor® polymerase chain reaction (PCR) assay (Roche Molecular Systems, Pleasanton, California) was performed with a lower limit of detection of 50 IU/ml.
A rapid virological response (RVR) is defined as having a HCV-RNA at week 4 of treatment below 50 IU/mL with a qualitative assay. A partial early virological response (pEVR) is defined as a 2$\log_{10}$ viral load decrease from baseline at week 12 of therapy while a complete EVR (cEVR) is defined as a qualitative undetectable (<50 IU/mL) HCV-RNA 12 weeks after initiation of treatment. A virologic non-response (null responder) is defined as a less then 2 $\log_{10}$ drop (defined by a quantitative PCR assay) in HCV viral load from baseline to week 12. For the description of the viral load decrease from baseline during the first 12 weeks of therapy, these definitions were applied. For further analysis of the first and second phase response, patients were classified as RVR or non-RVR, i.e. EVR plus null responders. A sustained virological response (SVR) is defined as a negative HCV viral load, detected by qualitative PCR, 24 weeks after

cessation of therapy. The first phase was defined as the first 48 hours (two days) of therapy and the second phase as the interval between day 2 and week 12 of therapy. The mathematical equations quoted below are used to describe
the HCV *viral load decrease* (Δ_{0-48}) from baseline

$$\Delta_{0-48} = \log_{10} V_{48} - \log_{10} V_0 \quad (1)$$

with V_{48} describing the viral load at 48 hours after the start of therapy and V_0 being the HCV-RNA load at baseline.
Subsequently, the *first phase slope* (λ_1) was described by

$$\lambda_1 = (LnVt - LnV_0)/2 \text{ days} \quad (2)$$

with Vt describing the HCV viral load at time point t with Ln being the natural logarithm.
The *second phase slope* (λ_2) was calculated by linear regression analysis using

$$\lambda_2 = n \Sigma t^*LnVt - (\Sigma t)^*(\Sigma LnVt) / n \Sigma LnVt^2 - (\Sigma LnVt)^2 \quad (3)$$

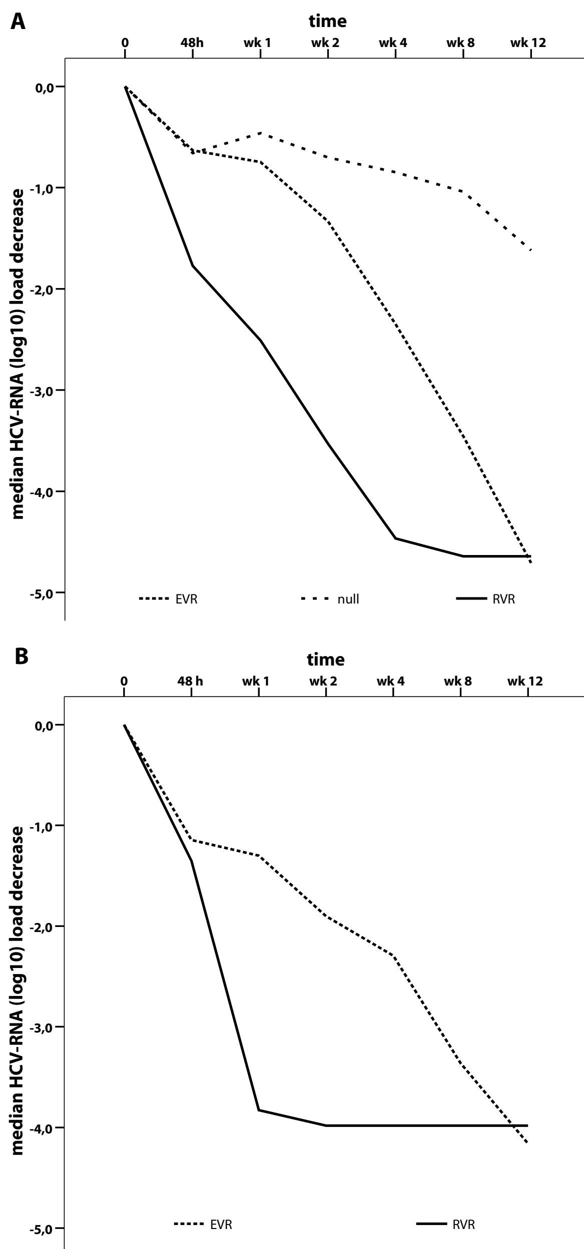
Furthermore, based on previously published kinetic models [8,27,28] the treatment efficiency factor ε was calculated by the equation

$$(1-\varepsilon)=V_{48}/V_0 \quad (4)$$

Statistical analysis

Continuous variables are expressed as median values (with range) whereas categorical variables are expressed as number of cases (percentages). Comparison between continuous variables from different response groups is performed non-parametrically using a Mann-Witney U-test. Comparing frequencies is done using Fisher's exact test. Univariate analysis is carried out using linear regression models and when no statistical significant difference is noted, multivariate analysis was not considered usefull and not performed. A p-value of <0.05 was considered statistically significant. All data are processed and analysed using SPSS (version 14; SPSS Inc. Chicago, IL, USA) and Microsoft Excel software (Microsoft Excel 2002 SP3, Microsoft Corporation, Seattle, WA, USA). Figures were generated in GraphPad Prism (version 4.0 for Windows, GraphPad Software, San Diego, California, USA).

Figure 1: Median decrease in HCV plasma viral load divided by viral response for both HCV mono-infected patients (panel A) and HIV/ HCV coinfected patients (panel B).



On the horizontal axis, the different time points (in weeks) are shown when blood samples were obtained. On the vertical axis, the median viral load decrease from baseline (log_{10}) is depicted. The solid black line (—) shows the rapid virological responders (RVR), the narrow dotted line (--) displays the early virological responders (EVR) and the wide dotted line (•••) represents the null responders (NR).

Results

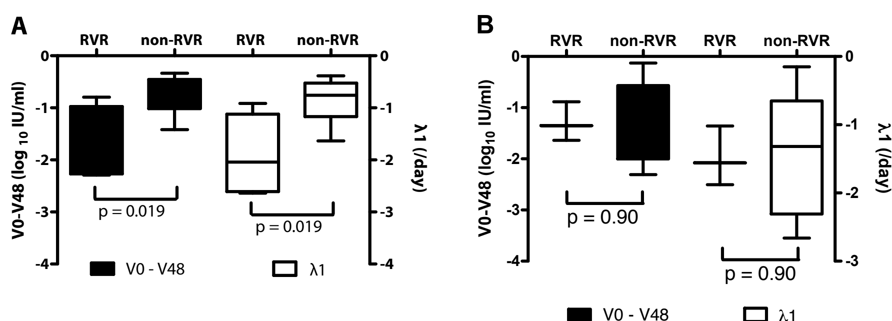
In this study, 23 chronic HCV-infected patients were included in this study, of whom 14 patients were HCV mono-infected while the other 9 were coinfected with HIV. Except for the age, there were no significant differences in patient characteristics (tabel 1). Five HCV-mono-infected patients achieved a RVR (36%), whereas 3 HIV/HCV coinfected patients did so (33%). There were more patients with a pEVR in the HIV/ HCV coinfected group than in the HCV mono-infected group (4 versus 1; p=0.08). Figure 1 shows the median drop in viral load (log_{10} IU/mL) from baseline per response group for both HCV mono-infected (1A) and HIV/HCV coinfected patients (1B) in the first 12 weeks of peginterferon-alfa-2a and ribavirin therapy. In the group of HCV mono-infected patients an overall SVR rate of 43% was achieved with 2 out of 6 EVR patients (33%), 4 out of 5 RVR patients (80%) and none of the null responders achieving a SVR. Among patients with a HIV/ HCV coinfection all 3 patients with an RVR (100%) and only 2 out of 6 patients with an EVR (33%) achieved a SVR leading to an overall SVR rate of 56%. This relatively high SVR rate in HIV/HCV coinfected patients was not related to differences between patient groups (SVR versus non-SVR) in age, body weight, baseline CD4 count, baseline HIV viral load, percentage of patients on HAART, baseline ALAT or stages of fibrosis (data not shown).

First phase viral kinetics

To characterize the first phase viral response in RVR patients, analysis of the viral load decrease ($\Delta_{0\text{-}48}$) in the first 48 hours after treatment initiation was performed. Patients were grouped in those achieving a RVR and those without a RVR (non-RVR) for both HCV mono-infected and HIV/HCV coinfected patients. Thirteen out of the 14 HCV mono-infected patients were available for this analysis, since one patient's first plasma sample was drawn after 24 hours after the start of therapy. In the HCV mono-infected patients, a larger viral load decrease ($\Delta_{0\text{-}48}$) in the first 48 hours of therapy was observed in the RVR-group (median of -1.77 log_{10} IU/mL± 0.66) when compared to the non-RVR group (median of -0.66 log_{10} IU/mL± 0.39; p=0.019; figure 2A). In the group of HIV/ HCV coinfected patients (n=9) no difference was observed in the first phase viral load decrease (p=0.90; figure 2B).
The first phase slope (λ_1) was calculated using equation (2) (methods). Confirming the data of the viral load decrease, a similar steeper slope was seen in the RVR group (λ_1 -2.04/ day± 0.76) when compared to the non-RVR group (λ_1 -0.76/ day± 0.41; p=0.019; figure 2A) in HCV mono-infected patients. Again, for the HIV/ HCV coinfected patients

no difference in the first phase slope was seen between RVR patients (λ_1 -1.56/ day± 0.44) and non-RVR patients (λ_1 -1.32/ day± 0.91; p=0.90; figure 2B).
It has been shown that the efficiency factor epsilon (ε) is associated with the sensitivity of the HCV virus for (peg)interferon-alfa. A significantly higher ε was seen in HCV mono-infected patients (98%± 7 versus 78%± 14; p=0.011) but not in the group of HIV/ HCV coinfected patients (96%± 6 versus 93%± 28; p=0.38) (data not shown).

Figure 2: Larger viral load decrease (V_0-V_{48}) and steeper slope (λ_1) in the first 48 hours of therapy in RVR-patients when compared to non-RVR patients in HCV mono-infected and HIV/HCV coinfected patient.

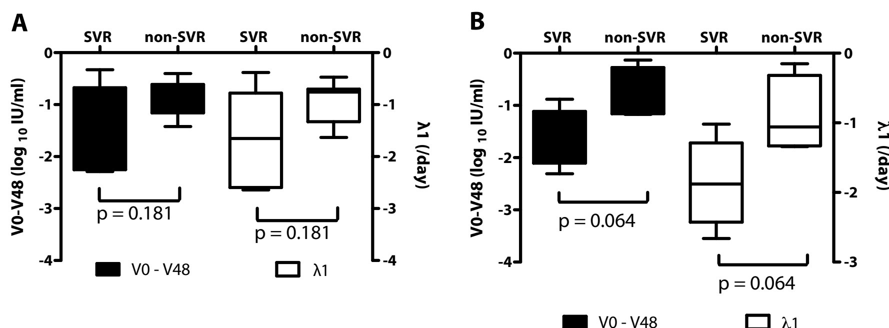


First phase viral load decrease (left y-axis) and first phase viral slope (right y-axis). Panel A: in HCV mono-infected patients a significant larger viral load decrease (p= 0.019) and steeper viral slope (p= 0.019) are seen in the first phase in RVR-patients when compared to non-RVR patients. Panel B: no difference in the first phase viral load decrease and first phase viral slope (p= 0.90) in the subgroup of HCV/ HIV coinfected patients. (Mann-Whitney test was used; V_{48}= viral load at 48 hours after the start of therapy; V_0= viral load at baseline; λ_1= viral slope in the first phase).

Sustained virological response in relation to the first phase viral kinetics

Achieving a RVR is the most important determinant for reaching SVR. Since RVR-patients are characterized by a greater viral load decrease and steeper viral slope in the first 48 hours of therapy, we analyzed these parameters in relation to the SVR. A larger but non-significant viral load decrease and slope were seen in HCV mono-infected patients when grouped in SVR and non-SVR (Δ_{0-48} -1.34 $\log_{10}$ IU/mL± 0.80 versus -0.66 $\log_{10}$ IU/ mL± 0.35; p=0.181 and λ_1 -1.65/ day± 0.93 versus -0.76/ day± 0.41; p=0.181; figure 3A). In HIV/ HCV coinfected patients a trend towards significance was seen with regard to differences in the viral load decrease and viral slope in the first 48 hours between patients reaching a SVR and those who did not reach a SVR (Δ_{0-48} -1.64 $\log_{10}$ IU/mL± 0.54 versus -0.92 $\log_{10}$ IU/mL± 0.48; p=0.064 and λ_1 -1.88/ day± 0.82 versus -1.06/ day± 0.56; p=0.064; figure 3B).

Figure 3: Patients achieving a SVR show a larger first phase viral load decrease (V_0-V_{48}) and a steeper first phase slope (λ_1) as compared to non-SVR patients.



First phase viral load decrease (left y-axis) and first phase viral slope (right y-axis). Panel A: HCV mono-infected patients achieving a SVR displays a larger median viral load decrease (p= 0.181) and a steeper viral slope (p= 0.181) during the first 48 hours of treatment when compared to those not achieving a SVR. Panel B: a trend towards significance in the viral load decrease (p= 0.064) and the viral slope (p= 0.064) during the first 48 hours in the subgroup of HIV/ HCV coinfected patients achieving a SVR. (Mann-Whitney test was used; V_{48}= viral load at 48 hours after the start of therapy; V_0= viral load at baseline; λ_1= viral slope in the first phase from baseline until 48 hours).

Table 1: Characteristics of the total patient population divided in HCV mono-infected and HIV/HCV coinfected patients.

Feature	HCV mono-infected (n=14)	HIV/HCV coinfected (n=9)	p-value
male / female	13 / 1	6 / 3	**ns**
age	48 (36 – 61)	43 (28 – 46)	**0.003**
weight (Kg)	78 (53 - 101)	70 (55 – 98)	**ns**
Response			
RVR	5 (36%)	3 (33%)	
cEVR	5 (36%)	2 (22%)	**ns**
pEVR	1 (7%)	4 (44%)	
null	3 (21%)	0	
genotype			
1	9 (64%)	8 (89%)	**ns**
4	5 (36%)	1 (11%)	
HCV viral load (Log_{10})	6.20 (4.63 – 7.09)	6.0 (4.93 – 7.19)	**ns**
ALT (IU/ml)	90 (18 – 385)	57 (31 - 177)	**ns**
CD4 (/mm3)	–	430	–
HIV-RNA <50 copies/ml	–	78%	–
SVR	**6 (43%)**	**5 (56%)**	**ns**

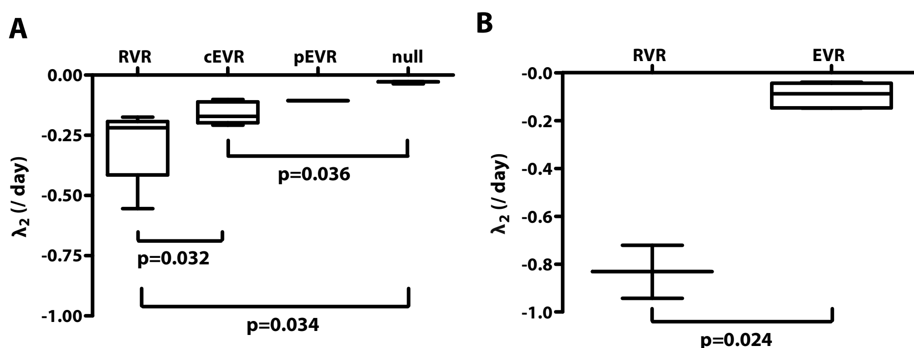
When appropriate the median value is given with the range between braquets. SVR = sustained viral response; RVR = rapid virological response (HCV-RNA < 50 IU/ml); pEVR = partial early virological response (>2 Log10 IU/ml drop in HCV-RNA from baseline at week 12 of therapy) and cEVR = complete early virological response (undetectable HCV-RNA at week 12 of therapy).

Second phase viral kinetics

The viral load decrease in the second phase has been shown to determine the virological response at week 12 after the start of therapy. The second phase slope (λ_2) was determined by linear regression analysis in both groups of patients using equation (3) (methods). In HCV mono-infected patients with a RVR a larger second phase slope (-0.22/ day± 0.15) was noted when compared to patients with a cEVR (-0.11/ day± 0.05; p=0.032) and null responders (-0.03/ day± 0.005; p=0.034; figure 4A). Likewise, patients with a RVR coinfected with HCV and HIV had a larger median λ_2 (-0.83/ day± 0.11) than EVR-patients (-0.09/ day± 0.05; p=0.024; figure 4B). Patient numbers were too small to subdivide them into cEVR and pEVR.

Evaluating the second phase in HCV mono-infected patients with an EVR, it appears that a possible third phase response is apparent (figure 1A). This shoulder phase exists between day 2 and week 2 after which a renewed viral decrease takes place.

Figure 4: Steeper second phase (λ_2) slope in RVR-patients compared to EVR patients and null responders.



Second phase viral slope (left y-axis). Panel A: in HCV mono-infected patients a significantly steeper second phase slope in patients with an RVR compared to pEVR patients and null responders (p= 0.032 and p= 0.034). Panel B: significant steeper second phase slope in RVR patients compared to EVR patients in HCV/ HIV coinfected patients (p= 0.024). HIV/ HCV coinfected patient groups were to small to subdivide into pEVR and cEVR. (Mann-Whitney test was used; λ_2= viral slope in the second phase from day 2 until week 12).

Factors correlating with the first phase viral kinetics

Finally, factors which may contribute to or influence the viral load decrease and the viral slope in the first phase were analyzed in a univariate manner. In both HCV mono-infected (p=0.02) and HIV/ HCV coinfected patients (p=0.001) at baseline the

viral load in the RVR-group was significantly lower than in non-RVR patients (data not shown). However, no correlation could be demonstrated between the baseline viral load and the first phase kinetics (Δ_{0-48} and λ_1) in both groups of HCV mono- and HCV/ HIV coinfected patients. Furthermore, in both groups of patients there was no correlation by univariate analysis between the first phase kinetics and the baseline viral load, baseline body weight, genotype 1 or 4 and baseline ALAT.

Discussion

In this study we demonstrate a larger viral load decrease (Δ_{0-48}) and steeper slope (λ_1) in the first 48 hours of pegIFN-alfa/ ribavirin therapy in HCV mono-infected patients achieving a RVR when compared to patients not achieving a RVR. Furthermore, in both HCV mono-infected patients and HIV/ HCV coinfected patients a larger viral load decrease and steeper slope are associated with achieving a SVR. This study also shows that the pegIFN-alfa efficiency factor ε is nearly 100% in HCV mono-infected patients achieving a RVR in contrast to around 80% in patients without a rapid virological response. No correlation between baseline viral load and the first phase first phase kinetics (Δ_{0-48} and λ_1) could be demonstrated. Moreover, there were no other correlations between the first phase viral kinetics and baseline ALT, coinfection with HIV, genotype 1 or 4 and baseline body weight. These results are consistent with previous publications on HCV viral load kinetics during (peg)interferon-alfa therapy. In a study in 30 HCV genotype 1-infected patients a difference was noted in the efficiency factor between fast and slow responders [15]. Furthermore, the second phase slope for null responders (-0.03 /day in our study) is similar to the rates in null responders reported by Di Bisceglie et al. [29]. When comparing the viral kinetics in HCV mono-infected and HIV/ HCV coinfected patients, conflicting data have been published with some confirming a biphasic HCV viral load decline [30] and others opposing this [9,11]. An absence of a first phase response and therefore a prolonged HCV viral load decrease has been attributed to the generally lower SVR rates achieved by peginterferon-alfa therapy in HIV/ HCV coinfected patients [31,32]. However, these analyses were not performed in patients achieving a RVR. In a cohort of HIV/ HCV coinfected patients, Ballesteros et al. [33] did not observe a difference in viral kinetics between HCV genotypes 1 and 4. Furthermore, faster plasma HCV viral load decrease in the first 48 hours of therapy was seen in patients achieving a SVR compared to those without a SVR. This latter observation is confirmed in our study. A higher SVR rate of 56% was reached in HIV/ HCV coinfected patients in our study [32,34]. This could not be explained by a difference

in parameters known to influence the outcome of therapy. Since the amount of liver fibrosis at baseline this is a known predictor for treatment success, this could be a possible explanation. However, liver histology was not available of all patients in this study and therefore remains a possible contributing factor.
In recent years, in HCV mono-infected patients a triphasic response is noted [35,36]. In these patients the first phase is followed by a plateau or shoulder phase lasting 4 to 28 days with no or minimal HCV RNA decline followed by again a viral decline. In our data there is also a suggestion of a shoulder phase between day two and week two, as been reported previously [35,36]. In line with the previously mentioned kinetic models, the second phase slope in our patients further discriminates between RVR, EVR and null responders, determining the clinical outcome at week 12. However, these models were based on daily subcutaneous interferon-alfa injections. With the new peginterferon-alfa formulations, a new kinetic model has recently been developed for peginterferon-alfa-2b [37].
This study was not designed and powered to investigate the 48 hour point as a new stopping rule. Though, this study has clearly shown a relationship between the first phase response and the achievement of a RVR and SVR in HCV mono-infected and HIV/HCV coinfected patients. This study, together with earlier retrospective studies [16,17] warrants larger clinical studies to evaluate the first phase viral load decrease as a tool to guide decisions regarding treatment (dis)continuation.
Several mechanisms may explain the observed differences between patients in first phase viral response. First, it may be caused by the differences in sensitivity of the hepatitis C virus to the interferon inhibiting effects. There is some evidence to suggest that mutations in the hepatitis C viral genome at baseline play a role in treatment outcome [38,39]. However, to date, no distinct baseline mutations in strains circulating in Western countries have been recognized to correlate with a RVR.
Secondly, the observed differences in first phase viral response may also be explained by differences in host response to interferon-alpha. Interferon-alpha is a cytokine that through binding with its cell surface receptor initiates an intra-cellular cascade of signal transduction and activation, leading to induction of multiple interferon stimulating genes (ISG) and thereby inhibition of viral replication [40,41]. Since, the peak concentration (Cmax) of peginterferon-alfa-2a is reached between 72-96 hours after the first dose and that the absorption half-life is 50 hours [42,43], a HCV viral load decrease in the first 48 hours after the start of therapy occurs primarily before peginterferon-alfa reaches its Tmax. It could be hypothesized that in patients with a RVR, the maximal concentration of peginterferon-alfa is not required in the first 48 hours to achieve a strong antiviral effect. It has recently been shown that patients with chronic hepatitis

C with a RVR during treatment with peginterferon-alfa-2b, up- or downregulate significantly more genes in both liver tissue and PBMC's than those patients without a RVR [44]. Furthermore, via Affymetrix microarray technology it was shown that rapid responders had a greater fold change in ISG compared to slow responders [45].
The role of ribavirin in the first weeks of treatment is unclear. Although steady-state levels of ribavirin are reached several weeks after the start of treatment [46], ribavirin exerts a moderate anti-viral effect (-0.5 to 1.6 $\log_{10}$ reduction) in the first 2 to 3 days after ingestion [47]. Both immunological enhancing properties as well as direct anti-viral effects have been described [48-50]. It was shown that when the efficiency (ε) is high, ribavirin has a negligible influence on viral load decrease, whereas the effect of ribavirin is strongly present in case of a low efficiency factor [51]. Taken together this suggests that in the first phase, ribavirin may play only a minor role whereas pegIFN-alfa predominantly exerts an effect on the viral load decrease in rapid viral responders.

