CHRONIC HEPATITIS C INFECTION: CLINICAL AND SOCIETAL EVALUATIONS

Daphne M. Hotho

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Clinical and Societal Evaluations

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Layout and printing: Optima Grafische Communicatie, Rotterdam, the Netherlands.

The work in this thesis was conducted at the Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center Rotterdam, the Netherlands.

ISBN: 978-94-6169-399-0

Printing of this thesis was supported by Erasmus Unversiteit Rotterdam, the department of Gastroenterology and Hepatology of the Erasmus MC Rotterdam, Nederlandse Vereniging voor Hepatologie, Merck, Sharp & Dohme B.V, Roche, Janssen Cilag, Hitachi Medical Systems en Zambon.

Chronic Hepatitis C Infection: Clinical and Societal Evaluations

Chronische Hepatitis C: klinische en maatschappelijke overwegingen

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
Vrijdag **21 juni 2013** om **11:30** uur

door

Daphne Marije Hotho

geboren te

Warnsveld

ERASMUS UNIVERSITEIT ROTTERDAM

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GENERAL INTRODUCTION

Chronic hepatitis C in Western countries

Chronic hepatitis C virus infection (HCV) affects 180 million people worldwide. Chronic HCV can cause hepatic decompensation, hepatocellular carcinoma (HCC), and portal hypertension[1]. Nowadays, chronic HCV is the leading indication for liver transplantation in developed countries. HCV is an enveloped single-stranded RNA virus that is transmitted in humans through blood-to-blood contact[2]. Acute infection leads to chronic infection in 50-80%[3]. In most cases, chronic HCV infection remains relatively asymptomatic until complications of disease present. Natural course of chronic HCV infection is highly variable with serious liver disease developing in one third of persons 20 years or less after infection and no progression in another third for 30 years or longer[4]. Factors that accelerate clinical progression include alcohol intake, which has a pronounced effect on the course of the disease, co-infection with HIV or HBV, male sex, and older age at infection[4-6]. Once cirrhosis is established, the risk of HCC is approximately one to four percent per year[7, 8]. HCC can occur without cirrhosis but is rare. For this reason, antiviral treatment is ideally provided in pre-cirrhotic liver disease. Furthermore, antiviral treatment is more effective in non-cirrhotic and, preferably less fibrotic stages of chronic HCV infection[1].

Antiviral treatment for chronic HCV infection

In contrast to hepatitis B virus infection (HBV) and human immunodeficiency virus (HIV), chronic HCV can be cured with antiviral therapy. Until very recently, antiviral therapy (AVT) consisted of peginterferon (PEG-IFN) and ribavirin (RBV)[9-12]. PEG-IFN/RBV for 24-48 weeks results in a sustained virologic response (SVR) of 41-93%. SVR means HCV-RNA non-detectability 6 months after PEG-IFN/RBV and is commonly used as a surrogate marker for cure[13].

There are six known HCV genotypes of which genotype (GT) 1 is the most frequent and unfortunately the least responsive to PEG-IFN/RBV. In GT-1 infected patients, SVR is achieved in only 41-52% of patients[9-12]. PEG-IFN/RBV is hampered by a long duration, high costs and substantial side effects of which flu-like symptoms, anemia and depression are the most frequently reported[9]. For these reasons, the identification of non-responders and responders to PEG-IFN/RBV pre-treatment has obtained increasing attention. Three landmark genome-wide association studies identified single nucleotide polymorphisms near the interleukin 28B (IL28B) region which were more frequent in responders to treatment. IL28B encodes interferon (IFN) λ3, a type III IFN involved in host antiviral immunity. Favorable variants of the two most widely studied IL28B polymorphisms, rs12979860 and rs8099917, are strong pretreatment predictors of early viral clearance and sustained viral response in patients with HCV GT-1 infection[14-17]. Thus, PEG-IFN/RBV is of only moderate success and the pool of HCV GT-1 non-responders is growing. In 2012, a new era has begun with the registration and reimbursement of the protease inhibitors telaprevir and boceprevir to PEG-IFN/RBV for the treatment of GT-1 infection[18]. With the addidtion of telaprevir or boceprevir, about 70% of naïve patients achieve SVR[19, 20]. In

previously treated GT-1 infected patients, SVR has been reported of 24-53% with the addition of telaprevir[21] and SVR rates of 59-66% with the addition of boceprevir[22]. Besides increased effectiveness, duration of therapy is generally shorter. Unfortunately, telaprevir and boceprevir carry significant side effects. With telaprevir anemia, gastrointestinal side effects and skin rashes are most frequently reported[19]; with boceprevir, flu-like symptoms remain most pronounced, as well as dysgeusia and anemia[20]. Another issue is the thrice a day dosing regimen of these additional agents. Both side effects and dosing frequency might influence compliance negatively and real life SVR rates are eagerly awaited. The 'ideal' HCV therapy would employ a few oral medications taken for a short duration, with minimal side effects and minimal drug-drug interactions. Therefore, the proof of concept of SVR achieved following an interferon-free regimen is of major clinical relevance[23, 24]. It is unclear when the first interferon-free regimen will become available for patients and physicians in daily clinical practice.