Conclusion

In conclusion, this study shows that in contrast to HIV/ HCV coinfected patients, genotype 1 or 4 HCV mono-infected patients with a RVR have a larger viral load decrease, a steeper viral slope and a higher efficiency factor in the first 48 hours after start of therapy compared to non-RVR HCV mono-infected patients. A similar difference in first phase viral load decrease and viral slope was seen in both HCV mono-infected and HIV/ HCV coinfected patients achieving a SVR compared to those without a SVR. The difference in first phase viral response is probably a combination of an individual's ability to upregulate intracellular pathways and ISG in combination with baseline mutations of the hepatitis C virus.

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Chapter 6

T-cell responses at baseline and during therapy with peginterferon-alfa and ribavirin are not associated with outcome in chronic hepatitis C infected patients

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Abstract

Background: Since the association between Hepatitis C virus (HCV)-specific T-cell responses both pre-treatment and during interferon-alfa based therapy and viral clearance is unresolved, a combined analysis of distinctive T-cell characteristics (proliferation and interferon-γ production) is important to clarify this issue.

Methods: Peripheral blood mononuclear cells (PBMC) collected in 22 chronic HCV infected patients at pre-treatment and at week 4 during pegIFN-alfa/ ribavirin therapy, were stimulated with overlapping peptide pools in a [^{3}H]-thymidine assay, an interferon-gamma–ELISA, and a sensitive 12-day T-cell expansion assay.

Results: Compared to the [^{3}H]-thymidine proliferation and interferon-gamma secretion assays, the 12-day T-cell expansion assay was more sensitive in detecting T-cell responses. No significant association was demonstrated between pre-treatment HCV-specific CD4+ or CD8+ T-cell responses and either a sustained virological response (SVR) or a rapid virological response (RVR). However, a skewing of individual responses towards the non-structural antigens was observed. During pegIFN-alfa/ ribavirin therapy, HCV-specific CD4+ and CD8+ T-cell responses declined similarly in both SVR/ RVR and non-SVR/ non-RVR patients.

Conclusion: No correlation was found between the magnitude of baseline HCV-specific T-cell responses and the outcome of pegIFN-alfa/ ribavirin therapy in terms of SVR and RVR. Moreover, the magnitude of HCV-specific T-cell responses declined in all patients early during treatment.

Introduction

Pegylated interferon alfa-2a or 2b (pegIFN-alfa) in combination with weight based ribavirin is currently the standard treatment for chronic hepatitis C virus (HCV) infected patients [1]. A sustained virological response (SVR), defined as an undetectable plasma HCV-RNA 24 weeks after cessation of therapy, is achieved in around 50% of patients infected with genotypes 1 and 4 [2]. Pre-treatment characteristics like baseline viral load [3], liver cirrhosis [4], coinfection with the human immunodeficiency virus (HIV) [5] and recently the interleukin-28B cc-genotype [6] are associated with outcome of therapy. Furthermore, achievement of a rapid virolgical response (RVR, i.e. plasma HCV-RNA with undetectable i.e. <50 IU/ml at week 4 of therapy) is regarded as a strong on-treatment predictor for SVR [7,8].
It has been suggested that cellular immune responses, modulated by pegIFN-alfa and ribavirin, play a role in forced viral eradication, based on the immunological properties attributed to these anti-viral compounds [9-12]. However, the role of HCV-specific T-cells before and during pegIFN-alfa/ ribavirin therapy is still controversial [13,14]. Some studies have shown that achievement of a SVR is associated with high baseline CD4+ and/ or CD8+ specific T-cell responses [15-18], while others have seen no such relationship [19-23]. Similarly, contradictory results have been reported on the role of HCV-specific T-cells during pegIFN-alfa/ ribavirin therapy showing either augmentation [11,21,24] or decline [15,18,19] of HCV-specific T-cells in relation to SVR. In patients achieving a RVR, higher percentages of baseline interferon-gamma (IFN-γ) producing CD8+ T-cells have been demonstrated compared to non-RVR patients [18].
There is no conclusive evidence whether HCV-specific immunity contributes to therapy induced viral clearance for several reasons. Good animal models are unavailable [25], frequencies of circulating HCV-specific T-cells are very low [26] and therefore hard to detect, and a consensus on the optimal *ex vivo* experimental cell culture protocols is lacking [27]. A robust and sensitive assay, able to detect low frequencies of HCV-specific T-cell responses, is needed to resolve the above mentioned controversies.
For this purpose, we examined the magnitude and breadth of HCV-specific T-cell responses at baseline and during therapy in patients with a HCV mono-infection using a sensitive 12-day expansion assay as has been previously reported by our group [28-30]. This assay measures both the IFN-γ production and the proliferative capacity of HCV-specific CD4+ and CD8+ T-cells simultaneously, allowing a more comprehensive analysis of the HCV-specific T-cell response. Using this assay, we found no relation between the magnitude of pre-treatment HCV-specific CD4+ or CD8+ T-cell responses and achievement of either a SVR or a RVR, albeit that skewing of these responses

was demonstrated against HCV non-structural antigens. In addition, irrespective of treatment outcome, HCV-specific T-cell responses declined in all patients early during treatment.

Materials and Methods

Patients

In this multi-centre cohort study, 22 patients diagnosed with chronic HCV mono-infection were consecutively enrolled [31], and prospectively sampled during standard treatment with either peginterferon alfa-2a (40 KD) (Pegasys® 180 µg/week; Roche, Basel, Switzerland) or peginterferon alfa-2b (12KD) (PegIntron® 1.5 µg/kg/week; Schering-Plough, Kenilworth, USA) in combination with weight based ribavirin (Copegus® or Rebetol®). Only patients with HCV genotypes 1 or 4 were included while patients with a coinfection with either hepatitis B or HIV were excluded. During treatment plasma HCV-RNA was measured using the qualitative Roche Amplicor® polymerase chain reaction (PCR) assay with a lower limit of detection of 50 International Units / milliliter (IU/ml). A rapid viral response (RVR) is defined as a qualitative undetectable HCV-RNA at week 4 of treatment (<50 IU/ml) whereas an early viral response (EVR) is defined as achieving either a $\geq 2\log_{10}$ viral load decrease from baseline or a qualitative undetectable (<50 IU/ml) HCV-RNA at week 12 after initiation of treatment. A sustained virolological response (SVR) was defined as a negative HCV viral load (<50 IU/ml) 24 weeks after discontinuation of therapy. All patients provided written informed consent and institutional ethical review boards at participating centers approved the protocol.

PBMC processing

Peripheral blood (approximately 25 ml) was collected before and at week 4 during therapy. Within 24 hours, peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll-Hypaque density gradient centrifugation. Cells were re-suspended in RPMI 1640 (Gibco Life Technologies, Breda, the Netherlands) to which 20% fetal calf serum and 1% penicillin and streptomycin were added (hereafter called medium). After adding 10% DMSO, PBMC were frozen to -80° C and stored thereafter at -180° C until further use. Except for the proliferation assay, all experiments were performed using frozen PBMC.

In vitro quantification of proliferation and cytokine production

Freshly isolated PBMC at a final concentration of $1x10^6$ cells/ml were cultured in quadruplets in 96-well round bottom plates in 200 µl culture medium in the presence of overlapping peptide pools (spanning the core, NS3, NS4 and NS5A and NS5B HCV genome; clone J4 genotype 1b; BEI Resources, Manassas, USA), anti-CD3 antibody (1 µg/mL; OKT-3; orthoclone), cytomegalovirus lysate or no stimulus. All cultures were performed in the presence of anti-CD28 (1 µg/ml; CD28.2; eBioscience) and anti-CD49d antibody (1 µg/ml; 9F10; eBioscience). After culturing for 3 days, 100 µl supernatant was harvested and replaced by 100 µl fresh culture medium. IFN-γ production was determined by a commercially available ELISA kit (eBioscience, San Diego, USA). After stimulation for 5 days, the cells were pulsed with 0.5 µCi/well of [^{3}H]-thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK), and harvested 16 hours later. Proliferation was determined as counts per minute (cpm) via liquid scintillation counting of the harvested cells. The cut-off value for a positive response was set as the median plus 2x the standard deviation (SD) of the medium.

HCV-specific CD4+ and CD8+ T-cell expansion assay

To stimulate both CD4+ and CD8+ T-cells, overlapping peptide pools (www.mimotopes.com) with a length of 18 amino acids (aa) and an overlap of 11 aa, spanning the whole HCV genome, were used. The peptides were pooled as follows: core (core/E1/E2/p7, aa 1-805 with a total of 116 peptides), non-structural protein 2 (NS2)(aa 806-1022, with a total of 31 peptides), NS3 (aa 1023-1645, with a total of 90 peptides), NS4 (aa 1646-1967, with a total of 49 peptides), NS5A (aa 1968-2415, with a total of 67 peptides) and NS5B (aa 2416-3011, with a total of 87 peptides). Peptide pools were dissolved in DMSO.
The 12-day expansion assay measured proliferative capacity and IFN-γ production as previously published [28,29]. Both pre-treatment and week 4 time points were assessed concurrently. PBMC were stimulated at $1x10^6$/ml with 2 µg/ml peptide pool (the DMSO concentration was never more than 1% in the final stimulation). Thereafter, cells were incubated in 96-well round bottom plates at $2x10^5$ cells/well at 37 ºC and 5% CO_2 and Interleukin-2 (IL-2) (20 U/ml) was added at days 3, 6 and 9. HCV peptides at a concentration of 2 µg/ml were added again on day 6. Cells were pooled, washed and counted on day 12, rested overnight in medium after which re-stimulation with HCV peptide pools for 6 hours in the presence of anti-CD28 (1 ug/ml) and anti-CD49d (1 µg/ml) as co-stimuli was performed. Concurrently, medium alone and PMA (10 ng/ml) with ionomycin (50 ng/ml) were used a negative and positive control respectively. After 1

hour, Brefeldin A (Golgiplug, BD Biosciences, San José, CA, USA) was added, followed by a further incubation period of 5 hours. Thereafter, cells were washed, permeabilized (FACS Permeabilizing Solution, BD) and stained with specific antibodies against CD3 (Pacific Blue, eBioscience), CD4 (PE Cy7, eBioscience), CD8 (APC AlexaFluor750, eBioscience) and IFN-γ (FITC, BD) for 20 minutes at 4 °C. After washing, cells were fixed with cellfix (BD) and at least 100,000 cells were acquired by flow cytometry (FACS, LRSII, BD). Using the forward and sideward scatter, the lymphocyte population was gated and analyzed by FACSDiva software (BD). The HCV-specific T-cell response (i.e. IFN-γ production) was calculated by subtracting the IFN-γ production in the unstimulated control and subsequently multiplied with the proliferation ratio (fold increase) of the expansion assay, resulting in a number of HCV-specific T cells per 10^6 PBMC, which is a combination of the (memory) T-cells initially present that survived, proliferated and differentiated into effector T-cell phenotype in each individual. A positive HCV-specific T-cell response was defined as a response of more than 200 out of 1 million PBMC since T-cell responses in healthy controls after culture with HCV peptide pools never exceeded 200 per million PBMC. Therefore, this was taken as a 'cut-off' for a positive HCV-response. All HCV-specific T-cell responses per patient (core, NS2, NS3, NS4 and NS5) were summed and displayed as the total HCV-specific T-cell response. For the analysis, patients were divided into SVR/ non-SVR or RVR/ non-RVR.

Surface marker staining

Direct *ex vivo* surface marker expression for T-cell subsets (CD27/CD45RO) and T-cell activation (CD38/HLA-DR) were analyzed pre-treatment and at week 4. After thawing and washing, cells were incubated for 20 minutes at 4 °C with a combination of antibodies against CD3 (PerCP, BD), CD4 (PE Cy7, eBioscience), CD8 (APC Alexa Fluor 750, eBioscience), CD38 (PE Cy7, eBioscience), HLA-DR (FITC, eBioscience), CD27 (FITC, BD) and CD45RO (APC, BD). After washing, cells were fixed with Cellfix and at least 200,000 cells were acquired by FACS (LRSII, BD). Using the forward and sideward scatter the lymphocyte population was gated and analyzed by FACSDiva software (BD).

Statistical analysis

Continuous data are presented as median values (with interquartile range (IQR)) analyzed using a Mann-Witney U-test whereas categorical variables are given as number of cases (percentage) analyzed using a Wilcoxon signed-rank test. Comparison of categorical variables was done using a Fisher's exact test. Spearmann's Rank correlation

and linear regression analysis were performed to examine the relationship between continuous variables and immunological parameters. A p-value ≤0.05 was considered as statistically significant and all tests used were two-sided. All data were analyzed using GraphPad Prism (version 5.0 for Windows, GraphPad Software, San Diego, USA).

Results

Of the 22 chronic HCV genotype 1 and 4 infected patients treated with pegIFN-alfa/ ribavirin, 11 patients reached a SVR (50%). Patient characteristics, shown in table 1, are similar when grouped into SVR and non-SVR. Factors possibly influencing the HCV-specific T-cell response like age, gender and extent of liver injury were not of

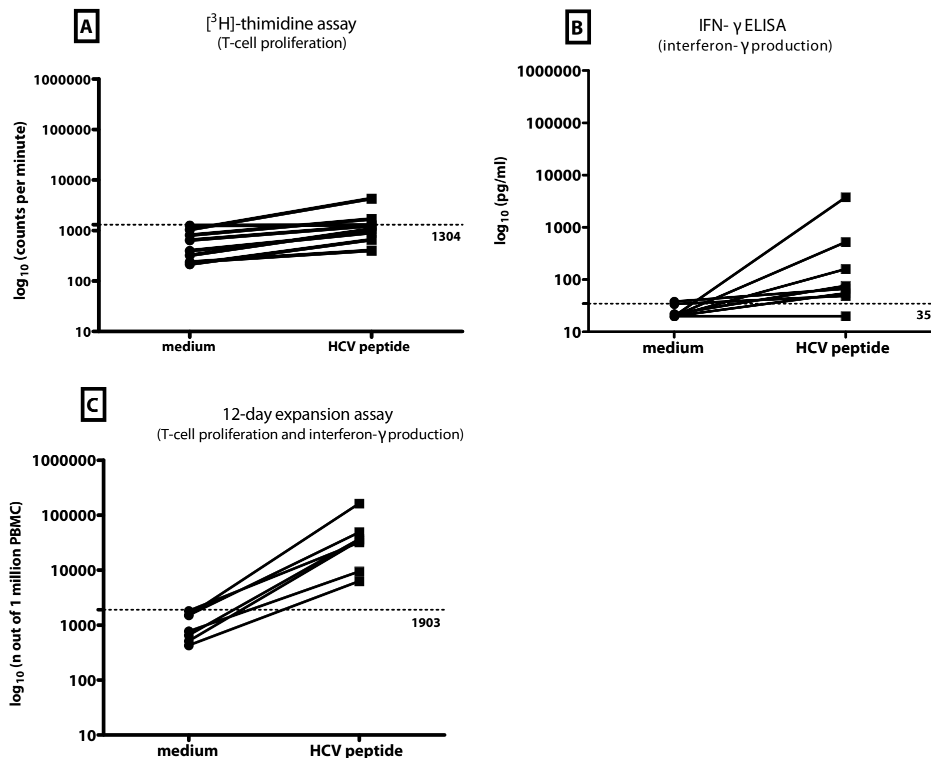
Table 1: Characteristics of patients grouped by clinical outcome

Feature	SVR (n=11)	non-SVR (n=11)	p-value
	General characteristics		
male / female	10 / 1	9 / 2	***ns***
Age (years)	48 (40 – 52)	47 (46-55)	***ns***
Weight (Kg)	78 (63 - 86)	78 (62 - 95)	***ns***
Caucasian ethnicity	11 (100)	11 (100)	***ns***
	HCV/ liver related characteristics		
Genotype			
1	8 (73)	9 (82)	***ns***
4	3 (27)	2 (18)	
HCV-RNA (Log10 IU/ml)	5.82 (5.55 – 6.57)	6.62 (5.50 – 6.68)	***ns***
ALT (IU/ml)	86 (30 – 135)	94 (48 – 120)	***ns***
Liver biopsy			
≤ F2	6 (55)	3 (27)	***ns***
F3-F4	1 (9)	3 (27)	
NP	4 (36)	5 (45)	
	Immunological characteristics		
CD38+/HLA-DR+			
CD4	0.30 (0.20-0.50)	0.60 (0.20-0.80)	***ns***
CD8	0.40 (0.30-0.70)	0.50 (0.20-1.30)	***ns***
CD8 subsets			
naive (CD27+CD45RO-)	32.2 (20.3-51.8)	39.4 (20.0-42.9)	***ns***
effector (CD27-CD45RO+)	37.6 (20.4-58.7)	22.3 (17.3-51.9)	***ns***
memory (CD27+CD45RO+)	27.8 (8.0-39.4)	36.7 (15.8-44.0)	***ns***
	Treatment outcome characteristics		
RVR	7 (64)	2 (18)	***0.08***
EVR	11 (100)	6 (55)	***0.04***

Continuous variables are shown as median values (interquartile range) while categorical variables are given as numbers (percentages). ns = not significant; NP = not performed.

significant influence on the outcome of treatment. In patients reaching a SVR, 64% also achieved a RVR and all reached an EVR. In contrast, in the non-SVR group only 18% achieved a RVR (p=0.08) and 55% reached an EVR (p=0.04). Furthermore, the baseline values of activated CD4+ and CD8+ T-cells (CD38/ HLA-DR double positive) as well as the percentages of T-cell subsets (defined by CD27 and CD45RO) were not different between SVR patients and non-SVR patients (table 1).

Figure 1: Higher HCV-specific T-cell responses were detectable using the T-cell expansion assay compared to proliferation and cytokine assay



To measure HCV-specific T-cell responses, three different T-cell assays were compared: [^{3}H]-thymidine assay (panel A), IFN-γ ELISA (panel B) and the 12-day T-cell expansion assay (panel C). PBMC were stimulated using the different protocols (see M&M section) without peptides (medium) (•) or with HCV peptides (■). All HCV-peptide responses were total responses directed against the total pool of HCV-peptides (i.e. core, NS2, NS3, NS4 and NS5). On the y-axis a log transformation is shown to account for the different scales of each assay. The dotted line (----) represents the threshold of each assay calculated as 2 times the standard deviation above the median of medium values. One patient's PBMC failed to grow in the expansion assay (no positive control) resulting in 7 evaluable patients for this assay.

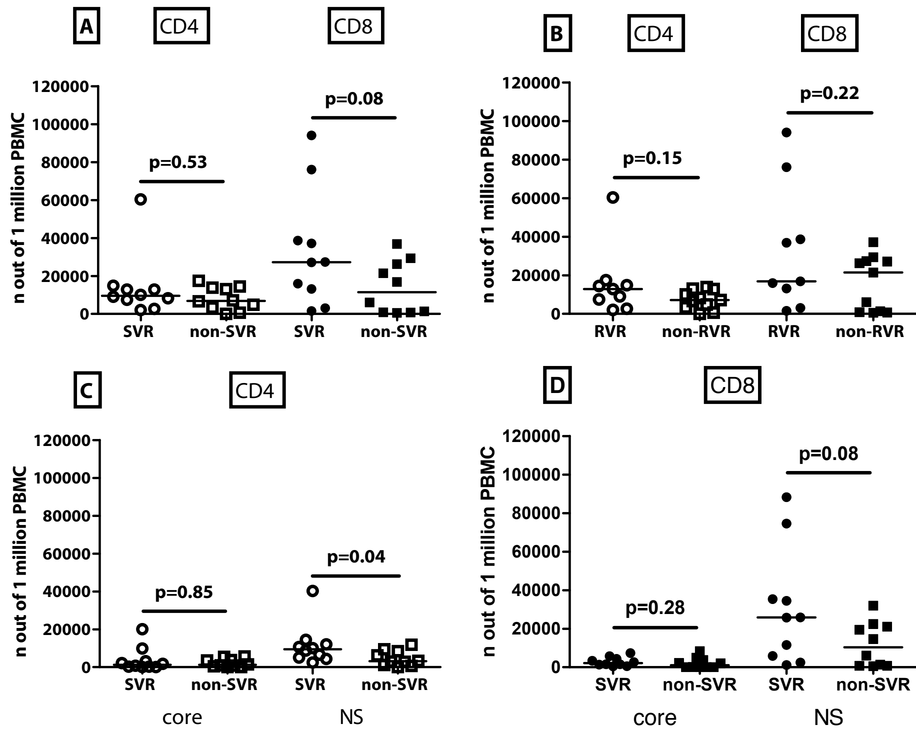
Higher HCV-specific T-cell responses were detectable using the T-cell expansion assay compared to proliferation and cytokine assay

Since direct *ex vivo* analyses have shown that very low numbers of HCV-specific T-cells are present [26], stimulation assays have been used to circumvent this problem [18,19,32]. In this study we compared the 12-day expansion assay with the [^{3}H]-thymidine proliferation assay and the IFN-γ ELISA as a more sensitive way to measure HCV-specific T cells. In a randomly selected subgroup of patients the majority (6 out of 8 patients) failed to show HCV-specific T-cell proliferation in the [^{3}H]-thymidine assay, although one of the remaining 2 patients displayed a relatively strong response (4304 cpm; figure 1A). Likewise, the IFN-γ ELISA showed low IFN-γ levels in supernatant after HCV peptide stimulation around the cut-off value (35 pg/ml) in 5 out of 8 patients (figure 1B) with a stimulation index (SI) of <3. In the remaining 3 patients moderate to good IFN-γ production was detectable with values ranging between 159 and 3,773 pg/ml (SI between 8 and 188). Finally, the 12-day expansion assay showed low background values and T-cell responses were detectable in all evaluable patients well above the cut-off value (SI ≥ 12; figure 1C). The 12-day expansion assay therefore allows for a sensitive detection of both HCV-specific CD4+ and CD8+ T-cell responses since it combines both production of IFN-γ and proliferation of (memory) T-cells into becoming effector T-cells.

Pre-treatment HCV-specific T-cell responses are not associated with outcome of therapy

To address the question whether pre-treatment HCV-specific T-cell responses were associated with therapeutic outcome, we measured HCV-specific CD4+ and CD8+ T-cells before start of therapy using the 12-day expansion assay in patients achieving a SVR (n=11) and those without a SVR (n=11). The pre-treatment HCV-specific CD4+ T-cell response was equally strong in patients reaching a SVR (9,564 with IQR 6,281-13,335) compared to those without a SVR (6,870 with IQR 2,699-14,001; p=0.53; figure 2A). Similarly, no difference in HCV-specific CD8+ T-cell responses was noted between SVR and non-SVR patients (27,237 (IQR 10,643-48,021) and 11,491 (IQR 829-26,992); p=0.08). Likewise, when patients were grouped according to achievement of a RVR or non-RVR, HCV-specific T-cell responses between the groups were not significantly different (figure 2B). Irrespective of treatment outcome, HCV-specific CD8+ T-cells responded better to stimulation with HCV antigens than HCV-specific CD4+ T-cells.

Figure 2: Pre-treatment total and individual HCV-specific T-cell responses in relation to outcome of therapy.



Total pre-treatment HCV-specific CD4+ (o/□) and CD8+ (•/■) T-cell responses (sum of core, NS2, NS3, NS4 and NS5A/B), measured with the 12-day T-cell expansion assay, are shown in relation to achieving a SVR (panel A) or a RVR (panel B) with pegIFN-alfa/ ribavirin therapy. Responses against individual HCV antigens (core or non-structural (NS, sum of NS2, NS3, NS4 and NS5A/B)) are shown for CD4+ (panel C) and CD8+ T-cells (panel D). On the y-axis the number of IFN-γ producing T-cells per million PBMC is shown. Of one patient in the SVR group too few pre-treatment PBMC were available for analysis while of 1 patient in the non-SVR group the PBMC failed to grow in the expansion assay (no positive control).

Subsequently, we assessed the focus of the T-cell response directed against individual HCV antigens in relation to the outcome of therapy. In patients achieving a SVR, preferential targeting of the non-structural HCV peptides by CD4+ T-cells was demonstrated (p=0.04) when compared to those patients not achieving a SVR (figure 2C). NS4 and NS5 being most significant for HCV-specific CD4+ T-cells (p=0.009 and p=0.05 respectively).

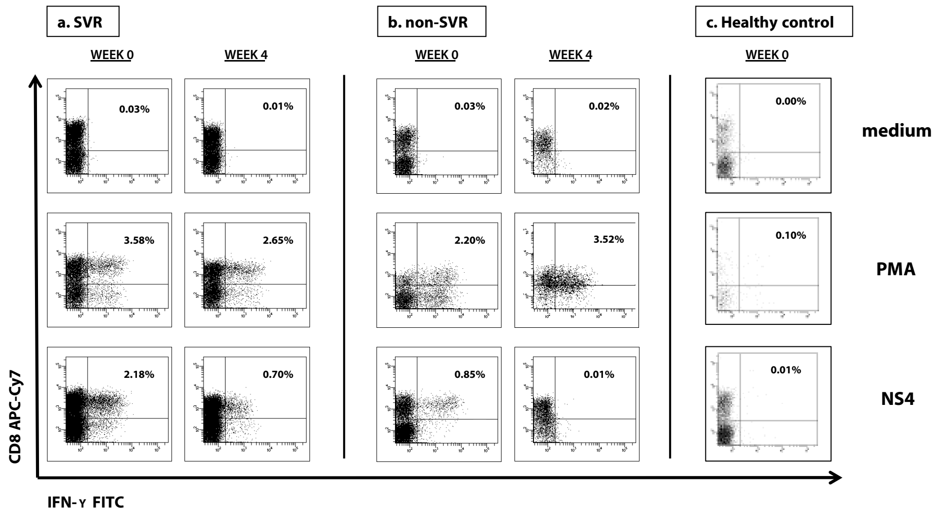
Several studies have analyzed the interdependence of CD4+ and CD8+ T-cell responses in the same individual either via depletion studies [26,32] or direct *ex vivo* quantification [20] showing a correlation between both responses. In agreement with these findings,

we also found a positive correlation between individual HCV-specific CD4+ and CD8+ T-cell responses (r=0.47 with p=0.04; Spearman Rank correlation; data not shown). There was no correlation between the HCV-specific CD4+ or CD8+ T-cell responses on the one hand and other baseline characteristics like ALAT and plasma HCV-RNA on the other hand (data not shown).

HCV-specific T-cell responses decline during pegIFN-alfa/ ribavirin treatment regardless of outcome of therapy

Next, we investigated the role of HCV-specific CD4+ and CD8+ T-cells in eradication of HCV during pegIFN-alfa/ ribavirin therapy by quantifying the number of HCV-specific T-cells at week 4 of therapy. This time point is used to define RVR which is an important virological parameter determining the chances of SVR [7,8]. Consequently, at this time point patients with detectable and those without detectable HCV-RNA were present allowing us to investigate the relationship between antigen presence and HCV-specific T-cell responses.

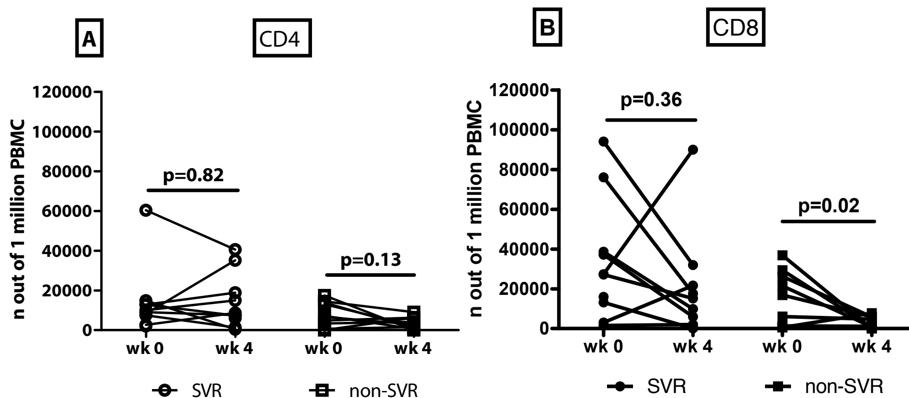
Figure 3: The effect of pegIFN-alfa/ ribavirin therapy on the percentage of IFN-γ producing HCV-specific CD4+ and CD8+ T-cell responses against HCV-NS4.



Flow cytometry dot plot showing a longitudinal analysis of the percentage of IFN-γ producing HCV-specific CD8+ T-cell responses against HCV-NS4 pre-treatment and at week 4 in a representative patient achieving a SVR (panel A), a patient without a SVR (panel B) and a healthy control (panel C). On the y-axis, CD8 APC-Cy7 and on the x-axis IFN-γ FITC is shown. The upper row displays the negative control (medium), the middle row is the positive control (PMA) and the bottom row shows the HCV-NS4 peptide.

To illustrate the HCV-specific T-cell responses over time, flow cytometry dotplots of IFN-γ producing CD8+ T-cells against NS4 are shown (figure 3) for both a SVR and a non-SVR patient. The percentage of IFN-γ producing HCV-specific CD8+ T-cells declined during therapy in both the SVR-patient (from 2.18% at baseline to 0.70% at week 4) and the non-SVR patient (from 0.85% at baseline to 0.01% at week 4). To account for the difference in T-cell proliferation, total HCV-specific T-cell responses were calculated by combining these T-cell proliferation ratios with the IFN-γ production. Regardless of the outcome of therapy, a decline in HCV-specific CD4+ and CD8+ T-cell responses was observed from baseline to week 4 (figure 4A and 4B). This decrease was more prominent in the CD8+ T-cell responses in non-SVR patients (median decline from 11,491 (IQR 829-26,992) to 1,011 (IQR 351-5,904); p=0.02)). The patient who showed a marked increase in HCV-specific CD8+ T-cell responses from baseline (27,345) to week 4 (89,956 per million PBMC) had no distinctive clinical, virological or immunological abnormalities that could explain this sharp increase (figure 4B). Similarly, when patients were grouped according to achievement of a RVR, a decline in HCV-specific CD4+ and CD8+ T-cell responses was observed as seen in SVR/non-SVR patients (data not shown).

Figure 4: HCV-specific T-cell responses decline during treatment regardless of achieving a SVR.



Longitudinal total HCV-specific T-cell responses, measured with the 12-day T-cell expansion assay, are displayed pre-treatment and at week 4 of pegIFN-alfa/ ribavirin therapy for CD4+ (panel A) and CD8+ (panel B) T-cells. The y-axis shows the number of IFN-γ producing T-cells per million PBMC. On the x-axis the different time point are shown (pre-treatment and week 4) while patients are grouped into SVR (o/•) or non-SVR (□/■). Removing the SVR-patient with the high HCV-specific CD8+ T-cell response at week 4 of therapy from the analysis resulted in a trend towards a significant decrease in T-cell responses at this time point (p= 0.08). Of 2 patients (1 SVR and 1 non-SVR) the PBMC failed to grow in the expansion assay (no positive control).

Since at baseline skewing towards the NS-peptides was associated with SVR, we evaluated the breadth of the response by the number of HCV NS-antigens targeted per patient at baseline and at week 4 of therapy. We observed that for both the CD4+ and CD8+ T-cell response the median number of targeted NS-antigens remained relatively conserved from baseline to week 4 in both SVR patients (3 to 4 and 4 to 3, respectively) and non-SVR patients (3 to 2 and 3 to 2, respectively; data not shown).

Discussion

This study evaluated the role of HCV-specific T-cell responses in PBMC of chronic HCV genotype 1 and 4 patients before and during pegIFN-alfa/ ribavirin therapy using a sensitive 12-day expansion assay. We are able to demonstrate that irrespective of clinical outcome, pre-treatment HCV-specific CD4+ and CD8+ T-cell responses exist and that they markedly decline during pegIFN-alfa/ ribavirin therapy in both SVR and non-SVR patients. Moreover, a similar pattern of HCV-specific T-cell responses was observed in patients achieving a RVR when compared to those without a RVR. Finally, skewing of the T-cell response against the NS-antigens was found to be associated with reaching a SVR.

T-cell proliferation and IFN-γ production are important parameters of an effective anti-viral response [33] which was generally analyzed using separate assays [16,17,22,34,35]. In recent years, several groups have used T-cell expansion in different assays trying to combine both features [18,19,32]. The 12-day T-cell expansion used in our study also combines both features of proliferation and IFN-γ production into one assay allowing simultaneous detection of CD4+ and CD8+ T-cell responses. The assay was first described by Reece et al. [36] as an *ex vivo* expansion assay in which IFN-γ secretion was assessed by ELISPOT. Our group has previously modified this 12-day expansion assay to allow the IFN-γ secretion to be measured by flow cytometry validating this method for Epstein-Barr virus infection [30,37], HIV [38] and HCV infections [28,29]. It combines the proliferation (and/or survival) of precursor (memory) HCV-specific T-cells after HCV-antigen stimulation and IFN-γ secretion upon specific re-stimulation for both CD4+ and CD8+ T-cell responses simultaneously. Compared to proliferation and IFN-γ production assays, the 12-day expansion assay showed an improved detection of HCV-specific T-cell responses. Although, this assay provides an estimate of the total HCV-specific T-cell response in a given patient, irrespective of HLA-type, it does not allow to dissect the specific epitopes which are recognized and results cannot be confirmed using tetramer-staining.

Compared to previous studies showing either no or low pre-treatment HCV-specific T-cell responses [11,17,21,22,24,39] and studies demonstrating strong and multi-specific T-cell responses to HCV at baseline associated with or without achievement of SVR [15,16,18-20,23,40-42], our study was distinctive. First, in this prospective study the use of a sensitive expansion assay greatly improved the detection of HCV-specific T-cell responses. Second, no association was found between either pre-treatment or on-treatment HCV-specific CD4+ and CD8+ T-cell responses and the outcome of pegIFN-alfa/ ribavirin therapy in terms of achieving a RVR, although the number of included patients was small. Furthermore, T-cell responses declined during anti-HCV treatment in all patients irrespective of clinical outcome.

The HCV-specific T-cell response in chronic HCV patients is highly regulated. It has become clear that multi-factorial mechanisms negatively regulate the HCV-specific T-cell response via mechanisms involving immunosuppressive cytokines and regulators such as PD-1 [43,44]. The enhanced sensitivity of the 12-day T-cell expansion assay could rely on lifting the regulation during the culture period, possibly as a result of the addition or production of growth factors, such as IL-2. Furthermore, when analyzing the different HCV-peptides in relation to SVR, a skewing of reactivity towards the non-structural HCV peptides is observed. This is in agreement with previous studies [45,46] showing an association between a vigorous T-cell response against NS3 and successful outcome of therapy. Furthermore, albeit not performed in our study, some studies have shown via tetramer analysis that specific epitopes in core and NS3 correlate with SVR [15,24]. Therefore, rather than looking at the total HCV-specific response, future studies, possibly using tetramers, should focus more on the detailed poly-functionality and quality as well as regulation of HCV-specific T-cell responses to identify pre-treatment factors responsible for successful therapy.

By evaluating the role of HCV-specific T-cells during pegIFN-alfa/ ribavirin therapy in both SVR and non-SVR patients a similar decline in HCV-specific T-cell responses from baseline to week 4 was demonstrated in both patient groups. Immunological parameters like T-cell activation or a shift in the numbers of T-cell subsets could not explain the therapy-induced decline of the HCV-specific T-cell response. This is in accordance with some other studies showing a decline in HCV-specific T-cell responses using IFN-γ production as direct effector function of T-cell activity [15,18,19]. In contrast, others have suggested augmentation of T-cells during therapy using T-cell proliferation assays with fresh PBMC and [^{3}H]-thymidine incorporation, thereby mainly describing T-cell memory function [11,21,24]. A possible explanation is that due to the decrease in viral load and thereby loss of antigen-triggering of HCV-specific T cells, these T-cell responses decline. This would suggest that, similar to other infections like HIV [47,48],

loss of antigen leads to a reduction of virus-specific T-cells and demonstrates no role for HCV-specific T-cell responses in forced viral clearance during pegIFN-alfa/ ribavirin therapy. This argument is supported by the observation that T-cell responses recover after treatment discontinuation in patients experiencing a relapse of HCV viremia [19,24,40]. One of our patients also experienced a relapse at the end of therapy with an increase in HCV-RNA levels subsequently followed by an increase in HCV-specific T-cell responses (data not shown).

Homing of HCV-specific T-cells to the liver during treatment is mentioned as an explanation for the low or absent T-cell responses measured in blood [22,49]. This hypothesis is supported by the observation that in cross-sectional studies higher percentages of HCV-specific T-cells are found in the liver than in peripheral blood [50,51]. However, peripheral T-cell responses are easily measurable in patients spontaneously clearing acute HCV [26,52,53]. Moreover, a recent study in chronic HCV patients demonstrated that several of the measured intra-hepatic epitope-specific T-cell responses were also found in PBMC only after *ex vivo* expansion of these T-cells [49,54]. This indicates that these responses present in the liver can also be measured in peripheral blood albeit at low frequencies. Therefore, the use of a sensitive expansion assay, as used in our study, enabled us to detect these small frequencies of peripheral HCV-specific T-cells. However, extrapolation of findings obtained using peripheral blood to the functionality of intra-hepatic T-cells should be made with caution, since the microenvironment of the liver may modulate the phenotype and activity of these cells [55].

Conclusion

Using a sensitive expansion assay, we found no correlation between pre-treatment HCV-specific T-cell responses and outcome (SVR or RVR) of pegIFN-alfa/ ribavirin therapy by assessing the total response to peptides spanning the entire HCV genome. However, skewing of the individual T-cell responses towards the NS-antigens was observed. Furthermore, irrespective of treatment outcome, HCV-specific T-cell responses declined during treatment in all patient groups. Although this suggests that total HCV-specific T-cell responses do not play a role in forced viral eradication, it is possible that HCV-specific T-cells at baseline initially maintain (or even increase) their effector function in the first weeks of therapy after which a decline sets in. Further studies to assess HCV-specific T-cell poly-functionality, kinetics and regulation in detail are needed.

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Chapter 7

No role for HCV-specific T-cell responses in forced viral clearance during HCV therapy in genotype 1 and 3 HIV/ HCV co-infected patients

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Abstract

Background: It has been suggested that HCV clearance during treatment with pegylated interferon (pegIFN-alfa) and ribavirin depends on killing of HCV-infected hepatocytes by HCV-specific T-cells. Since limited data are available on HCV-specific T-cell responses during HCV treatment in HIV-infected individuals, who have lower treatment success rates, we conducted a thorough analysis of the HCV-specific T-cell responses early during treatment in HIV/HCV coinfected patients with different HCV genotypes.

Methods: HCV-specific T-cell responses were measured during the first 12 weeks of treatment using a sensitive assay for detection of antigen-specific memory T-cells using overlapping peptide pools corresponding to the infecting genotype of the patient.

Results: In total, 28 HIV/ HCV co-infected patients were included, of whom 18 were infected with HCV genotype 1 and 10 were infected with HCV genotype 3. Neither baseline HCV-specific CD4+ T-cells nor CD8+ T-cells were associated with a sustained viral response. During the first 12 weeks of HCV treatment, we observed a decline in both magnitude and breadth of HCV-specific CD4+ and CD8+ T-cell responses. Even more, genotype 3 infected patients tended to have a stronger decline in HCV-specific T-cell responses than genotype 1 infected patients, which paralleled the stronger decline in viral load.

Conclusion: Neither HCV-specific T-cells at baseline nor augmentation of HCV-specific T-cells during therapy appear to play a major role in forced viral clearance in HIV/ HCV co-infected patients, even in patients infected with a genotype associated with a good response to therapy.

Introduction

Spontaneous clearance of hepatitis C virus (HCV) occurs in 15-40% of individuals [1]. Treatment of chronic HCV-infection with pegylated interferon (pegIFN-alfa) and ribavirin leads to a sustained virological response (SVR, undetectable HCV-RNA 6 months after discontinuation of therapy) in 50-90% of patients [2,3]. The most important baseline predictor for SVR known so far is HCV genotype; both HCV mono-infected and HIV/ HCV co-infected individuals with genotype 2 and 3 respond better to therapy than those with genotype 1 and 4 [4].

Decline of HCV-RNA levels during combination therapy is biphasic and mathematical modeling of HCV kinetics has suggested that the slope of HCV-RNA decline in the first 48 hours is most likely determined by the half life of free virus (i.e., blocking of new virus production), while the slope thereafter is determined by the half life of infected cells (i.e., killing of infected hepatocytes by cytotoxic T lymphocytes (CTL)) [5,6]. In HCV mono-infected patients higher proliferative capacity of HCV-specific CD8+ T-cells before therapy and higher pre-treatment IFN-γ production by CD4+ T-cells were associated with SVR, which may reflect better capacity to kill infected targeT-cells during forced clearance of HCV [7,8].

Approximately 30% of HCV-infected patients are HIV co-infected due to shared routes of transmission [9]. HIV/ HCV co-infection is associated with lower rates of SVR after pegIFN-alfa/ ribavirin treatment [10], suggesting that the host immune system plays an important role in treatment-induced viral clearance. Furthermore, HIV/ HCV co-infection is associated with faster progression to liver-related morbidity and mortality [11,12].

A strong HCV-specific T-cell response (predominantly IFN-γ production) targeting multiple HCV proteins was shown to be associated with spontaneous viral clearance in HCV mono-infected patients [13,14]. Since there is hardly any data available on HCV-specific T-cell responses during treatment with pegIFN-alfa/ ribavirin in HIV/ HCV co-infected patients, we performed a comprehensive analysis of HCV-specific T-cell responses during treatment and compared individuals infected with HCV genotypes that respond good (HCV genotype 3) and genotypes that are less responsive to HCV treatment (HCV genotype 1).

Materials and methods

Patients and HCV treatment

Twenty-eight HIV-infected patients with HCV genotype 1 or 3 co-infection were studied prospectively. Twenty patients were included in a randomized, open label, pilot study and were treated for chronic HCV with combination therapy consisting of pegIFN-alfa and ribavirin. Ten patients were treated with pegIFN-alfa-2b (Pegintron, Schering-Plough) 1.5 μg/kg/wk (standard-arm) and 10 patients were treated with induction pegIFN-alfa-2b (regimen: 3.0 μg/kg/wk during the first 4 wks, 2.0 μg/kg/wk during the next 4 wks and 1.5 μg/kg/wk during the remaining 40 weeks) plus oral ribavirin at a daily dose of 1000 mg (bodyweight <75 kg) or 1200 mg (bodyweight ≥75 kg). The remaining 8 patients were included from a study (Privicop) that was designed for immunological and viral studies during HCV treatment in which patients were treated with standard doses pegIFN-alfa-2a (180 μg/wk, Pegasys, Roche) and weight-based ribavirin [15]. Institutional review boards at participating centers approved the protocol. All patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Viral assays

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

Response definitions

Rapid virological response (RVR) was defined as undetectable viral load at week 4 of treatment using a qualitative assay (<50 IU/ml). *Early virological response* (EVR) was defined as undetectable viral load or at least 2 log HCV-RNA load decline at week 12 of treatment compared to baseline. *Sustained virological response* (SVR) was defined as undetectable HCV-RNA 24 week after treatment discontinuation.

HCV peptide pools

For stimulation and expansion of HCV-specific T-cells, pools of overlapping peptides spanning the complete HCV proteome were used. To study differences in T-cell responses between HCV genotype 1 and 3 infected individuals, HCV genotype 1a and 3a peptide pools were used (kindly provided by www.beiresources.com), consisting of 14- to 18-mers, overlapping by 11 amino acids. Peptides were dissolved in DMSO and pooled in 6 pools (corresponding to Core (115 peptides), NS2 (31 peptides), NS3 (50 peptides), NS4 (49 peptides), NS5A (67 peptides) and NS5B (87 peptides)) in such a way that each peptide was present in the final pool at a concentration of 1 mg/ml.

HCV-specific T-cell expansion

To analyze HCV-specific T-cell responses, we used a sensitive expansion assay, previously shown to be superior to [^{3}H]-incorporation assays and cytokine production assays [16]. Cryopreserved PBMC were thawed and expanded during 12 days using overlapping HCV genotype 1 or 3 peptide pools (2 μg/ml, DMSO concentration never exceeding 1%) in the presence of recombinant IL-2 (360 IU/ml). After 12 days, expanded cells were washed, counted and rested overnight. On day 13 cells were restimulated using peptide pools from the corresponding HCV genotype to assess IFN-γ production.

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

IFN-γ producing T-cells were enumerated using intracellular cytokine staining (ICCS). Briefly, PBMC were stimulated for 6 hours with HCV Core, NS2, NS3, NS4 and NS5 peptide pools (2 μg/ml) and both αCD28 (2 μg/ml) and αCD49d (2 μg/ml) as co-stimuli, after 1h 1:1,000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, USA) was added. As a negative control, PBMC were cultured with medium and co-stimulation alone. As a positive control PBMC were stimulated with 10 ng/ml PMA, 2 μg/ml ionomycin and co-stimulation. After stimulation, cells were washed, permeabilized (FACS Permeabilizing Solution, BD), washed and stained with antibodies specific for CD3, CD4, CD8 and IFN-γ (eBioscience, San Diego, CA, USA). After fixation (Cellfix, BD) min. 25,000 events were acquired on a LSRII flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analyzed using FACSDiva software (BD).

Results were expressed as the percentage of IFN-γ producing T-cells, which was calculated by subtracting the percentage of IFN-γ producing T-cells in medium from the percentage of IFN-γ producing T-cells in peptide pool (re-)stimulated conditions. We calculated the number of HCV-specific T-cells as the product of the percentage of IFN-γ-producing T-cells and the proliferation rate, which we defined as the total number of cells recovered after 12 days divided by the total number of cells put in to culture at day 0 [14,17,18]. The number of IFN-γ producing HCV-specific T-cells per million PBMC is a combination of (memory) cells initially present and the ability of these cells to survive, proliferate and differentiate to (IFN-γ-producing) *effector* T-cells during the 12-day culture period [14,18].

Statistical analysis

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

Results

Baseline characteristics of the patient population and clinical outcome

In total, 28 HIV-infected patients were included; 18 were co-infected with HCV genotype 1 and 10 were co-infected with HCV genotype 3. In each group, 5 patients were treated with induction treatment and the remaining patients were treated with standard dose regimens of pegIFN-alfa-2a or pegIFN-alfa-2b. Twenty patients were treated with HAART (table 1). The median CD4+ T-cell count (430 cells/ml (IQR 280-740 cells/ml) was not different between HAART-treated and untreated patients (p=0.44, data not shown). The baseline $\log_{10}$ HCV load was lower in HCV genotype 3 compared to HCV genotype 1 infected individuals (p=0.055; table 1).
Of 28 included patients, 22 (78.6%) achieved an EVR and of those, 11 (50%) achieved a SVR. As expected, patients infected with HCV genotype 3 were more likely to achieve an EVR (10/10 genotype 3 infected patients versus 12/18 genotype 1 infected patients, p=0.039) and a SVR (6/10 genotype 3 infected patients versus 5/18 genotype 1 infected patients, p=0.090) compared to patients with HCV genotype 1 (table 1). There was no difference in achieving a SVR between the different regimens (3/10 in

patients treated with standard dose pegIFN-alfa-2b, 4/8 in patients treated with standard dose pegIFN-alfa-2a and 4/10 in patients treated with induction pegIFN-alfa-2b, data not shown).

Table 1: Baseline characteristics of included patients.

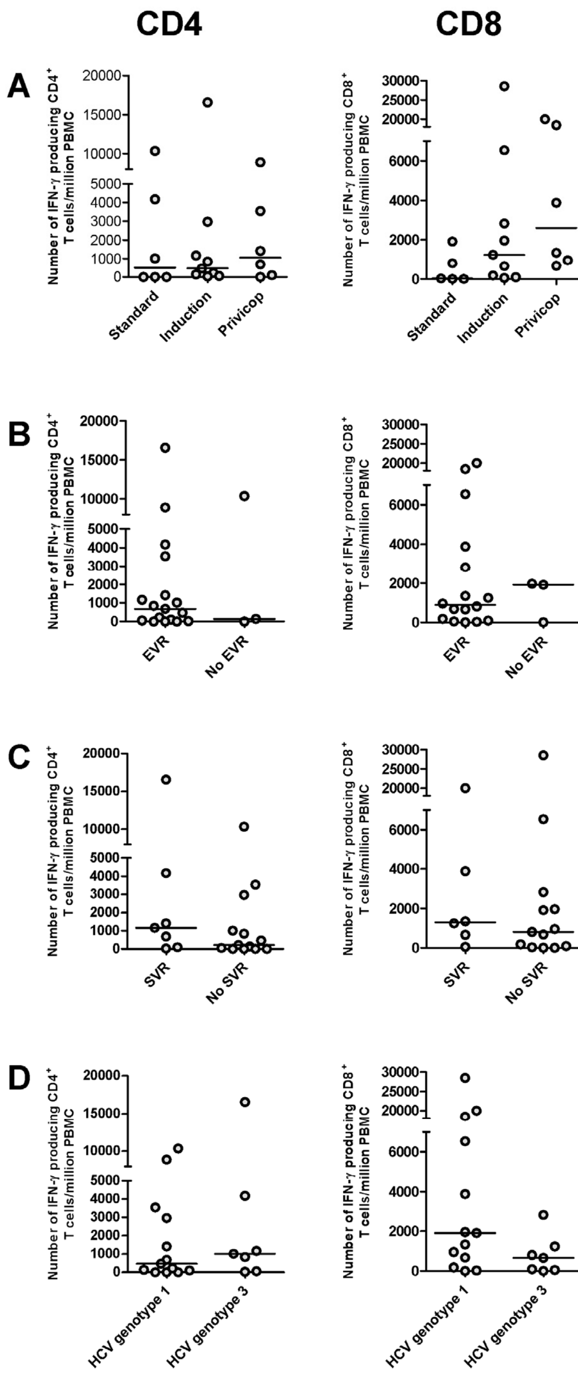
	HCV genotype 1 n=18	HCV genotype 3 n=10	P value*
General characteristics			
Female sex (%)	4 (22.2%)	1 (10%)	0.42
Median age (IQR)	42 (34-46)	42 (39-47)	0.81
Treatment related characteristics			
Standard of care (n)	13	5	
Peg-IFN-alfa-2a (Pegasys ®)	8	-	
Peg-IFN-alfa-2b (Pegintron ®)	5	5	
Induction arm (n)	5	5	
At least treated up to week 12 (%)	83.3%	100%	0.17
EVR (%)	12 (66.7%)	10 (100%)	0.039
SVR (%)	5 (27.8%)	6 (60.0%)	0.094
HCV/liver-related characteristics			
Median baseline HCV viral load (IU/ml, IQR)	6.34 (5.97-6.48)	5.78 (5.39-6.23)	0.055
Median ALT (IU/ml) (IQR)	68 (51-88)	126 (85-152)	0.25
F3-F4 liver fibrosis in biopsy	5/8	1/5	0.22
HIV-related characteristics			
Median CD4 count (IQR)	430 (370-740)	280 (250-540)	0.37
Treated with HAART (n)	11	9	0.11
Undetectable HIV viral load (n)	11	8	0.31
HCV-specific T-cell responses			0.89
Detectable CD4 response	8/13	5/8	
Detectable CD8 response	10/13	4/7	
Detectable both CD4 and CD8	7/13	4/7	

* Pearson χ^2 for proportions, Kruskall-Wallis for continuous variables.

Baseline HCV-specific T-cell responses

In some studies on HCV mono-infected individuals, baseline T-cell characteristics like proliferative capacity of HCV-specific CTL and IFN-γ production of CD4+ T-cells were associated with SVR [7,8]. Therefore, we analyzed HCV-specific T-cell responses using an assay that measures a combination of these properties [14,18], which was shown to be more sensitive than previously used assays and provided us the opportunity to be able

Figure 1: HCV-specific CD4+ nor CD8+ T-cells are baseline predictors for early (EVR) or sustained virological response (SVR).



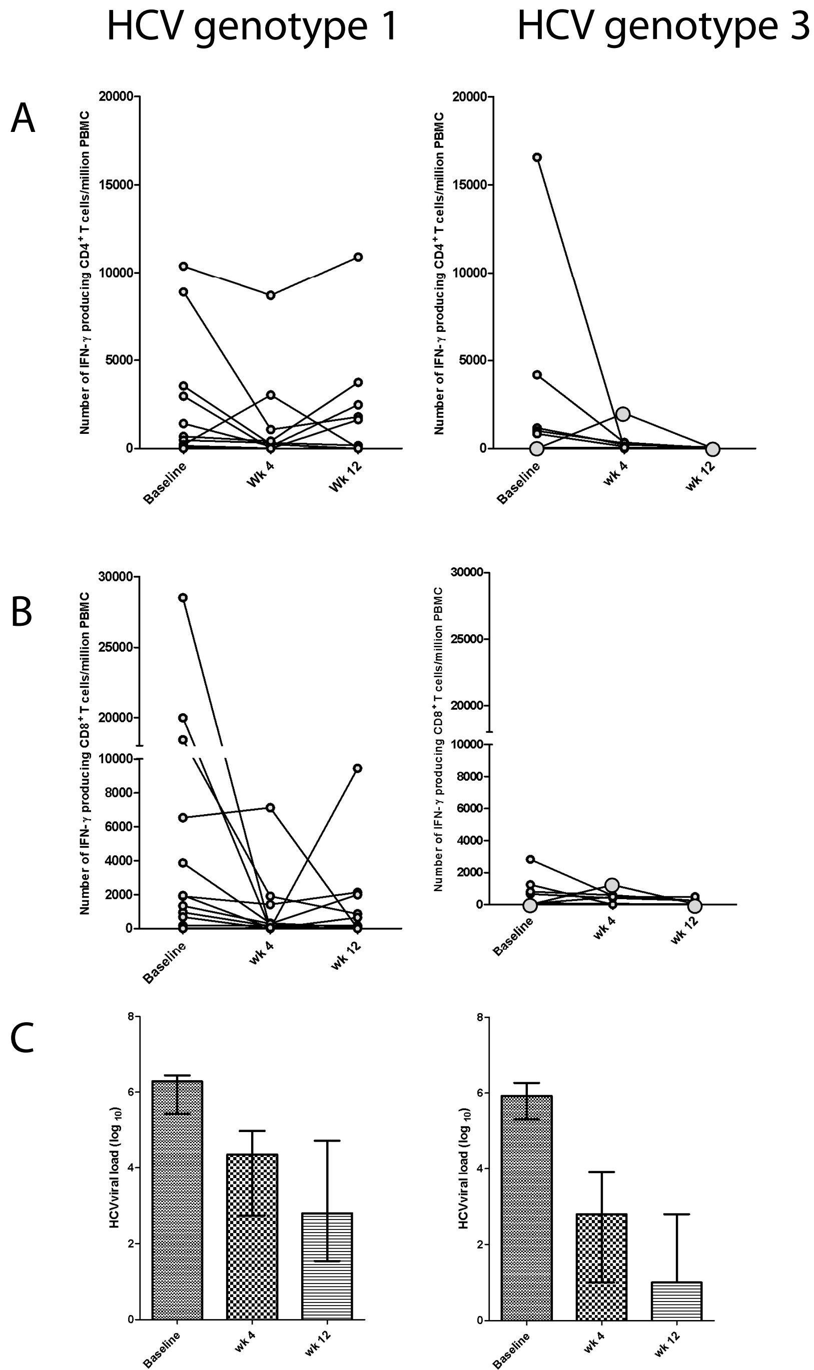
A) Baseline HCV-specific CD4+ (left panel) and CD8+ T-cell response (right panel) as measured after 12-day expansion in the 3 different study groups: standard arm: treated with pegIFN-alfa-2b and ribavirin, induction arm: treated with pegIFN-alfa-2b induction scheme and ribavirin, and Privicop: treated with pegIFN-alfa-2a and ribavirin.
B) Baseline HCV-specific CD4+ (left panel) and CD8+ T-cell response (right panel) as measured after 12-day expansion in patients achieving an early virological response (EVR) and those who do not.
C) Baseline HCV-specific CD4+ (left panel) and CD8+ T-cell response (right panel) as measured after 12-day expansion in patients achieving a sustained virological response (SVR) and those who do not.
D) Baseline HCV-specific CD4+ (left panel) and CD8+ T-cell response (right panel) as measured after 12-day expansion in patients infected with HCV genotype 1 and 3.

to detect HCV-specific T-cells also during therapy [16]. The experiments could be carried out in 21 of 28 (75%) patients. HCV-specific CD4+ T-cell responses could be detected in 13/21 (61.9%) and HCV-specific CD8+ T-cell responses in 14/20 (70.0%) patients (table 1). The magnitude of the total HCV-specific CD4+ as well as CD8+ T-cell response was not different between treatment groups (Kruskall Wallis, p=0.98 and p=0.32, respectively; figure 1A). Furthermore, HCV-RNA loads at baseline were comparable between the three treatment regimen groups: 6.4, 5.9 and 6.2 for standard pegIFN-α2a, standard pegIFN-α2b and induction pegIFN-α2b, respectively (data not shown). Although, compared to the other treatment groups, ALT levels were lower in the group treated with standard pegIFN-α2a, baseline HCV-specific T-cell responses were comparable. Therefore, we subsequently analyzed the groups together. In the analyzed patients, only 3 individuals did not achieve an EVR. These individuals had similar HCV-specific CD4+ and CD8+ T-cell responses to those not achieving an EVR (p=0.87 and p=0.91 respectively; figure 1B). Seven patients achieved a SVR, 13 did not and one was lost to follow-up. HCV-specific T-cell responses at baseline were not different between those who achieved a SVR and those who did not (p=0.39 and p=0.69 for CD4+ and CD8+ T-cell response, respectively; figure 1C).
The magnitude of the total HCV-specific CD4+ T-cell response was not significantly different between HCV genotype 1 and HCV genotype 3 infected patients (p=0.39; figure 1D). The HCV-specific CD8+ T-cell response tended to be lower in genotype 3 than in genotype 1 infected patients, although this did not reach statistical significance (median 1912 (IQR 429-12477) versus 658 (IQR 54-1242) IFN-γ producing CD8+ T-cells/ million PBMC, respectively, p=0.10; figure 1D).

Fate of HCV-specific T-cell responses during treatment

Longitudinal HCV-specific T-cell responses were examined at week 4 and 12 of treatment, corresponding to time points of RVR and EVR, respectively. During HCV

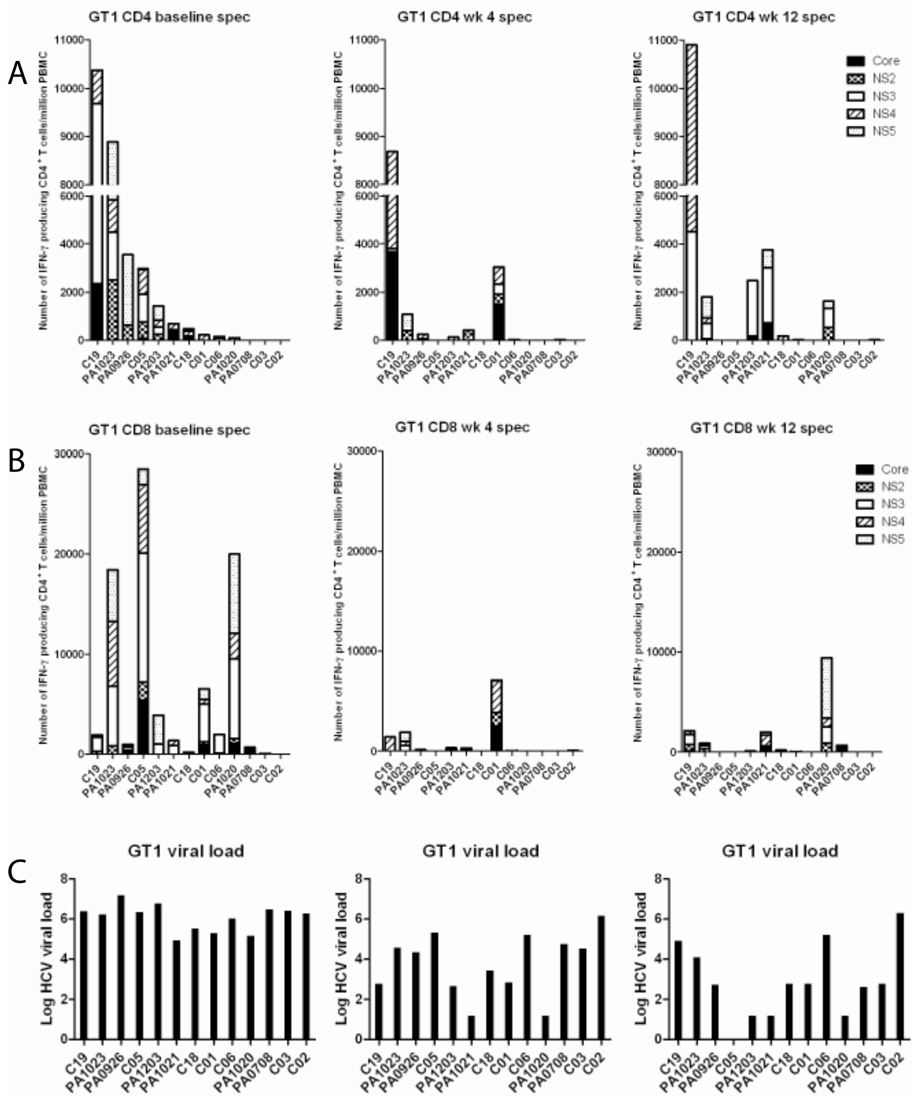
Figure 2: Decline of HCV-specific T-cell responses during pegIFN-alfa and ribavirin therapy parallels viral load kinetics.



A) Fate of HCV-specific CD4+ cells in genotype 1 infected patients (left panel) and genotype 3 infected patients (right panel) as measured after 12-day expansion at baseline, week 4 and week 12. Patient C17 is indicated by the enlarged symbols.
B) Fate of HCV-specific CD8+ cells in genotype 1 (left panel) and genotype 3 infected patients (right panel) as measured after 12-day expansion at baseline, week 4 and week 12. Patient C17 is indicated by the enlarged symbols.
C) Log_{10} viral load during HCV treatment with pegIFN-alfa and ribavirin in time in HCV genotype 1 (left panel) and genotype 3 (right panel) infected individuals at baseline, week 4 and week 12.

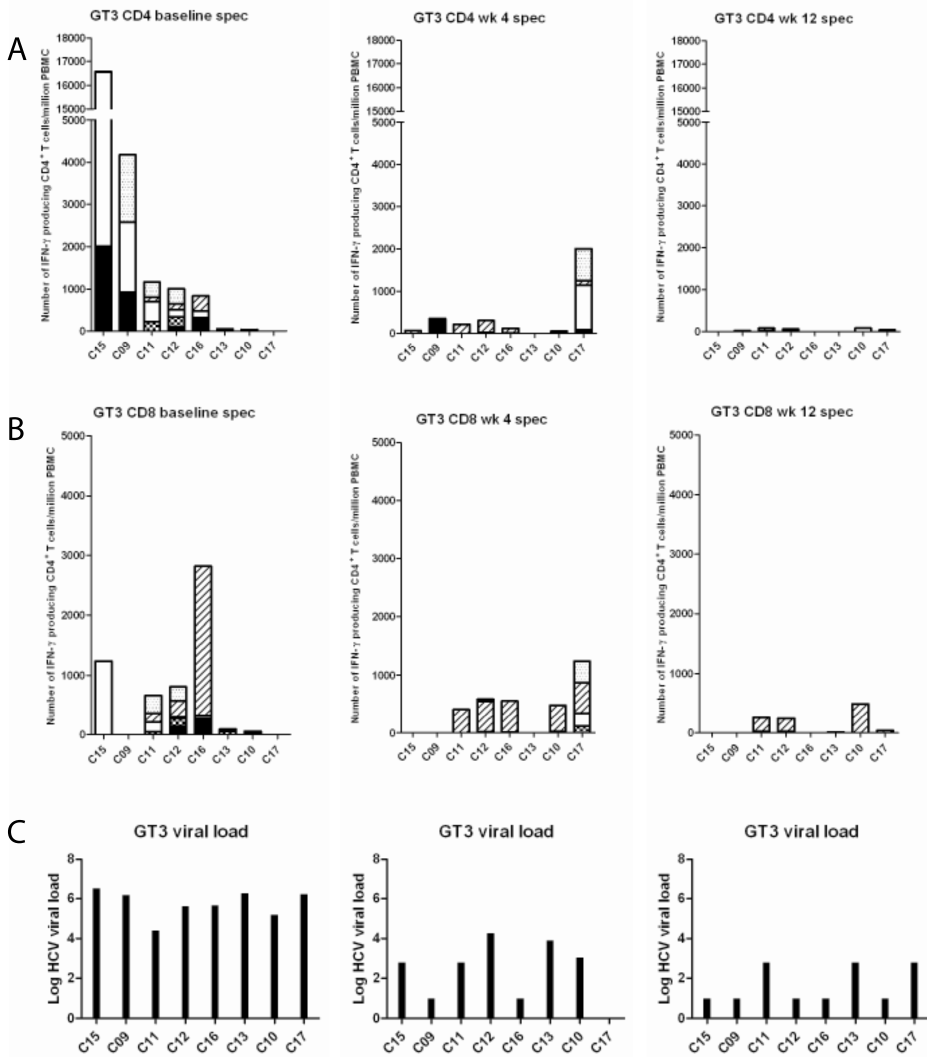
treatment both HCV-specific CD4+ T-cell (figure 2A) and CD8+ T-cell responses (figure 2B) declined in both HCV genotype 1 and genotype 3 infected individuals.
In genotype 1 infected patients the overall HCV-specific CD4+ T-cell responses declined from 468 to 68 CD4+ T-cells/ million PBMC (IQR 0-912 CD4+ T-cells/ million PBMC) at week 4 and then increased again to 900 CD4+ T-cells/ million PBMC (IQR 22-2794) at week 12. The HCV-specific CD8+ T-cells declined from 1912 to 103 CD8+ T-cells/ million PBMC (IQR 0-1144 CD8+ T-cells/ million PBMC) at week 4 and 173 CD8+ T-cells/ million PBMC (IQR 0-1995 CD8+ T-cells/ million PBMC) at week 12 (figure 2A).
In genotype 3 infected patients, the decline in the first 4 weeks was more pronounced, parallel to the steeper decline in viral load (figure 2C). The HCV-specific CD4+ T-cell responses declined from 922 to 214 CD4+ T-cells/ million PBMC (IQR 58-350 CD4+ T-cells/ million PBMC) at week 4 and decreased further to 50 CD4+ T-cells/ million PBMC (IQR 33-70) at week 12. HCV-specific CD8+ T-cells declined from 658 to 517 CD8+ T-cells/ million PBMC (IQR 307-747 CD8+ T-cells/ million PBMC) at week 4 and decreased further to 243 CD8+ T-cells/ million PBMC (IQR 24-372 CD8+ T-cells/ million PBMC) at week 12 (figure 2B).
The median viral load decline in the first 4 weeks of treatment was lower in genotype 1 infected patients (2.11 (IQR 1.66-3.56)) than in genotype 3 infected patients (3.10 (IQR 2.15-4.39), p=0.17), in the first 12 weeks of treatment this was 3.62 (IQR 2.13-4.46) and 4.50 (3.49-4.89), respectively (p=0.15; figure 2C).
When studying in more detail HCV-specific CD4+ and CD8+ T-cell responses in HCV genotype 1 infected individuals we observed that these responses could still be detected (i.e., ≥200 IFN-γ producing T-cells/ million PBMC) after 12 weeks of treatment in 5 and 3 patients, respectively (figure 3A and B). This in contrast to HCV genotype 3 infected patients, where in all individuals especially HCV-specific CD4+ T-cells had declined to undetectable levels (Pearson χ^2 p=0.037; figure 4A and B). The total HCV-specific CD4+ and CD8+ T-cell response at week 12 was lower for genotype 3 infected patients compared to genotype 1 infected patients (median 50 and 900 IFN-γ pro-

Figure 3: Decline in HCV genotype 1 specific CD4+ and CD8+ T-cell magnitude and breadth.



A: Fate of HCV-specific CD4+ cells per study participant as measured after 12-day expansion at baseline (left panel), week 4 (middle panel) and week 12 (right panel).
B. Fate of HCV-specific CD8+ cells per study participant as measured after 12-day expansion at baseline (left panel), week 4 (middle panel) and week 12 (right panel).
C Log$_{10}$ viral load per study participant at baseline (left panel), week 4 (middle panel) and week 12 (right panel).

Figure 4: Decline in HCV genotype 3 specific CD4+ and CD8+ T-cell magnitude and breadth.

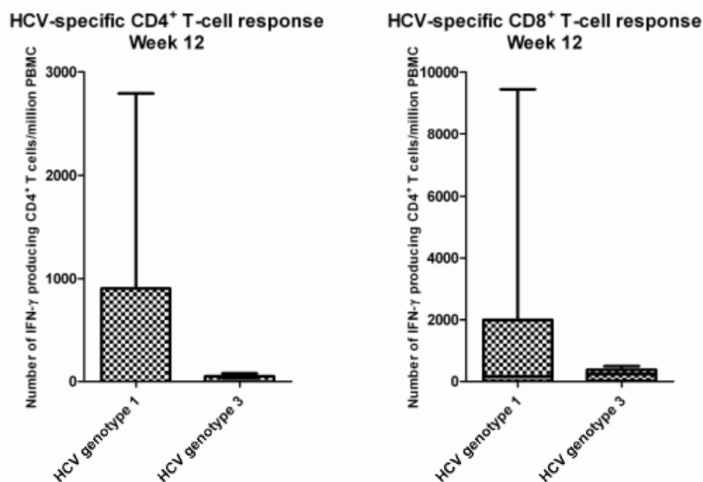


A: Fate of HCV-specific CD4+ cells per study participant as measured after 12-day expansion at baseline (left panel), week 4 (middle panel) and week 12 (right panel).

B. Fate of HCV-specific CD8+ cells per study participant as measured after 12-day expansion at baseline (left panel), week 4 (middle panel) and week 12 (right panel).

C Log_{10} viral load per study participant at baseline (left panel), week 4 (middle panel) and week 12 (right panel).

Figure 5: Lower HCV-specific CD4+ and CD8+ T-cell responses after 12 weeks of therapy in genotype 3 versus genotype 1 infected patients.



Median response and interquartile range of HCV-specific CD4+ (left panel) and CD8+ T-cell response (right panel) at week 12 after start of treatment.

ducing CD4+ T-cells, respectively), although this did not reach statistical significance (figure 5).

Breadth of the HCV-specific T-cell responses during treatment

Since in spontaneous viral clearance there is an association with breadth of the HCV-specific T-cell response, we evaluated how many HCV proteins were targeted per patient at each time point. At baseline a median of 2 proteins was targeted by HCV-specific CD4+ T-cell responses, while CD8+ T-cells targeted a median of 1.5 proteins (data not shown). The median number of targeted proteins was not significantly different between individuals infected with genotype 1 compared with those infected with genotype 3. The breadth of the response decreased in most individuals with a detectable HCV-specific CD4+ (n=13) or CD8+ (n=14) T-cell response at baseline (figure 3A and B; 4A and B).

Interestingly, patient C19 showed a decline in HCV-specific CD4+ T-cell responses at week 4, however the CD4+ T cell response rebounded to baseline level at week 12. This patient showed an initial viral response to treatment (with detectable HCV-RNA, but under the detection limit of the bDNA assay at week 4). However HCV-RNA was above the bDNA limit from 8 week onwards. In the genotype 3 group, while in all patients

HCV-specific T-cell responses decreased considerably, patient C17 still showed detectable T-cell responses at week 4. Although the T-cell response declined afterwards to undetectable levels, this patient had a relapse after treatment discontinuation (figure 2B).

Discussion

It was proposed that the second phase of the decline of HCV during treatment with pegIFN-alfa and ribavirin is caused by an increase of (HCV-specific) CTL [6]. To date, only very limited data are available on HCV-specific T-cell responses during HCV treatment, especially in HIV-infected individuals who have lower treatment success rates. Also in HCV mono-infected patients predictors for treatment outcome and the effect of HCV treatment on HCV-specific T-cells are still not well understood [19-25]. Therefore, we conducted a thorough analysis of the HCV-specific T-cell responses early during treatment in HIV/HCV coinfected patients.
Unfortunately, in our study we were not able to identify baseline predictors for SVR, which could be due to the heterogeneity of the study population, although we tried to make the group as homogeneous as possible by analyzing the effect of variation in HCV-therapy, genotype and HAART. In addition, we used an assay which measures multiple properties of a T-cell response which is associated with effectiveness. Using this assay, we were able to show kinetics of HCV-specific T-cells during therapy. One previous study in HCV mono-infected patients showed an augmentation of HCV-specific T-cell responses during treatment with pegIFN-alfa and ribavirin [26]. Although the used assay in our study has proven to be very sensitive for the detection of low frequencies of antigen-specific T-cells [14,17,27,28], we did not observe an increase in HCV-specific T-cell responses during treatment. In contrast, we observed a decrease of HCV-specific T-cells during treatment in nearly all patients. This suggests that the immunomodulatory properties of pegIFN-alfa and ribavirin on HCV-specific T-cells might actually be limited in forced viral clearance, at least in HIV/ HCV co-infected patients. Even more, we observed undetectable HCV-specific T-cell responses more often in genotype 3 compared to genotype 1 infected patients at week 12 of therapy. This implies that the immune response declines in parallel with the viral load, and that there is no boosting of the immune response in these patients. This is not completely unexpected, since HIV-specific T-cell responses have also been shown to decrease after initiation of HAART [29,30]. Capa *et al*. have also shown in a similar HIV/HCV coinfected population that HCV-specific T-cell responses decline during HCV treatment [31].

In our study we observed higher ALT levels in genotype 3 infected patients compared to genotype 1 infected patients. However, this seemed to be caused by lower ALT levels in patients treated with pegIFN-alfa–2a, who were all infected with HCV genotype 1. Possibly, this disparity is related to a different distribution of fibrosis and/or inflammation in both treatment studies. Unfortunately, we have only liver biopsies in 13/28 patients and therefore cannot exclude this. Also, different HAART regimens in both treatment studies could have led to the observed difference in ALT levels.
The experiments described in this study were performed on peripheral T-cells. It would be interesting to study the dynamics of intra-hepatic T-cells, especially since Neumann-Haefelin *et al* have shown an enrichment of HCV-specific T-cells in the liver compared to the peripheral compartment [32]. Thus, an apparent decline of HCV-specific T-cells in the blood might reflect preferential specific recruitment or homing of such cells into the liver. Although liver biopsies are performed in the Netherlands according to AASLD and national guidelines [10,33], a biopsy is not mandatory before start of treatment. Future studies might consider fine needle aspiration biopsies, which are much less invasive for the patient.

Conclusion

We did not observe an enhancement of HCV-specific T-cell responses in this cohort of HIV/ HCV genotype 1 and 3 co-infected patients. We did observe different kinetics of the HCV-specific T-cell responses in genotype 1 and 3 infected individuals, and these responses paralleled the decline in viral load. Therefore, augmentation HCV-specific T-cells does not appear to play a major role in forced viral clearance in HIV/ HCV co-infected patients.

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Chapter 8

Low dose of the novel caspase-inhibitor GS-9450 demonstrates no effect on peripheral T-cell apoptosis markers

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In preparation

Abstract

Background: Chronic hepatitis C virus (HCV) infection is characterized by development of liver fibrosis caused by a combination of liver inflammation, stellate cell activation and hepatocyte apoptosis. Increased rates of apoptosis and activated caspases have been shown in HCV-infected patients. GS-9450, a novel caspase-inhibitor, has demonstrated hepatoprotective activity in fibrosis/ apoptosis animal models. This study evaluated the effects of GS-9450 on peripheral T-cell apoptosis in chronic HCV-infected patients.

Methods: As sub study of the GS-US-227-0102, a double-blind, placebo-controlled phase 2a trial evaluating the safety and tolerability of GS-9450, apoptosis of peripheral CD4+ and CD8+ T-cells was measured using activated capase-3, activated caspase-8 and CD95 (Fas). Blood samples were drawn at baseline, day 14 after therapy and at 5 weeks off-treatment follow-up in the first cohort of 10 mg.

Results: In contrast to the placebo-treated patients, GS-9450 caused a 46% decrease in ALT-values from baseline to day 14 in all treated patients (118 to 64 U/L) rising again to 140 U/L (19%) at 5 weeks off-treatment follow-up. In all patients, during treatment and follow-up, percentages of activated caspase-3+, activated caspase-8+ and CD95+ on peripheral T-cells were not significantly influenced by placebo or GS-9450 treatment. Interestingly, compared to healthy controls, higher percentages of caspase-3+CD4+ T-cells (median 0.4% and 9.7%; p=0.002) and caspase-3+CD8+ T-cells (median 1.0% and 3.3%; p=0.01) were demonstrated in HCV-infected patients at baseline. Similarly, higher rates of CD95 and caspase-8 expression by CD4+ and CD8+ T-cells were seen in HCV-infected patients compared to healthy controls.

Conclusion: The low dose of caspase-inhibitor GS-9450 lowered ALT-values in all HCV-infected patients without affecting peripheral T-cells apoptosis rates. However, at baseline percentages of activated caspase-3+, activated caspase-8+ and CD95+ T-cells were higher in chronic HCV-infected patients compared to healthy controls.

Introduction

Persistence of the Hepatitis C Virus (HCV) in the liver leads to the development of liver fibrosis over the course of decades [1]. This process of liver fibrosis progression is a complex interplay between activated T-cells, activated stellate cells and apoptosis of injured hepatocytes [2]. Apoptosis or programmed cell death is a physiological phenomenon that occurs in most organs in the human body and is a key element in the host defense against viral pathogens [3]. The main signaling pathway to induce apoptosis in hepatic diseases and normal healthy livers is the death receptor pathway consisting of different receptors of the Tumor Necrosis Factor family being TNF-related apoptosis-inducing ligand (TRAIL), TNF-alpha and Fas (CD95/ APO-1) [4]. Fas ligand (FasL) is expressed by natural killer cells and cytotoxic T lymphocytes (CTLs, i.e. CD8+ effector T-cells) and Fas-FasL interaction is the main effector mechanism of CTLs inducing apoptosis of virus infected hepatocytes [5,6]. Recently, in a HCV genotype 1b transgenic mouse model an increased rate of apoptosis of peripheral CD4+ and CD8+ T-cells was noted when compared to healthy control mice [7]. Furthermore, this was associated with an up-regulation of FasL on the hepatocytes suggesting that the hepatic micro-environment, with up-regulation of FasL, promotes increased T-cell apoptosis and thereby contributes to viral persistence. Apoptosis is gradually being more recognized as an important factor in liver fibrosis development [8-10]. Caspases, proteolytic enzymes belonging to a family of intracellular cysteine proteases, play an important role in this apoptotic process. After interaction with the cell surface Fas-receptor, intra-cellular activation of death domains (FADD) result in proteolytic cleavage pro-caspase-8 into its active form caspase-8 [11]. This process leads down-stream to the cleavage of pro-caspase-3 into its active form caspase-3, the central protease in the apoptosis pathway. The role of apoptosis in chronic HCV is currently not well understood. Liver biopsy studies in patients with chronic HCV have shown an increased presence of apoptotic hepatocytes [12,13]. Moreover, the percentage of apoptotic hepatocytes, demonstrated immunohistochemically as caspase-3 positive cells, has been shown to correlate with the amount of liver fibrosis [14,15].

GS-9450 is an irreversible inhibitor of caspase-8, -9, and -1 and has demonstrated hepatoprotective activity in both fibrosis assays and apoptosis animal models (unpu-lished). Furthermore, a phase-1 trial dosing GS-9450 for 14 days in healthy volunteers proved to be safe and well tolerated [16]. Recently, the results of a phase-2a study, evaluating the safety and tolerability of GS-9450 have been presented [17]. However, since a baseline liver biopsy was not mandatory for entering the phase-2 study and in the previous phase-1 study the effect of GS-9450 on the T-cells was not evaluated, as a

sub study during the phase-2 study we analysed the effects of GS-9450 on peripheral T-cell apoptosis during GS-9450 therapy.

Patients and Methods

Clinical study

The GS-US-227-0102 study was a phase 2a trial evaluating the safety and tolerability GS-9450, a potent irreversible inhibitor of caspase-8, -9, and -1. This novel drug is currently developed by Gilead Inc. (Durham, NC, USA). Ascending doses of GS-9450 were evaluated in this randomized placebo-controlled study [17]. For the first cohort of 10 mg GS-9450, 8 patients were included from the Netherlands, 6 receiving GS-9450 and 2 receiving placebo. Inclusion was based on the presence of a chronic HCV infection and ALT or AST > 1.5 x the upper limit of normal (ULN; in our centre 35 U/L). Furthermore, patients had to have previously failed conventional anti-HCV therapy, were unable to tolerate it, or had contraindications for treatment with (peg)interferon-alfa/ribavirin. Important exclusion criteria were decompensated liver disease or evidence of hepatocellular carcinoma (based on liver biopsy within the previous 2 years), coinfection with hepatitis B virus (HBV) or human immunodeficiency virus (HIV) and current or near future pregnancy. A laboratory sub study of this trial was designed for this first cohort, evaluating the effects of GS-9450 on peripheral T-cells.

PBMC processing

Peripheral blood (approximately 30 ml) was collected at baseline (i.e. day 0 of the study), at week 2 (i.e. day 14 of the study) and at week 7 (i.e. 5 weeks off-treatment follow-up). Within 24 hours, peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll-Hypaque density gradient centrifugation. Cells were re-suspended in RPMI 1640 (Gibco Life Technologies, Breda, the Netherlands) to which 20% fetal calf serum and 1% penicillin and streptomycin were added (hereafter called medium). Except for the caspase-8 experiments, all assays were performed on fresh PBMC. As controls, blood of 5 healthy individuals (HC) was requested from the local blood bank (screened and negative for hepatitis B, HIV and hepatitis C).

Apoptosis analysis by FACS

PBMC in medium were washed and resuspended in PBA (phosphate buffered saline containing 0.16% BSA and 0.1% sodium-azide) to a concentration of 2 million PBMC/ ml and divided in aliquots of 200 uL. Cells were incubated for 15 minutes at 4 °C with 3 different sets of antibodies against i) activated caspase-3 (FITC, BD Biosciences, San José, CA, USA), ii) activated caspase-8 (FITC, Biovision, Mountain View, CA, USA) and iii) activation/ subsets (CD38 (PE Cy7, eBioscience) and HLA-DR (FITC, eBioscience) / (CD27 (FITC, BD) and CD45RO (APC, BD)). Added to all these 3 different sets were antibodies against CD3 (pacific Blue,), CD4 (PE Cy7, BD), CD8 (APC Cy7, eBioscience, San Diego, USA) and CD95 (PE, BD). Cells for caspase-expression were washed and permeabilized with permalysis buffer (BD) and stained with antibodies for activated caspase-3 or activated caspase-8 for 15 minutes at 4 °C. After washing, cells were fixed with Cellfix (BD) and at least 200,000 cells were acquired by Fluorescence Activated Cell Sorting (FACS, LRSII, BD).
Using the FACSDiva software (BD), we constructed a CD4 PE Cy7 and CD8 APC Cy7 scatter plot, gated CD3+ T-cells, in order to identify all CD3+CD4+ and CD3+CD8+ T-cells. Within these populations we gated on activated caspase-3 or -8 and CD95 to obtain the percentage of caspase or CD95 expressing CD3+CD4+ or CD3+CD8+ T-cells.

Statistical analysis

Continuous data are presented as median values (with interquartile range (IQR)) and analyzed non-parametrically using a Mann-Whitney U-test for baseline values and a Friedman test for longitudinal analysis. Categorical variables are given as numbers of cases (percentage). A p-value ≤0.05 was considered as statistically significant and all tests used were two-sided. All data were analyzed using GraphPad Prism (version 5.0 for Windows, GraphPad Software, San Diego, USA).

Results

Eight patients were randomized to receive either 10 mg GS-9450 (n=6) or placebo (n=2). Patient characteristics are given in table 1 showing that the majority of patients were male (75%) with a median age of 50 years. As mandatory for inclusion, all patients had elevated ALT-values with a median of 118 U/L. Baseline HCV viral loads varied

from 1,500 IU/ml to 1,350,000 IU/ml with a median of 409,000 IU/ml. Since inclusion of all genotypes was allowed, 4 patients (50%) were infected with genotype 1, 2 (25%) with genotype 4 and 2 (25%) with genotype 3. Treatment with either GS-9450 or placebo was generally well tolerated with fatigue, headache and nausea being the most reported side-effects.

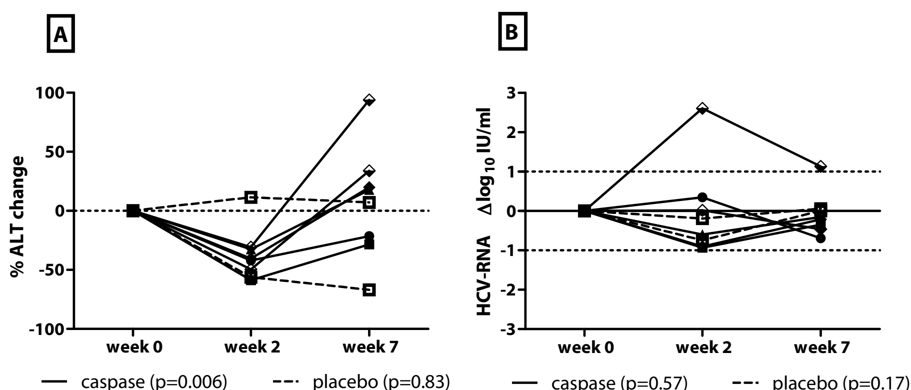
The effectiveness of 10 mg of GS-9450 was studied in a 14-day dosing trial. Irrespective of the allocated treatment group, ALT-values declined in 7 of 8 study subjects during the first 14 days of the study (figure 1A). In patients receiving GS-9450, median ALT-values dropped from 118 U/L to 64 U/L (-46%) rising again to 140 U/L (+19%) at week 7 (i.e. 5 weeks of treatment follow-up)(p=0.006; Friedman test). In one of the patients receiving placebo ALT-values did not change during the treatment period. In the other patient, ALT-values decreased from 178 U/l at baseline to 78 U/L (-56%) at day 14 and further declined to 59 U/L (-67%) at week 7. During the 14-day trial HCV viral loads remained stable within 1 $\log_{10}$ in all but one patient (figure 1B). This patient

Table 1: Characteristics of the patients treated with either GS-9450 or placebo

Patient	Age (years)	sex	HCV-RNA (IU/ml)	Genotype	ALAT (U/L)	Side-effects	Treatment
1001	49	male	438,000	1b	121	dizziness and nausea	10 mg
1002	56	female	1,200,000	4a/4c/4d	109	fatigue, somnolence and feeling hot	10 mg
1003	43	male	39,000	4a/4c/4d	178	hyperhydrosis, affect liability, flank pain and tooth infection	placebo
1004	51	female	894,000	3a	209	headache, palpitations, dry skin, flank pain and parosmia	10 mg
1005	40	male	196,000	1a	171	vomiting, affect liability, nausea, headache, sore throat, rhinitis and fatigue	10 mg
1006	40	male	380,000	3a	115	affect liability	10 mg
1007	54	male	1,500	1a/1b	65	headache and gastro-enteritis	10 mg
1008	56	male	1,350,000	1a	70	nausea, headache, fatigue and back pain	placebo
median (IQR)	**50** (51-56)	NA	**409** (78,250-1,124,000)	NA	**118** (80-176)	NA	NA

NA = not applicable

Figure 1: Longitudinal analysis of ALT and HCV-RNA during therapy.



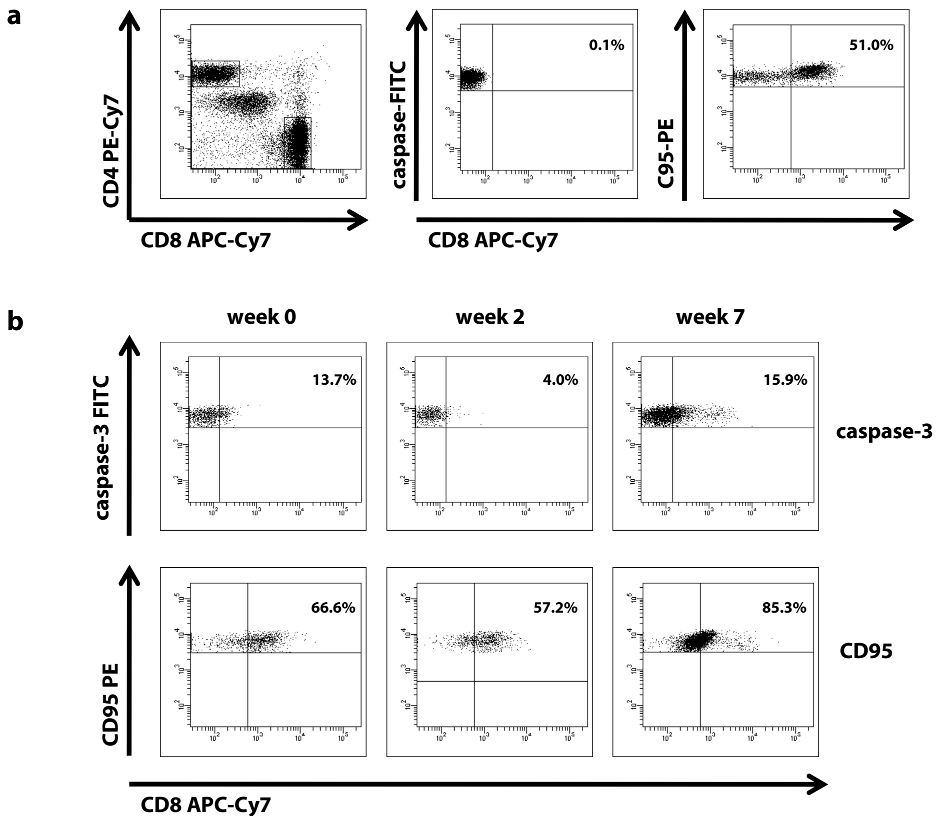
Changes (%) in plasma ALT (panel A) and change in HCV-RNA values (panel B) from baseline during the duration of the study. On the y-axis are the % change in ALT (U/l) and the Δ HCV-RNA (log_{10} IU/ml) depicted. On the x-axis the weeks of the study are shown. Patients treated with the caspase-inhibitor GS-9450 are represented by the solid black lines and black dots and those receiving placebo are represented by the black dotted lines and open squares.

experienced a 2.61 Log_{10} increase in HCV-RNA during the 14-day treatment period which declined but did not return to baseline value during follow-up.

Baseline expression of caspase-3, caspase-8 and CD95 on peripheral T-cells

Since a mouse model has suggested an interaction between HCV-infected hepatocytes and T-cell apoptosis contributing to viral persistence and liver biopsies were not performed at baseline, we were interested in the effects of GS-9450 on peripheral T-cells in chronic HCV patients in this study. Since the caspase-inhibitor GS-9450 targets activated caspase-8 and activated caspase-3 is the main caspase protein in the apoptosis pathway, these were chosen for the analysis. In order to be able to interpret the expression of activated caspase-3, activated caspase-8 and CD95 by peripheral T-cells, we first analyzed these markers in 5 healthy controls (HC). A representative flow cytometry dotplot of a healthy control demonstrating both CD4+ and CD8+ populations as well as the expression of activated caspase-3 and CD95 on CD8+ T-cells is shown in figure 2A. Furthermore, expression of activated caspase-3 and CD95 is shown on CD8+ T-cells of a patient (figure 2B). The median percentage of intracellular activated caspase-3 expression in these HC was 0.4% (IQR 0.1-1.1) for CD4+ T-cells and 0.1% (IQR 0-0.9) for CD8+ T-cells. Compared to these HC, higher rates of activated caspase-3 were detected in the HCV-infected patients in CD4+ (median 9.7% with IQR 6.2-25.5; p=0.002) and CD8+ (median 3.3% with IQR 1.5-8.5; p=0.01) T-cells (figure

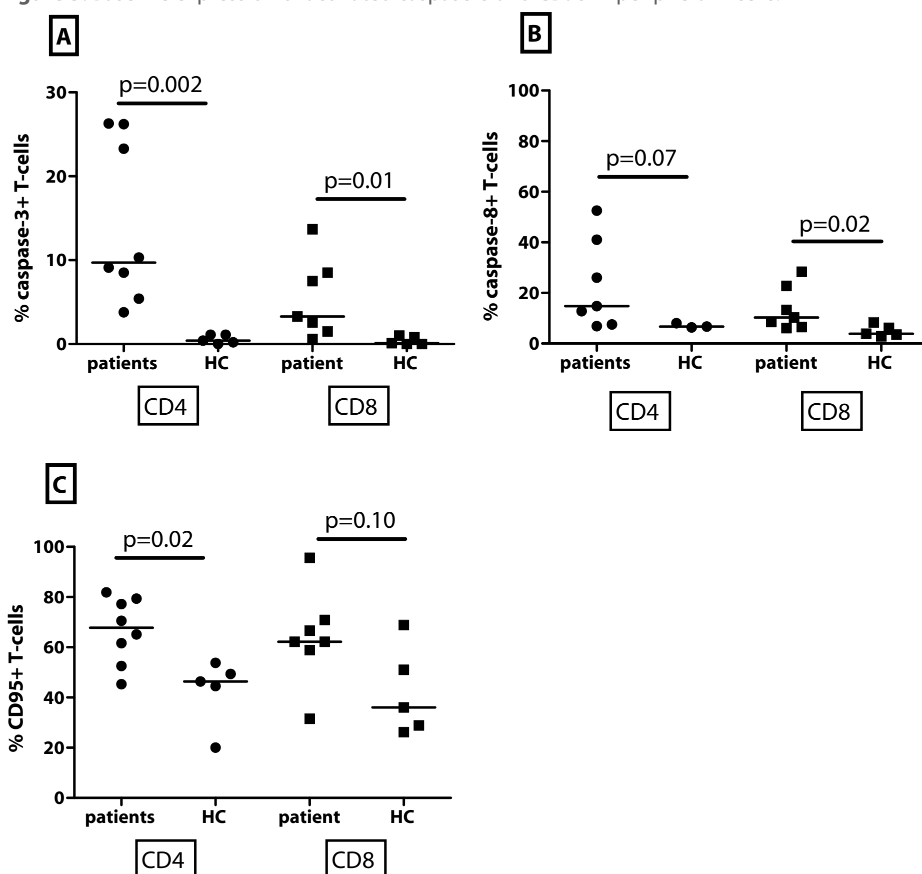
Figure 2: Flow cytometry analysis of caspase-3 and CD95 expression.



Panel A: representation of a scatter plot defining a patient's CD4+ and CD8+ T-cell populations on the left. The middle and right plots represent expression of activated caspase-3 and CD95 in a healthy control's CD8+ T-cells (x-axis).
Panel B: Flow cytometry dot plot showing a longitudinal expression of activated caspase-3 and CD95 in a chronic HCV-infected patient treated with the caspase-inhibitor GS-9450. The upper row depicts the caspase-3 expression while the lower row shows the CD95 expression in CD8+ T-cells (y-axis). From left to right, the study weeks 0, 2 and 7 are depicted.

3A). Expression of activated caspase-8 by CD8+ T-cells was also significantly higher in chronic HCV-patients compared to HC (median 10.3 (IQR 6.5-22.8) and 3.90 (IQR 3.2-7.3); p=0.02) with a trend towards significance for the CD4+ T-cells (14.8 (IQR 7.5-41.0) and 6.7 (IQR 6.4-8.0); p=0.07; figure 3B). Finally, surface expression of CD95 on CD4+ T-cells was higher in the patients compared to HC (median 67.8% (IQR 54.8-78.9) and 46.4% (IQR 32.3-51.6); p=0.002; figure 3C). However, this difference was not significant in CD8+ T-cells between patients (median 62.2% (IQR 58.9-70.9)) and HC (median 36% (IQR 27.6-60); p=0.10).

Figure 3: Baseline expression of activated caspase-3 and CD95 in peripheral T-cells.



Comparison of activated caspase-3 (panel A), activated caspase-8 (panel B) and CD95 (panel C) expression on peripheral T-cells between patients in the GS-9450 study at baseline and healthy controls (HC). On the y-axis the percentages of activated caspase-3, activated caspase-8 or CD95 are depicted. The black dots (•) represent CD4+ T-cells and the black squares (■) the CD8+ T-cells. Of 1 patient FACS analysis of CD8+ T-cells failed resulting in 7 patients being evaluated. CD4+ T-cells of 2 HC were unavailable for caspase-8 analysis.

Longitudinal analysis of caspase-3 and CD95 expression in peripheral T-cells

Next, we analyzed the expression of activated caspase-3, activated caspase-8 and CD95 during the 14-day dosing period. Percentages of activated caspase-3+ CD4+ T-cells decreased in all patients (figure 4A). At 5 weeks off-treatment follow-up, in the majority of patients activated caspase-3 expression returned to baseline values or even increased in the 2 patients receiving placebo during the dosing period. Activated

caspase-3 expression in CD8+ T-cells was more variable during the 14-days on treatment, decreasing in 4 of 8 patients (50%) and increasing in the others (figure 4B). Five weeks after discontinuation, expression of activated caspase-3 by CD8+ T-cells returned to baseline values in most patients.

Since analysis of activated caspase-8 expression was evaluated on frozen PBMC, we investigated the effects of freezing/ thawing on activated caspase-8 expression. Comparison of activated caspase-8 expression between frozen and fresh PBMC in healthy controls did not show significant differences between the two methods (p=0.64; Wilcoxon signed rank test). Activated caspase-8 expression in CD4+ T-cells decreased in all but one patient, returning to baseline in 3 patients or declining further in another 4 patients at week 7 (figure 4C). Similarly, expression of activated caspase-8 by CD8+ T-cells showed a decrease in 5 of 8 patients returning to baseline in 4 of them (figure 4D). A mostly similar expression of activated caspase-3 and activated caspase-8 was noted in both placebo-treated and GS9450-treated patients suggesting no effect of the caspase-inhibitor on these apoptosis markers in peripheral T-cells during therapy. Differences in expression of CD95 by CD4+ T-cells during the study were less pronounced with 5 patients showing minimal changes while 3 patients showed a decrease in CD95 expression (figure 4E). In CD8+ T-cells CD95 expression decreased in 5 of 8 patients from baseline to week 2 and even further at week 7 (figure 4F).

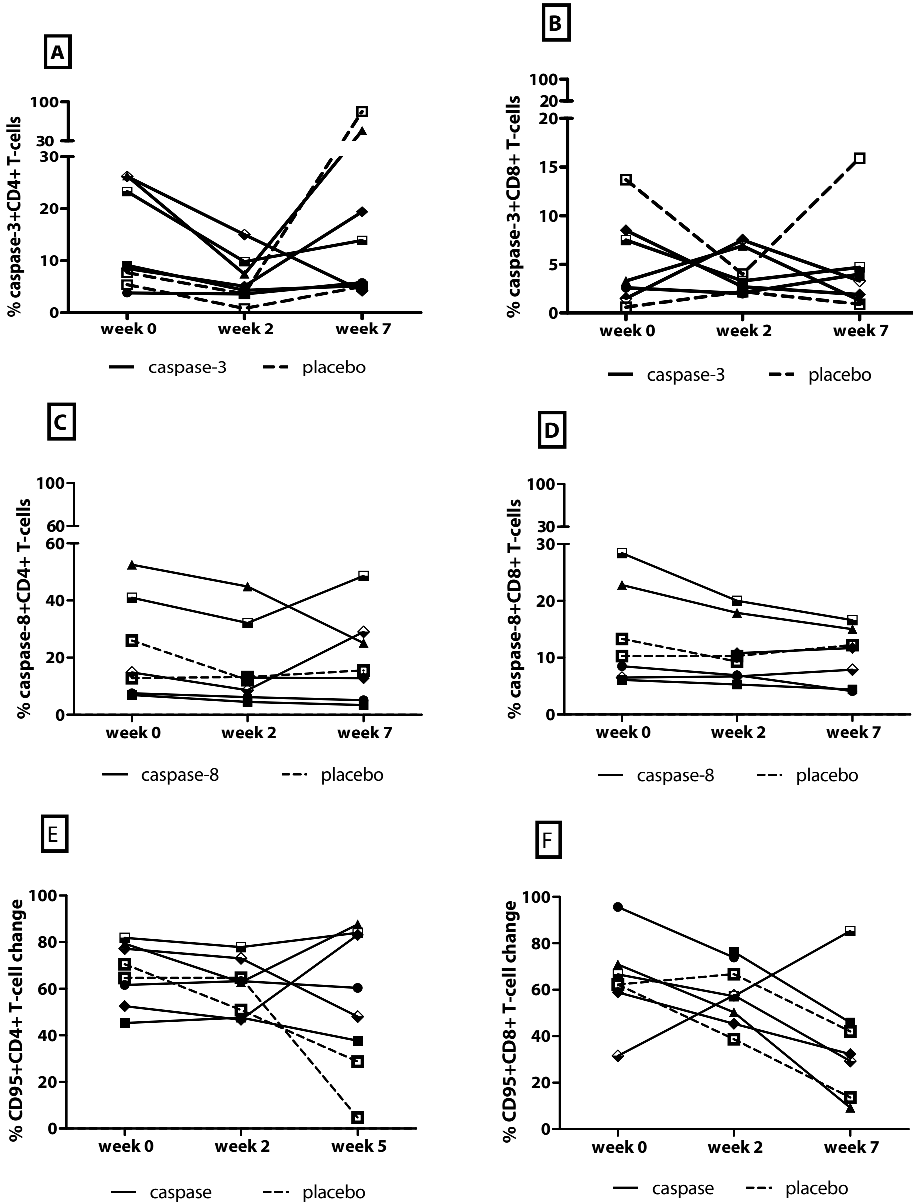
There was a significant correlation between the expression of activated caspase-3 and activated caspase-8 (spearman r=0.40 and p=0.008) or CD95 (spearman r=0.34 and p=0.02) when CD4+ and CD8+ T-cells at weeks 0, 2 and 7 were analysed together.

Discussion

In this study we evaluated the effects of the caspase-inhibitor GS-9450 on peripheral T-cells during a 14-day treatment period. Although treatment with GS-9450 resulted in lower ALT-values, it did not affect either the HCV viral load or the peripheral T-cells apoptosis rates. However, we demonstrate that at baseline, percentages of activated caspase-3+, activated caspase-8 and CD95+ T-cells are higher in chronic HCV-infected patients compared to HC.

The effect of GS-9450 on lowering the ALT-value was strong (nearly 50% reduction) suggesting a decrease in apoptotic hepatocytes and thus prevention of release of aminotranferase enzymes. In addition, cessation of alcohol consumption was a study requirement and might explain in part the ALT-decrease in these patients. This is supported by the observation that the ALT-values of one placebo-treated patient

Figure 4: Longitudinal analysis of activated caspase-3, caspase-8 and CD95 expression.



Longitudinal analysis of CD4+ and CD8+ T-cells expressing activated caspase-3+ (panel A and B), activated caspase-8 (panel C and D) and CD95+ (panel E and F) during therapy. On the y-axis percentages of activated caspase-3, activated caspase-8 or CD95 are shown and on the x-axis the weeks of the study are depicted. The black solid lines (—) represent the patients receiving GS-9450 and the black dotted lines (--) represent those taking placebo. One patient's CD8+ T-cells at baseline (see figure 3) and another patient's CD8+ T-cells at week 7 were not available for analysis.

decreased during therapy and even further declined after placebo discontinuation at the time ALT-values in GS-9450 treated patients increased again. A competitor caspase-inhibitor, of which development recently has been stopped, showed a similar decline in ALT-values (in a dose-depended matter) in chronic HCV patients in both a 14-day and a 12-week dosing trial [18,19]. In all these caspase-inhibitor studies an overshoot of aminotransferase activity occurred after discontinuation of the drug. This could possibly be prevented by tapering instead of abrupt discontinuation and should be investigated in future studies.

Treatment with GS-9450 did not affect T-cell apoptosis markers, as measured by activated caspase-3, activated caspase-8 and CD95 expression. This might be explained by the pre-clinical animal and in vitro studies, demonstrating the hepatic selectiveness of GS-9450. However, the exact mechanism of hepatic uptake of the drug has not been unraveled to date. Another possible explanation for this absence could be that the dose of 10 mg GS-9450 was too low to exert an effect on peripheral T-cell apoptosis.

An interesting observation in this study was the significant differences in activated caspase-3, activated caspase-8 and CD95 expression by peripheral T-cells between chronic HCV patients and healthy controls. This could not be explained by a difference in T-cell activation markers (HLA-DR/ CD38) or T-cell phenotypes (CD27/ CD45RO) since no difference between HC and HCV-infected patients could be demonstrated for these T-cell features (data not shown). The reason for the higher rate of apoptosis in chronic HCV patients is currently unknown. It has been shown by Toubi et al. [20] that both spontaneous and dexamethasone-induced apoptosis was increased in chronic HCV-infected patients compared to healthy individuals. They also showed that dexamethasone-induced apoptosis of peripheral T-cells was correlated with increased ALT-values and decreased NF-κB levels. Furthermore, experimental HCV murine models have suggested that both HCV core and non-structural proteins enhance apoptosis of activated CD4+ and CD8+ T-cells via up-regulation of Fas-FasL interaction [7,21]. This may suggest that HCV is capable of down-regulating specific immune responses by inducing T-cell apoptosis and therefore contributing to persistence of a chronic HCV infection.

This study has several limitations. First, as staging of liver fibrosis was not part of the study, correlation between liver fibrosis and apoptosis-markers could not be performed. This would have been of interest given the earlier publications demonstrating a relation between intra-hepatic caspase expression and the degree of fibrosis [14]. When a correlation between the stage of liver fibrosis and peripheral T-cell apoptosis at baseline could be demonstrated, this might be a valuable marker for the

evaluation of liver fibrosis. However, no correlation was shown between ALT-values and either activated caspase-3, activated caspase-8 or CD95 expression (data not shown). Furthermore, the number of patients in this study was small possibly explaining the absence of significance in baseline CD95+CD8+ T-cells between HCV-infected patients and HC. Finally, the caspase-8 analyses were performed on frozen T-cells. Freezing of cells is known to induce apoptosis in cells [3,14]. However, the differences in activated caspase-8 expression between fresh and frozen PBMC was limited (data not shown) and longitudinal changes in expression of activated caspase-3 and activated caspase-8 was similar. Therefore, we think that the effects of freezing in this study is negligible. However, further studies on the role of activated caspase-3 and activated caspase-8 by peripheral T-cells is needed.

Conclusion

During a 14-day dosing period, the caspase-inhibitor GS-9450 decreased ALT-values without affecting the expression of activated caspase-3, activated caspase-8 and CD95 expression by peripheral T-cells. However, expression of activated caspase-3, activated caspase-8 and CD95 were found to be higher in patients with chronic HCV compared to healthy controls. Taken together, by inhibiting hepatocyte apoptosis, progression of hepatic fibrosis could possibly be halted and therefore could be of value in a subgroup of chronic HCV-infected patients with fibrosis without alternative therapeutic options.

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Chapter 9

General discussion and future perspectives

Introduction

In 2004, when starting the research that has led to this thesis, the first pre-clinical studies with novel anti-Hepatitis C Virus (HCV) inhibitors were published and experts in the field predicted the end of peginterferon-alfa (pegIFN-alfa) and ribavirin therapy within the next coming years. Currently, we are at a turning point in history eagerly awaiting the introduction of the HCV protease inhibitors telaprevir and boceprevir into clinical practice [1]. Until proven superior though, pegIFN-alfa/ ribavirin combination therapy remains the only option for the treatment of HCV. Intriguingly, despite its use for many years, still major uncertainties exists about the working mechanism of both pegIFN-alfa and ribavirin [2,3]. This is especially important for patients with "hard-to-treat" genotype 1 or 4 with or without the human immunodeficiency virus (HIV) coinfection. Therefore, the focus of this thesis is on these "hard-to-treat" patients evaluating factors that possibly influence the outcome of therapy.

Acute hepatitis C

In contrast to the high sustained virological response rates (SVR, i.e. undetectable HCV-RNA 24 weeks after treatment discontinuation) in acute HCV mono-infected patients, the optimal treatment regimen for acute HCV in HIV-infected patient is being debated [4,5]. Although it was hypothesized in **chapter 2** that pegIFN-alfa monotherapy would be as effective as pegIFN-alfa/ ribavirin therapy in HIV-infected patients with acute HCV, the cohort study (**chapter 3**) demonstrated low SVR rates and high rates of non-response to pegIFN-alfa monotherapy. This resulted in the recommendation that combination therapy with pegIFN-alfa/ ribavirin is the preferred treatment regimen in HIV-infected patients with acute HCV (**chapter 4**). Defining the optimal therapeutic regimen for acute HCV in HIV-infected patients is very important given the rising incidence of acute HCV among HIV-infected man having sex with men (MSM) in many different countries worldwide [6-12]. Phylogenetic analyses resulted in the discovery of large international networks in which high risk sexual behavior (fisting and unprotected anal intercourse) and ulcerative sexual transmitted diseases were risk factors for acute HCV transmission (i.e. permucosal transmission) [10,13]. This is a new phenomenon compared to the traditional risk groups, i.e. iv drug users sharing contaminated needles and healthcare workers at risk of needle stick injuries. Opposing routes of transmission (permucosal via the rectum versus directly into the bloodstream) might activate the immune system in a different way possibly explaining the lower SVR rates

even with pegIFN-alfa/ ribavirin combination therapy in HIV-infected patients with acute HCV compared to the high SVR rates with pegIFN-alfa monotherapy in acute HCV mono-infected patients.

Priming of the immune response

To date most studies have focused on the cellular immune response during an acute HCV infection showing that successful clearance of acute HCV-infection is associated with a strong and vigorous HCV-specific CD4+ and CD8+ (i.e. cytotoxic T lymphocytes or CTLs) T-cell response targeting multiple HCV epitopes [14-17]. However, only a small number of studies have evaluated the initial priming of CD8+ T-cells. Two distinct mechanisms have been proposed; either T-cells are not primed at all (primary CD8+ T-cell failure) or T-cells are primed but vanish quickly (CD8+ T-cell exhaustion) [18]. In case of priming of naive CD8+ T-cells, the important question is whether they are primed in the liver or in the local lymph nodes.
Both HCV and HIV are RNA-viruses transmitted via sexual intercourse. Since many studies have investigated the early priming of the immune response after HIV transmission, this is used as model to try and explain CD8+ T-cell priming in acute HCV infections. After HIV has crossed the mucosal barrier, dendritic cells (DCs) in the submusoca transport the virion via its cell surface receptor DC-SIGN to the regional lymph nodes where priming of the cellular immune response takes place [19]. A supposedly similar mode of transport might occur after permucosal infection of acute HCV given the reported binding of HCV envelope proteins E1 and E2 to the DC receptor DC-SIGN [20]. In this situation, presentation and priming to naive CD8+ T-cells will take place in the local regional lymph node after which the T-cells migrate to the site of 'inflammation'. This is distinctively different from the priming of the immune response in patients acquiring their acute HCV infection from iv drug use. Then the virions are injected directly into the bloodstream after which binding to HCV-specific receptors on the hepatocytes occurs. Currently, it is unclear how effectively hepatocytes, functioning as antigen presenting cells (APCs), are capable of priming CD8+ T-cells [21] or whether other APCs like Kupffer cells, liver sinusoidal endothelial cells (LSEC) and DCs play a role in CTL priming. Likewise, it is unknown whether differences in priming result in a delayed CTL response leading to differences in outcome of the acute infection.
Clinical studies evaluating the spontaneous clearance rate of acute HCV did not report a difference between populations composed of iv drug users or HIV-positive MSM [15,22-26]. This either suggests that priming of the immune response does not influence the outcome of an acute HCV infection or that a difference in priming is leveled

by other factors known to influence spontaneous viral clearance like demographic characteristics (ethnicity, gender and age) or the recently discovered host-related factors like IL-28B polymorphisms [27].

IL-28B polymorphisms and outcome of therapy

An interesting development is the recent discovery of several single nucleotide polymorphisms (SNPs) in the interleukin-28B (IL-28B) gene, located on chromosome 19 [28]. It is now firmly established in chronic HCV genotype-1 infected patients as a strong predictor for treatment-induced clearance [29,30] or non-response [31,32] to pegIFN-alfa/ ribavirin therapy. Evidence of the impact of IL-28B SNPs on the outcome of acute HCV infection is emerging, but studies are contradictory. Thomas et al. analyzed the SNP rs12979860 in a cohort of 388 patients who spontaneously cleared the HCV infection and compared this with a cohort of 620 HCV-infected patients in which the HCV-infections persisted [33]. Patients carrying the C/C-genotype were 3 times more likely to clear the HCV infection compared to patients with the C/T and T/T genotypes (combined odds ratio (OR) =0.33, $P<10^{-12}$). In both groups of patients (clearance and persistence) a minority of patients were coinfected with HIV (19.3% and 24.4% respectively) but by multivariate analysis confounding of HIV was excluded suggesting a similar positive role in spontaneous viral clearance for the SNP rs12979860 C/C genotype in HIV-infected patients with acute HCV. Together with the findings of the cohort study described in **chapter 3**, two recent studies could not confirm this positive association between the rs12979860 C/C-genotype and treatment outcome [34,35].
Importantly, our study is the first to report on the influence of another SNP rs8099917 in HIV-infected patients with an acute HCV infection, showing that the presence of the G-allele (G/G and G/T) is associated with non-response to pegIFN-alfa monotherapy. Although, this rs8099917 SNP has also been found to be associated with non-response in chronic HCV-infected patients treated with pegIFN-alfa/ ribavirin, further studies are needed to determine the value of IL-28B SNPs in predicting the outcome of acute HCV in HIV-infected patients [31,32].
The IL-28B gene encodes for interferon-λ3 (IL-λ3), a relatively newly described cytokine, together with IL-λ1 (IL-29 gene) and IL-λ2 (IL-28A gene) belonging to the IL-λ family [36]. Via binding of IFN-λ to its receptor, the JAK-STAT pathway is activated leading to induction of antiviral, anti-proliferative, anti-tumor and immune responses [37]. Compared to IFN-alfa, IFN-λ is a weak antiviral cytokine in vitro and its contribution to viral clearance is unknown [38]. A recent publication by Hondo et al. demonstrated that in chronic HCV-infected patients with a non-response to pegIFN-alfa/ ribavirin

therapy, up-regulation of interferon stimulating genes (ISG) was associated with the G-allele of the SNP rs8099917 (figure 2). Unfortunately, due to ethical considerations, it's very difficult to repeat these investigations in patients with acute HCV since there is no stringent reason to perform a biopsy. However, the observation by Hondo et al. highlights the importance of host-related factors (IL-28B and ISG regulation) in the outcome of pegIFN-alfa/ ribavirin therapy.

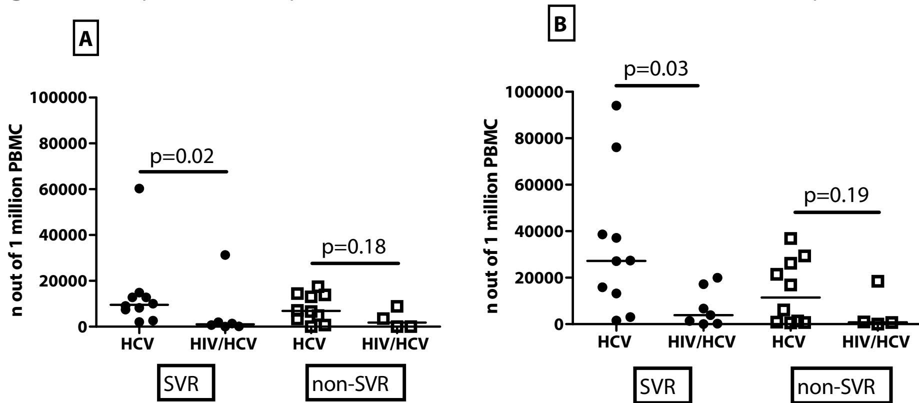
Chronic Hepatitis C

In contrast to the fairly high SVR rates achieved with treatment for acute HCV, successful forced viral clearance with pegIFN-alfa/ ribavirin for chronic HCV genotypes 1 and 4 is lower around 30-50% depending on the presence of an HIV-coinfection [39-42]. It has been suggested that a higher frequency of regulatory T-cells [43], HCV escape mutations [44] and continued exposure of HCV-antigens to T-cells leading to exhaustion [45] are explanations for the low SVR rates in chronic HCV-infection. The latter, i.e. responsiveness of HCV-specific T-cell responses at baseline and during pegIFN-alfa/ ribavirin therapy was the subject of **chapters 6 and 7**. Numerous studies already evaluated a possible association between outcome of therapy and HCV-specific T-cells both at baseline and during treatment [46-56]. Although both studies described in this thesis did not to show such an association they were distinctive because of the use of a novel sensitive expansion assay.

The role of interleukin-2 in HCV

In **chapter 6** we compared the sensitivity of the [^{3}H]-thymidine assay and our novel 12-day expansion assay clearly demonstrating a superior sensitivity of the latter assay. Its strength is its capability to measure both the IFN-γ production and the proliferative capacity of HCV-specific CD4+ and CD8+ T-cells simultaneously, allowing a more comprehensive analysis of the HCV-specific T-cell response. Although total HCV-specific T-cell responses were not related to outcome of pegIFN-alfa/ ribavirin therapy (SVR or RVR), strong HCV-specific T-cell responses were detected *in vitro* in several patients. The question is why these HCV-specific T-cells are capable of *in vitro* proliferation while *in vivo* they fail to clear the HCV infection. The most widely used explanation is the so-called "exhaustion" of T-cells due to chronic antigen stimulation [57]. This phenomenon of T-cell exhaustion was first described using the lymphocytic choriomeningitis virus model (LCMV) showing that chronic antigenic stimulation led

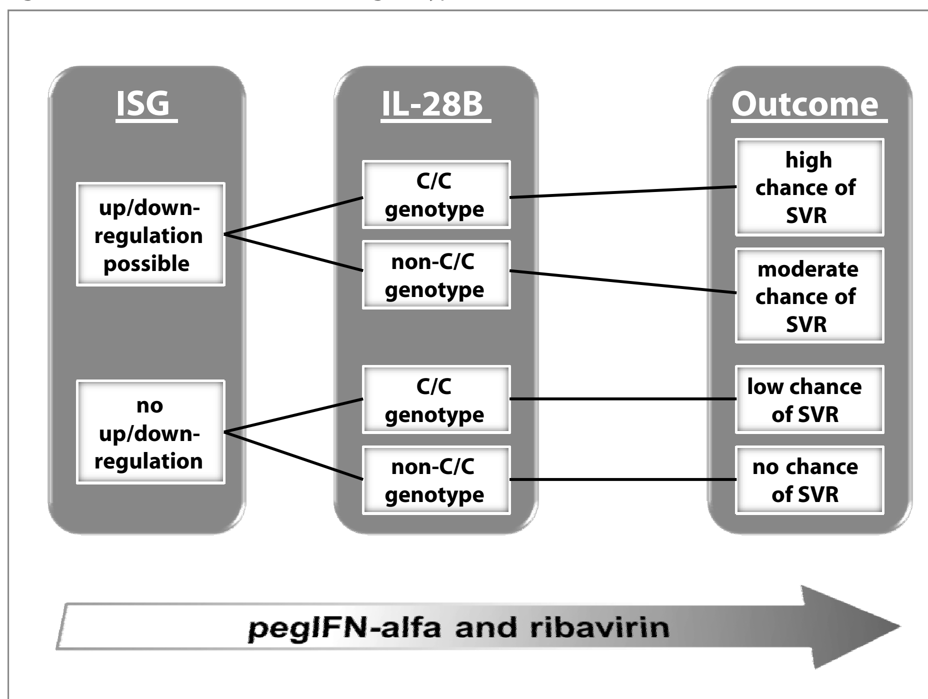
Figure 1: HCV-specific T-cell responses in HVC mono-infected and HIV/ HCV coinfected patients.



Comparison of both the CD4+ and CD8+ HCV-specific T-cell responses from HCV mono-infected and HIV/ HCV coinfected patients in the Privicop-study divided by achievement of a SVR or non-SVR (data merged from studies described in chapters 6 and 7).

to the reduced production of interleukin-2 (IL-2) subsequently followed by loss of T-cell function as measured by a reduced capacity to produce tumor necrosis factor (TNF) and IFN-γ [58]. Loss of IL-2 production by T-cells was also demonstrated in chronic HCV-infected patients [59,60]. Therefore, it could be hypothesized that adding IL-2 during the 12-day expansion process lifts the ban on HCV-specific T-cell proliferation thereby restoring IFN-γ production. This would imply that previously used proliferation assays like carboxyfluorescein diacetate succinimidyl ester (CFSE) dye dilution assay, during which T-cells are expanded in vitro for 6 days without the use of IL-2, would be less successful in their ability to measure proliferation of T-cells upon stimulation with HCV antigens. Indeed, several studies using the CFSE dye dilution assay failed to show a significant T-cell reactivity at baseline [50,56,61]. Moreover, in the study by Pilli et al. [49] far higher HCV-specific T-cell responses were reported using a 10-day expansion protocol using IL-2 addition compared to the standard CFSE assay. Although directly comparing CFSE dye dilution assay to the 12-day expansion assay is difficult because of the different readout systems (a stimulation index (SI) versus IFN-γ production by T-cells, respectively), these observations contribute to the possibility that addition of IL-2 is important for revival of *in vivo* pre-existent but suppressed T-cell responses. Despite the provision of IL-2 during the expansion, still distinct differences are observed in T-cell proliferation between infected patients. For example, previous data in HIV-positive carriers of the Epstein-Barr virus (EBV) showed correlations between the number of EBV-specific T-cells after expansion and the EBV viral load, suggesting

Figure 2: Proposed prediction algorithm for the chance of achieving a SVR in HCV-infected genotype 1 infected patients based on the host characteristic interferon stimulating genes (ISG) regulation and interleukin-28B (IL-28B) genotype.



The most important pre-treatment predictors of a successful outcome of pegIFN-alfa/ ribavirin therapy (right column) in HCV genotype 1-infected patients are ISG regulation (left column) and the IL-28B polymorphism (middle column). They are considered far more important than other predictors like presence of cirrhosis, ethnic background and age.

that clinically relevant differences (i.e. suppression of viral load) can still be detected [62].

Interestingly, preliminary trials testing the therapeutic efficacy of recombinant IL-2 (rhIL-2) in chronic HCV-infected patients demonstrated reductions in ALT-levels [63] as well as a possible increased SVR rate [64]. A few other trials have been conducted but not (yet) published (http://www.clinicaltrials.gov/ct2/results?term=interleukin-2+AND+hepatitis+C).

While using the same 12-day expansion assay, lower HCV-specific T-cell proliferative responses were found in HIV/ HCV-coinfected patients (**chapter 7**) compared to the HCV mono-infected patients (**chapter 6**) from the privicop-study (figure 1A and B). This has been also shown in previous studies, although again using other T-cell assays (CFSE and ELIspot) [56,65-67], suggesting that either higher concentrations of IL-2 are

needed in the assays of HIV/ HCV coinfected patients or that other factors determine the ability of HCV-specific T-cells to proliferate *in vitro*. A recent study in chronic HCV-infected patients by Bengsch et al. [68] showed *in vitro* that exhausted CD8+ T-cells co-expressed several inhibitory receptors (PD-1, 2B4, CD160 and KLRG1) in part contributing to the failure of mounting a successful CD8+ T-cell response. Interestingly, in this study the CFSE assay was amended by the addition of rhIL-2. Along the same lines, blocking the programmed death receptor-1 (PD-1, i.e. expressed on T-cells inhibiting its function after interaction with the ligand PD-1L on hepatocytes, DCs and kupffer cells), was recently shown to improve CD4+CD25+FoxP3+ T-regulatory function after the addition of IL-2 in vitro [69]. To firmly establish the enhancing role of IL-2 in vitro expansion assays, short period T-cell proliferation with and without addition of IL-2 should be investigated. Thereafter, future studies should focus more on the previously mentioned inhibitory receptors associated with an exhausted T-cell phenotype in relation to outcome of pegIFN-alfa/ ribavirin therapy.

Role of natural killer cells and dendritic cells in therapy induced viral clearance

A limited role for HCV-specific T-cells has been demonstrated in the 2 chapters in the thesis. This raises the question whether other cell types play a role in viral clearance during pegIFN-alfa/ ribavirin therapy. Two possible candidates are DCs and Natural killer cells (NK-cells) because of their ability to produce cytokines known to play a role in viral clearance. The latter are frequently found in the normal healthy liver contributing to the defense against viral pathogens via cytolysis of infected cells, production of cytokines like IFN-γ and activation of T-cells [70]. Considerable uncertainty exists about the role of NK-cells in chronic HCV in general and during pegIFN-alfa/ ribavirin therapy induced viral clearance in particular. In chronic HCV-infected patients the frequency of NK-cells in both peripheral blood [71,72] and in the liver [73] is decreased compared to healthy controls. In part this could be explained by the observation that the HCV envelope protein E2 is able to cross-link with the NK-cell surface receptor CD81, one of the HCV entry receptors [74,75]. However, a recent study by Yoon et al. [76] showed no alteration in NK-cell function after in vitro exposure to HCV. Furthermore, the $CD56^{dim}$ NK-cell populations, known to exert cytolytic effector cell functions, seem not to be affected by the lower NK-cell frequency [71]. Recently, Ahlenstiel et al. [77] demonstrated that expression of the degranulation marker CD107a but not the production of IFN-γ increased after *in vitro* stimulation with IFN-alfa suggesting a role for NK-cells in direct cell-mediated killing. Until more studies become available, with the use of small

animal models, uncertainty remains about the contribution of NK-cells to therapy-induced viral clearance.

Plasmacytoid dendritic cells (pDCs) are a highly specialized subset of DCs, shown to be abundant in the liver and capable of producing large amounts of type I interferons [78-80]. However, in chronic HCV-infected patients, both the frequency of pDCs and their ability to produce IFN-alfa upon stimulation have been shown to be reduced [81]. Inhibition of DC effector function is suggested to occur via interference with HCV proteins core and NS3 [82]. However, these observations have been contradicted by others showing both normal frequencies and functional capacity of pDCs in chronic HCV-infected patients [83,84]. A recent study by Ulsenheimer et al. revealed that both the frequency as well as IFN-alfa production by DCs in patients sampled after achieving a SVR with pegIFN-alfa/ ribavirin therapy nearly equaled those found in healthy controls suggesting that restoration of NK-cell numbers and function is possible after successful clearance of HCV. Therefore, the role of pDCs in the intra-hepatic endogeneous IFN-alfa production and thereby its contribution to viral clearance needs to be further explored.

The role of (peg)interferon-alfa in current and future therapies

Working mechanism of pegIFN-alfa

Mathematical modeling of HCV viral decline has shown a biphasic pattern of HCV-RNA elimination during pegIFN-alfa/ ribavirin therapy [85,86]. The first phase, describing the inhibition of viral production, is characterized by a rapid viral decline and occurs in the first 24 to 48 hours (till day 2) after the start of therapy [85,87-89]. It is generally accepted that these first 48 hours reflect the sensitivity of the virus to (peg)IFN-alfa. The second phase is slower and more variable in time lasting from day 2 to several weeks after treatment initiation and correlates with the elimination of HCV infected hepatocytes [90]. This rapid virological decline during the first 48 hours of therapy is in sharp contrast to the peak concentration of peginterferon-alfa 2a (Cmax) reached between 72-96 hours after the first dose and the absorption half-life of 50 hours [91,92]. This observed difference between the very early effects of pegIFN-alfa (i.e. decline in the first 24 to 48 hours) and its pharmacokinetic parameters question the classical views of pharmacokinetics in which the peak concentration or the trough level of pegIFN-alfa is important to exert its antiviral and immunomodulatory action. Therefore, it might be that not the dose of (peg)IFN but the host's ability to coop with

these pegIFN-injections is the most important factor in the working mechanism of pegIFN-alfa.

Two different arguments support this hypothesis. First, some clinical trials have shown that induction therapy with high dose (peg)IFN-alfa did not result in higher rates of SVR [93,94]. Similarly, data from the IDEAL-trial, a head-to-head comparison between pegIFN-alfa-2b 1.5 μgr (clinically used) and pegIFN-alfa-2b 1.0 μgr (official registration) demonstrated that the latter dose was equally effective in terms of SVR rates [95]. Second, IFN-alfa is known for its direct anti-viral action through transcriptional activation of interferon-stimulating genes (ISG) leading to blocking of viral replication and degradation of viral RNA [96,97]. Numerous ISG have been identified clustering in certain pathways involved in IFN-signaling, inflammation, proteolysis or apoptosis [98,99]. A paired liver biopsy study in 16 patients by Sarasin-Filipowicz et al. [100] showed that already 4 hours after the first pegIFN-alfa injection (ribavirin was started after the liver biopsy to prevent confounding) the number of up- or downregulated ISG (>2-fold) was higher in patients achieving a RVR compared to those without a RVR (221 versus 31 genes; p= 0.001). The same authors evaluated baseline liver biopsies of 112 chronic HCV-infected patients showing increased ISG expression in patients with a poor response to future pegIFN-alfa based therapy while those patients with a rapid response lacked pre-activation of ISG in liver biopsies at baseline [101]. Interestingly, this pre-activated endogenous IFN-system was seen more often in patients infected with genotypes 1 and 4 compared to genotypes 2 and 3 possibly in part explaining the difference in SVR rates after pegIFN-alfa therapy between the HCV genotypes [101]. Taken together, not the maximum concentration of pegIFN-alfa but the (in)ability to induce an ISG response during the first hours after a low dose of pegIFN-alfa seems very important in determining the outcome of therapy (figure 2).

Due to a lack of useful cell culture models and an immunocompetent small animal model, research into the working mechanism of IFN-alfa in relation to viral clearance is hampered [102]. Though chimpanzees can be chronically infected with HCV, they do not respond to IFN-treatment making this model unsuitable for future studies into the relationship between IFN-alfa dosage and ISG regulation [103]. Furthermore, serial liver biopsies in the first hours after pegIFN-alfa treatment initiation, in a large enough patient populations, is not feasible due to ethical and patient issues. An interesting future clinical study would be to tailor pegIFN-alfa dosage based on the presence or absence of baseline activated ISG in liver biopsy, i.e. low dose pegIFN-alfa in patients with minimal upregulated ISG versus standard dose pegIFN-alfa for those patients with highly activated ISG at baseline.

New therapies for hepatitis C

Within the next couple of years, several new classes of antiviral agents, Specifically Targeted Antiviral Therapy for hepatitis C (STAT-C), will enter clinical practice [104]. The NS3/4A protease inhibitors, telaprevir and boceprevir, currently the most advanced in their development, have shown promising results in phase-2 trials increasing the SVR rate with 20% in genotype 1 patients [105,106]. By binding of the inhibitor to the active site of the NS3/4A protein, this complex efficiently terminates the mode of action of the HCV NS3/4A protein thereby halting viral replication [1]. Interestingly, the HCV NS3/4A protein has also been shown to play an important role in disrupting the intracellular signaling pathways by interfering with the TLR3 and RIG-I pathways [107,108]. Therefore, blocking the function of the NS3/4A protein with the new STAT-C inhibitors, would also halt the inhibiting effects of the NS3/4A protein on the intracellular signaling possibly leading to restoration of the innate intracellular signaling pathway. Supposedly, this could also lead to restoration of the adaptive immune response since upregulation of ISG has been shown to result in production of cytokines and chemokines like CXCL9, a T-cell chemoattractant [109]. These supposed mechanisms of action of the novel protease inhibitors gives rise to potential new therapeutic strategies.
The first step has been taken by the execution of the INFORM-1 trial [110] showing a pronounced viral load decrease to undetectable levels in most patients after 14 days of therapy with a combination of a protease inhibitor and a polymerase inhibitor. Similarly to the rapid development of HIV viral resistance to for example zidovudine monotherapy [111], already short term experience with these novel HCV protease inhibitors has revealed that viral mutations of the NS3/4A protein rapidly occur with monotherapy. Therefore, a combination of several STAT-C drugs is necessary to efficiently block HCV replication and thereby prevent development of HCV resistance mutations [104].

Is future treatment with low dose or a short course of pegIFN-alfa possible?

PegIFN-alfa is infamous for its severe side-effects like depression, severe weight loss, flu-like symptoms and bone marrow suppression resulting in leucocytopenia [112]. With the development of the new STAT-C inhibitors, a future therapeutic regimen with similar efficacy but without pegIFN-alfa might seem possible. However, in order to speculate about this possibility, the following 2 assumptions have to be made. First, as discussed before, there is a limited role for HCV-specific T-cell responses during pegIFN-alfa/ ribavirin therapy and therefore removing pegIFN-alfa would not affect

T-cell responses and thus outcome of therapy. The lack of T-cell boosting could be explained by a drop in HCV-RNA levels and thus a diminished antigenic stimulation of the T-cell receptor leading to a decline in T-cell proliferation [113]. Although no studies to date have investigated the HCV-specific T-cell response in patients treated with the novel STAT-C inhibitors, a similar pattern of HCV-specific T-cell responses might be expected since these agents efficiently block viral replication leading to a drop in HCV-RNA. The second assumption is that only a low percentage of hepatocytes are infected with HCV thereby allowing the liver to effectively regenerate. However, there is considerable uncertainty about the percentage of HCV-infected hepatocytes with studies ranging from below 20% [114] to virtually 100% [115] depending on the used technique. Anyhow, independent of the number of injured hepatocytes it has been shown that the liver has a good regenerative capacity since hepatocytes undergoing apoptotic changes cause differentiation of progenitor cells into hepatocytes [116,117]. Next to these 2 assumptions, the 2 following condition have to be met as well. First, HCV replication has to be blocked in total so that no resistance mutations can occur. The previously mentioned INFORM-trial proved that this is possible since no HCV resistance mutations occurred after a 14-day treatment period [110]. Second, as discussed before, a lack of pre-activated ISG with the ability to upregulate these ISG upon pegIFN-alfa stimulation, has been shown to be important to determine the outcome of therapy.

Combining these presumptions it could be suggested that efficient inhibition of viral replication can be achieved with multiple STAT-C drugs leading to termination of HCV production in the small number of hepatocytes allowing the liver to clear the infected hepatocytes via apoptosis and hepatic regeneration. As discussed previously, an important side-effect of inhibiting HCV replication is that the intracellular signaling pathways (RIG-I and TLR-3) and possibly the adaptive immune system will be restored via efficient upregulation of ISG contributing to viral clearance. Therefore, a low dose or a short course of pegIFN-alfa might be possible in combination with effective STAT-C inhibitors. Future studies should examine the efficacy of a combination of HCV protease and polymerase inhibitors in combination with a limited 4 week course of low dose pegIFN-alfa.

Caspase-inhibition in hepatitis C

Besides the new STAT-C molecules, also caspase-inhibitors are being developed for the treatment of patients with chronic hepatitis C with the rationale that the

development of liver fibrosis is caused by hepatocyte apoptosis, a tightly regulated process executed by enzymes called caspases [118-120]. Mainly, HIV/ HCV coinfected patients with moderate to severe liver fibrosis and unsuccessful pegIFN-alfa/ ribavirin treatment in the past, are now at risk of liver fibrosis progression. In **chapter 8** the safety and efficacy of GS-9450, a novel caspase-inhibitor, is described showing a pronounced decrease of ALT values during a 14-day course of therapy while no increased rate of activated caspase-3, activated caspase-8 and CD95 expression on peripheral T-cells was observed.

Toxicity of caspase-inhibition

In order to halt HCV-induced liver fibrogenesis, a longer duration of caspase-inhibitor treatment should be investigated. In our study, chronic HCV-infected patients were only treated for 14 days. However, recently the competitor caspase-inhibitor PF-03491390, was demonstrated to be safe, well tolerated and able to lower ALT-values during a 12-week dosing trial [121]. Despite this, some concern exists about the possibility of caspase-inhibitors to promote hepatocarcinogenesis. However, the main receptor for induction of apoptosis is the Fas-deathrecepter pathway [122] and animals deficient in the Fas-receptor are not prone to develop cancer [123]. Unfortunately, the development of both GS-9450 and its competitor PF-03491390 have both been halted due to toxicity issues (personal communication).

Increased peripheral apoptosis rates

An interesting finding of the caspase-study described in **chapter 8** was the increased rate of activated casaspe-3, activated caspase-8 and CD95-expression in T-cells in chronic HCV-infected patients compared to healthy controls. Previous publications have demonstrated increased expression of Fas Ligand (FasL) on peripheral T-cells [124,125]. Furthermore, a correlation between FasL expression and increased expression of the homing receptor chemokine (C-X-C motif) receptor 3 (CXCR3) suggests that activated T-cells expressing FasL are involved in promoting liver inflammation [126]. After exerting their effector function intra-hepatically, these activated T-cells go into apoptosis resulting in massive loss of T-cells in patients with chronic hepatitis C [127]. Therefore, it is of interest to examine the stage of development of the peripheral caspase-3+/ caspase-8+ T-cells of the patients in the caspase-study. Did they escape the liver after exerting their effector function or are they on their way to home to the liver? This is the subject of future research.

Future research perspectives

The goal of performing research is to try and confirm or discard a formulated hypothesis. However, by doing so novel research question arise leading to new ideas for future research. This thesis is no different. For the near future investigations will focus on two specific areas of interest.

First, the observation of increased expression of activation (CD95) and apoptosis (caspase-3 and -8) on peripheral T-cells in a small number of chronic hepatitis C infected patients is intriguing. A further more extensive in-depth analysis of activation and apoptosis markers has to be performed in a larger set of these patients. If the preliminary observations are confirmed then it would be of interest to establish a possible association between expression of peripheral T-cells apoptosis rates and liver-infiltrating T-cells. To investigate the latter, combined expression of homing markers and apoptosis markers on T-cells should be performed. Furthermore, a possible association between the stage of liver fibrosis and the rate of peripheral T-cell apoptosis rates needs to be examined. To be able to reliably perform these analyses, a cross-sectional study requiring liver biopsies is mandatory. Several different liver diseases (i.e. viral hepatitis, non-alcoholic steato-hepatitis (NASH) and primary biliary cirrhosis (PBC)) share a common final pathway resulting in liver fibrosis and cirrhosis. Therefore, analyzing the expression of activation markers and apoptosis markers on peripheral T-cells in other liver diseases besides chronic hepatitis C would be of interest.

Second, recent publications on the importance of IL-28B in determining the outcome of anti-HCV therapy have sparked new interest into the association between host-related factors and success of therapy. Studying gene up- and downregulation in peripheral T-cells before and during therapy might shed more light on the role and function of these cells in viral clearance. Especially, regulation of genes involved in apoptosis and T-cell activation should be analyzed. Furthermore, this would provide the opportunity to establish the proposed relationship (as described in figure 2) between IL-28B polymorphisms and up- or down-regulation of ISG in terms of outcome of HCV therapy.

Conclusion

Chronic HCV affects millions of people worldwide with major impact on the morbidity and mortality of the people affected. In HCV-infected patients coinfected with HIV, an

accelerated progression of liver fibrosis is seen, even with successful suppression of HIV due to combination antiretroviral therapy (cART). Current therapeutic regimens with pegIFN-alfa and ribavirin are only moderately successful, especially in "difficult to treat" patients with genotype 1 or 4 and with a HIV coinfection. Despite the lack of reliable small animal models, progress has been made with regard to the interplay between pegIFN-alfa and ribavirin on the one hand and HCV and the immune system on the other hand. The studies described in this thesis have contributed in different ways to these issues. The acute HCV cohort study (**chapter 3**) contradicted earlier theoretical reasoning (**chapter 2**) about the efficacy of pegIFN-alfa monotherapy adding important data in favor of using pegIFN-alfa in combination with ribavirin for the treatment of acute HCV in HIV-infected patients (**chapter 4**). The study evaluating the viral load change in the first 48 hours after the start of pegIFN-alfa/ ribavirin therapy (**chapter 5**) generated a new hypothesis with regard to the working mechanism of pegIFN-alfa. The immunological studies evaluating the role of HCV-specific T-cells in both HCV mono-infected and HIV/ HCV coinfected patients demonstrated that HCV-specific T-cells do not seem to be involved in therapy-induced viral clearance (**chapter 6 and 7**). Finally, higher rates of activated caspase-3, activated caspase-8 and CD95 were observed in chronic HCV-infected patients compared to healthy controls (**chapter 8**) which leads to new research areas in the field of T-cell apoptosis. In the next couple of years, very exciting new developments are expected in the field of viral hepatitis C. The development of an immunogenic mouse models will revolutionize the research into HCV pathogenesis leading to major improvement in the understanding of HCV cell entry, immune evasion and treatment-induced viral clearance. Furthermore, the introduction of HCV protease- and polymerase-inhibitors will have major therapeutic implications revolutionizing the future anti-HCV therapy in terms of regimen and duration. This will hopefully lead to easy eradication of HCV thereby minimizing its effects on the human population.

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Chapter 10

Nederlandse samenvatting

Introductie

Hepatitis is een ontsteking van de lever, afgeleid van de Griekse woorden *hepar* (lever) en *itis* (ontsteking), die door vele verschillende ziekten veroorzaakt kan worden. Het hepatitis C virus (HCV), ontdekt in 1989 als veroorzaker van de ziekte non-A non-B hepatitis", is een van de meest voorkomende oorzaken van een hepatitis. Wereldwijd zijn naar schatting 170 miljoen mensen geïnfecteerd met HCV, waarvan er tussen de 12.000 en 60.000 in Nederland wonen. Er zijn meerdere subtypen (genotypen) van HCV bekend die qua virale opbouw zo'n 30% van elkaar verschillen. In tegenstelling tot genotypes 2 en 3, zijn HCV genotypes 1 en 4 met de huidige standaard behandeling van peginterferon-alfa (wekelijkse injecties) en ribavirine (dagelijks tabletten) moeilijk te behandelen. De kans op een succesvolle behandeling van chronische HCV met klaring en dus genezing van het virus (i.e. sustained virological response rate (SVR)) is veel lager voor genotypes 1 en 4 dan voor genotypes 2 en 3 (50% versus 80%). Daarbij is de behandelduur van chronische HCV genotypes 1 en 4 twee keer zo lang (48 weken in plaats van 24 weken). HCV en het humaan immunodeficiency virus (HIV) worden beiden via bloed-bloed contact overgedragen. Ongeveer 30% van de HIV-patiënten is daarom ook geïnfecteerd met HCV. In vergelijking met chronische HCV patiënten ligt het percentage van een succesvolle behandeling bij HIV/ HCV genotype 1 of 4 gecoinfecteerde patiënten nog lager (rond de 30%). Patiënten met een HCV genotype 1 of 4 met of zonder HIV-infectie, worden daarom gezien als moeilijk behandelbaar. Ze vormen de patiëntengroep waarop de studies in dit proefschrift zijn gebaseerd.

Dit promotieonderzoek is onder te verdelen in 3 hoofdlijnen: huidige behandeling van acute hepatitis C, huidige behandeling van chronische hepatitis C en toekomstige behandelingen van chronische hepatitis C. Allereerst wordt in de **hoofdstukken 2, 3 en 4** de optimale behandeling van acute HCV bij HIV-patiënten bestudeerd. Vervolgens worden in de **hoofdstukken 5, 6 en 7** de immunologische en virologische veranderingen onderzocht tijdens de behandeling van chronische HCV. Als laatste wordt in **hoofdstuk 8** de veiligheid van een nieuw medicijn geëvalueerd dat ontwikkeld wordt ter voorkoming van lever fibrose (verlittekening van de lever) bij patiënten met chronische HCV.

Behandeling van acute hepatitis C

De eerste 6 maanden (vanaf het moment van infectie met HCV), worden gedefinieerd als acute hepatitis C. Als HCV in die periode niet door het lichaam opgeruimd wordt, dan wordt er gesproken van chronische HCV. Klassiek wordt HCV overgedragen via bloed-bloed contact en komt daarom het meest voor bij intraveneus drug gebruikers en patiënten die een met HCV besmette bloedtransfusie hebben gekregen. Uit studies blijkt dat behandeling met peginterferon-alfa (pegIFN-alfa) monotherapie (dus zonder ribavirine) bij 90% van deze patiënten met acute HCV leidt tot genezing (SVR) van de infectie. De laatste jaren is er een epidemie van acute HCV onder HIV-patiënten waarbij vermoed wordt dat seksuele transmissie een belangrijke rol speelt. Door gebrek aan kwalitatief goede studies, is de optimale behandeling van acute HCV bij HIV-patiënten onduidelijk. Er wordt zowel pegIFN-alfa monotherapie en pegIFN-alfa in combinatie met ribavirine gebruikt waarbij de meest gerapporteerde SVR percentages variëren van 50-80%. Dit is beduidend lager dan de eerder vermelde 90% na pegIFN-alfa monotherapie bij patiënten met een acute HCV mono-infectie.
In **hoofdstuk 2** wordt beargumenteerd dat pegIFN-alfa monotherapie ook de eerste keus behandeling kan zijn voor HIV-geïnfecteerde patiënten met acute HCV. Enerzijds omdat er geen overtuigend bewijs is dat pegIFN-alfa/ ribavirine combinatietherapie een hogere SVR geeft dan pegIFN-alfa monotherapie. Anderzijds kunnen patiënten waarbij de 1^{e}-lijns behandeling (i.e. pegIFN-alfa monotherapie) faalt, dan opnieuw behandeld worden, waarbij dan ribavirine wordt toegevoegd (combinatie-therapie). Hiervan is aangetoond dat er dan alsnog een redelijke kans bestaat op het behalen van een SVR. De effectiviteit van pegIFN-alfa monotherapie voor de behandeling van acute HCV bij HIV-patiënten is onderzocht in de grootste cohort studie tot nu toe (**hoofdstuk 3**). Het resultaat is een lage SVR en een hoog percentage patiënten dat niet respondeert op de therapie. Een mogelijke verklaring voor het hoge aantal non-responders in deze studie is het voorkomen van een rs8099917 IL-28B polymorfisme (een nucleotide verandering in het DNA). Het G/G-genotype van dit IL-28B polymorfisme is hierbij geassocieerd met falen van de pegIFN-alfa monotherapie.
Deze studie draagt bij aan de inmiddels groeiende overtuiging dat pegIFN-alfa/ ribavirine combinatietherapie de eerste keuze van behandeling is voor HIV-patiënten met een acute HCV. Dit heeft geleid tot de eerste Nederlandse richtlijn voor de behandeling van acute HCV in HIV-patiënten (**hoofdstuk 4**).

Behandeling van chronische hepatitis C

Virale kinetiek

De behandeling met pegIFN-alfa/ ribavirine is langdurig (meestal 48 weken) en gaat gepaard met veel bijwerkingen zoals algehele malaise, gewichtsverlies, koorts-achtige symptomen na de injectie, depressie en bloedbeeld afwijkingen (anemie, leucopenie en trombopenie). Het voorspellen van het succes van de behandeling is daarom belangrijk en gebeurt nu op week 4 en week 12 van de behandeling. Wanneer nog eerder dan week 4 voorspeld kan worden of de behandeling wel of niet succesvol is, is dit zowel kosten-besparend alsook belangrijk voor de duur van de bijwerkingen. In **hoofdstuk 5** wordt onderzocht of de afname van de hoeveelheid virus (HCV viral load) al 2 dagen na de start van de behandeling (na 48 uur) voorspellend is voor het succes van de therapie. Door de soms kleine patiëntenaantallen zijn niet alle uitkomsten significant. Desalniettemin wordt aangetoond dat een snelle daling van de viral load, 48 uur na de start van de behandeling, geassocieerd is met een succesvolle uitkomst (SVR) bij patiënten met een chronische HCV-infectie zonder en met een HIV-coinfectie. Dit snelle effect op de viral load kan niet verklaard worden door de farmacokinetische eigenschappen van pegIFN-alfa (bereikt maximale concentratie pas na 72 tot 96 uur en heeft een absorptie halfwaardetijd van 50 uur) en ribavirine (steady state bereikt na weken). Mogelijk is een lage concentratie van beide middelen al voldoende om een anti-viraal effect te bewerkstelligen. Recente studies hebben laten zien dat bij patiënten die een snelle daling van de viral load hebben, genen van HCV-geïnfecteerde levercellen duidelijk actiever zijn, in vergelijking tot patiënten die geen snelle daling van hun virale load hebben. Met andere woorden, activatie van de cellulaire genen in reactie op pegIFN-alfa/ ribavirine is belangrijk voor de uitkomst van de behandeling. Dit wordt verder beschreven in de discussie (**hoofdstuk 9**).

Immunologie

T-lymfocyten (T-cellen) zijn witte bloedcellen die via het uitscheiden van stoffen (cytokinen) belangrijk zijn in de verworven afweer (cellulaire immuun respons) tegen virussen. De afgelopen jaren is er veel onderzoek gedaan naar de rol van deze T-cellen tijdens de behandeling van chronische hepatitis C, met tegenstrijdige bevindingen als resultaat. Allereerst kan dit verklaard worden uit de zeer lage aantallen HCV-specifieke T-cellen die in de chronische fase in het bloed van de patiënt aanwezig zijn, waardoor er geen grote aantallen T-cellen voor onderzoek voorradig zijn. Verder zijn er

verschillende meetmethoden in het lab beschikbaar, zoals proliferatie assays, ELIspot assays en directe kleuringen, wat vergelijking van studies moeilijk maakt. Als laatste is er geen geschikt diermodel beschikbaar voor *in vivo* onderzoek van HCV-specifieke T-cellen. In **hoofdstuk 6 en 7** wordt met behulp van een nieuwe zeer gevoelige methode (12-day expansion assay) geprobeerd het aantal HCV-specifieke T-cellen te profileren (vermenigvuldigen) door middel van stimulatie met HCV-peptiden (kleine stukjes HCV), waarna de mogelijkheid tot productie van cytokinen (IFN-γ) wordt gemeten (uitgedrukt als T-cel respons). Van patiënten met een HCV mono-infectie en van patiënten met een HIV/ HCV coinfectie zijn T-cellen afgenomen voor de start van (baseline) en tijdens de pegIFN-alfa/ ribavirine behandeling (week 4 en 12). De T-cell responsen op baseline verschillen per patiënt maar zijn als groep niet geassocieerd met de uitkomst van de behandeling. Tijdens pegIFN-alfa/ ribavirine behandeling nemen de T-cel responsen af in alle patiënten, ongeacht het wel of niet bereiken van een SVR. Deze bevindingen suggereren dat HCV-specifieke T-cellen niet van invloed zijn op het uiteindelijk wel of niet klaren van de HCV-infectie tijdens pegIFN-alfa/ ribavirine therapie.

Nieuwe behandelingen voor hepatitis C

Met de komst van nieuwe anti-HCV medicijnen (protease- en polymerase-remmers), zal de behandeling van HCV in de komende jaren radicaal veranderen. Veel patienten, die eerder niet succesvol gereageerd hebben op pegIFN-alfa/ ribavirine behandeling en op dit moment al matige tot ernstige fibrose (verlittekening van de lever) hebben, zijn in afwachting van deze nieuwe medicijnen. Tot die tijd lopen deze patiënten echter een sterk verhoogd risico op het ontwikkelen van leverfalen of een hepatocellulair carcinoom (leverkanker). Het proces van lever fibrose wordt veroorzaakt door apoptose (geprogrammeerde celdood) van hepatocyten, waarbij eiwitten genaamd caspases een belangrijke rol spelen. Een nieuwe ontwikkeling is de mogelijkheid om de activiteit van deze intra-hepatische caspases te remmen met een zogenaamde caspase-remmer. In **hoofdstuk 8** wordt de klinische studie beschreven waarin patiënten met chronische HCV en sterk verhoogde alanine-aminotransferase (ALAT, enzym dat vrijkomt uit kapotte hepatocyten) gedurende 14 dagen behandeld worden met een caspase-remmer. Tijdens de behandeling treedt er een verlaging op van de ALAT-waarden. Eerder was al aangetoond dat deze ALAT als afgeleide maat kan dienen voor het percentage apoptotische hepatocyten. Daarbij toonde deze studie aan dat het effect van de caspase-remmer specifiek is voor de lever, aangezien

er geen veranderingen in de apoptose van periphere T-cellen wordt gezien tijdens behandeling. Interessant is echter wel de observatie dat de studie-patiënten met chronische hepatitis C voor start van de caspase-behandeling een significant hoger percentage apoptotische T-cellen hadden, in vergelijking tot gezonde vrijwilligers. De oorzaak hiervoor is nog niet bekend en wordt in een nieuwe studie verder uitgezocht.

Conclusie

Met de komst van de nieuwe protease- en polymerase-remmers treedt er een interessant nieuw tijdperk aan in de behandeling van hepatitis C. PegIFN-alfa en ribavirine zullen echter de komende jaren nog onderdeel blijven uitmaken van het behandelingsschema. Onderzoek naar het virologisch en immunologisch werkingsmechanisme van pegIFN-alfa en ribavirine is belangrijk voor de plaatsbepaling van beide middelen in toekomstige regimes. In de discussie (**hoofdstuk 9**) wordt hier uitgebreider op ingegaan. Daarin wordt gepostuleerd dat een lage dosis pegIFN-alfa voldoende is om in de patiënt intra-hepatische genen te activeren die bepalend zijn voor de uitkomst van de behandeling. Ook andere nieuwe patient-factoren zoals polymorfismen in het IL-28B gen zullen belangrijk worden in de behandeling van hepatitis C. Er zijn de komende jaren belangrijke ontwikkelingen die de behandeling van HCV radicaal zullen gaan veranderen. Het belooft een interessante tijd te worden.

De belangrijkste conclusies uit dit proefschrift zijn:

- PegIFN-alfa monotherapie als behandeling voor acute HCV bij HIV-patiënten resulteert in een laag succespercentage.
- Bij patiënten met een chronische HCV is een snelle daling van viral load al 2 dagen na de start van pegIFN-alfa/ ribavirin behandeling voorspellend voor het succes van de behandeling.
- De HCV-specifieke T-cel respons voor en tijdens behandeling met pegIFN-alfa/ ribavirin is niet gecorreleerd met de uitkomst van de behandeling. Dit geldt voor zowel chronische HCV mono-infecties en HIV/ HCV coinfecties.
- Ten opzichte van gezonde vrijwilligers hebben patiënten met chronische hepatitis C een verhoogde percentage apoptotische T-cellen in hun bloed.

List of publications

J.E. Arends, S. van Assen, C.J. Stek, A.M.J. Wensing, J.H. Fransen, I.M. Schellens, S.N.M. Spijkers, T. Mudrikova, D. van Baarle, H.G. Sprenger and A.I.M. Hoepelman.Peginterferon-alfa monotherapy leads to low response rates in HIV-infected patients with acute hepatitis C.
Submitted.

J.E. Arends, F.A.E. Lambers, J.T.M. van der Meer, G. Schreij, C. Richter, K. Brinkman and A.I.M. Hoepelman on behalf of the Netherlands society for AIDS physicians (NVAB).
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Submitted.

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"For it is commonly said: accomplished labours are pleasant" – Cicero

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Curriculum vitae

Joop Arends werd op 10 september 1974 geboren te Oron in Nigeria. Na het behalen van zijn VWO diploma in 1993 aan het Sint-Maartens College in Maastricht werd hij uitgeloot voor de studie geneeskunde. Hij ging geneeskunde studeren aan de Universiteit van Antwerpen (vroeger RUCA) waar hij zijn 1e kandidatuur behaalde. In 1994 werd hij nageplaatst voor de studie geneeskunde aan de Universiteit van Maastricht (UM) waar hij in 2000 zijn artsexamen behaalde. Tijdens zijn studie onderzocht hij "antigen retrieval" technieken op de afdeling pathologie van het Veterans Affairs (VA) Hospital in Memphis (USA) en onderzocht hij op de afdeling pathologie van het University College London (UCL) de expressie van MAdCAM-1 in milt-lymfomen. Tenslotte verrichte hij als student-assistent aan de UM onderzoek op het gebied van MALT-lymfomen onder begeleiding van dr. Bot en dr. H. Schouten. Vervolgens ging hij als arts-assistent geneeskunde niet in opleiding werken op de afdeling Interne Geneeskunde in het Onze Lieve Vrouwe Gasthuis in Amsterdam. Na een 5 maanden durende reis door Zuid-Amerika startte hij in april 2002 in het OLVG met de opleiding tot Internist onder leiding van dr. P.H.J. Frissen. In april 2004 vervolgde hij zijn opleiding in het Universitair Medisch Centrum Utrecht (UMCU) onder leiding van prof. dr. E. van der Wall en later prof. dr. D. Biesma. Tussen 2006 en 2009 volgde hij het aandachtsgebeid Infectieziekten onder leiding van prof. dr. I.M. Hoepelman. Naast de opleiding tot medisch specialist verrichtte hij promotieonderzoek op het gebied van hepatitis C onder leiding van prof. dr. I.M. Hoepelman en dr. D. van Baarle. Dit werd tussen 2007 en 2008 onderbroken door een onderzoeksjaar in het immunologisch laboratorium onder leiding van prof. dr. F. Miedema. Sinds 2009 is hij werkzaam als Internist-Infectioloog binnen het cluster Interne Geneeskunde en Infectieziekten van de divisie Interne Geneeskunde en Dermatologie (DIGD) van het UMC Utrecht.

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