Antiviral treatment: diagnosis and treatment in special populations

Since the screening of blood(-derived) products (i.e. 1992) for HCV, iatrogenic transmission has become rare in developed countries and injecting drug use (IDU) has become the major route of transmission[25]. Unfortunately, injecting drug users (IDUs) generally tend to keep out of hospital care. Treatment of a patient with PEG-IFN/RBV requires knowledge of virologic response and its frequent and severe side effects. This is even more so with the addition of boceprevir/telaprevir. Interestingly, evidence suggests that treatment of active IDU with antiviral therapy may be one of the best ways to contain the burden[26]. Therefore, treatment of HCV in IDU is of great importance from a public health perspective. Opioid substitution therapy seems to provide a beneficial framework for antiviral therapy resulting in SVR rates comparable to non-IDUs[27]. Unfortunately, HCV screening in addiction treatment centers is not always as frequent as it should be and treatment uptake among these patients is low[27, 28]. Besides patient characteristics that hamper well-timed diagnosis and treatment uptake, physicians might be reluctant to initiate therapy as well. Interferon-induced depression in HCV-infected patients has been reported to occur more often in patients suffering from pre-existent psychiatric illness. Although the addition of telaprevir/boceprevir might shorten duration of therapy, one case of suicide has been reported already[19]. Unfortunately, ways to identify patients prone for IFN-induced depression have been suggested but are not univocal.

AIMS AND OUTLINE OF THE THESIS

With this thesis, we aimed to improve treatment of chronic HCV in active substance users. The first part of this thesis focuses on possible hampers of initiation of antiviral treatment in HCV-infected substance users. Abdominal ultrasound is part of the diagnostic evaluation before an HCV-infected patient can initiate antiviral therapy. In **Chapter 1**, we investigated common bile duct dilatation, an alarming symptom which is often seen in patients who are prescribed methadone. Although comparable SVR rates have been described in patients who do use substances

such as heroin and/or cocain, the influence of active substance use as such has never been studied in relation to antiviral therapy. **Chapter 2** describes our attempt to overcome this final hamper in the treatment of chronic HCV in active substance users. The second aim of this thesis is to improve current antiviral therapy for chronic HCV. Depression is one of the main reasons for early drop-out from PEG-IFN/RBV for chronic HCV. We aimed to identify patients prone for IFN-induced depression and studied the effect of prophylactic escitalopram by conducting a post-hoc analysis of a randomized-controlled trial in HCV-infected patients (**Chapter 3**). We studied a new direct acting antiviral agent in a phase I clinical trial (**Chapter 4**). Further, we investigated durability of the virologic endpoint 'SVR' after antiviral treatment with a direct acting antiviral agent (**Chapter 5**). Thirdly, we assessed clincial importance of a hepatitis E virus infection in HCV-infected subjects. Hepatitis E virus infection has obtained increasing attention as a relatively common, asymptomatic infection in the general population and as a possible cause of chronic hepatitis in immunocompromised subjects. **Chapter 6** describes epidemiology of HEV in chronic HCV-infected patients and clinical characteristics of subjects affected.

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CHAPTER 1

COMMON BILE DUCT DILATATION: A COMMON FINDING WITHOUT UNDERLYING PATHOLOGY

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ABSTRACT

Background & Aim. Common bile duct dilatation (CBD(D)) is a clinical finding that warrants exclusion of malignancy. However, other elements, e.g. medication use, have been linked to CBDD. We studied prevalence of CBDD and the clinical implications of CBDD. **Methods**. From July 2010 until May 2011, CBD was measured in all patients referred for ultrasonography (US) (CBDD >= 6.0 mm). Patients with a history of biliary or pancreatic surgery were excluded. **Results.** CBDD was present in 34 out of 530 patients (7%). Univariable analysis demonstrated an association between methadone (p<0.01, odds ratio (OR): 81.68 (32.98-202.28)), antidepressants (p<0.01, OR: 4.52 (1.57-13.01)), benzodiazepines (p<0.01, OR: 8.41 (3.72-19.04)) and antipsychotics (p<0.01, OR: 9.75 (3.60-26.44)) and CBDD. Within methadone users (MU) (n=46), none of the other psychotropic drugs were associated with CBDD (n=26). Median CBD diameter: MU: 6.8 mm (95%confidence interval (C.I.) 6.2-7.9), non-MU: 3.3 mm (95%C.I. 3.3-3.7) (p<0.01). Eighteen of 34 patients (1 MU, 6 non-MU) with CBDD underwent additional radiological examination (repeat US, magnetic resonance (cholangiopancreatic) imaging, computed tomography, contrast-enhanced imaging or endoscopic US); no biliary or pancreatic malignancy could be demonstrated in MU, hepatocellular carcinoma was found in one patient, non-MU. The remaining patients were followed up; none developed clinical worsening and/or jaundice (MU: median follow-up: 9 months, range: 1-18 months; non-MU: median follow-up: 16 months, range: 1-17 months). **Conclusion**. Asymptomatic CBDD is highly prevalent among MU and, in the absence of cholestasis not associated with pathology.

INTRODUCTION

In abdominal imaging, common bile duct dilatation ((CBD)D) without further symptoms or signs of gallbladder stones is an infrequent finding generally considered as alarm symptom. CBDD may be caused by malignancy of the papilla of Vater, the CBD or pancreas. Therefore, this finding warrants exclusion of malignancy[1]. With abdominal ultrasonography (US), a normal CBD cannot always be visualized. If visible and measurable, the generally accepted upper limit of normal of the diameter of the CBD is 6 millimeter[2]. Regarding normal limits, some factors have been described to increase the diameter of the CBD and, of these, age and cholecystectomy are the most consistent ones[3, 4 2, 5]. Narcotics have been found to increase sphincter of Oddi pressure as well and could thereby induce bile duct dilatation. In few cases, CBDD has been described in relation to use of methadone[6-9]. However, the exact prevalence of CBDD among patients on prescription of methadone is unknown. Furthermore, it is unclear, if more prevalent, whether CBDD should be regarded as an alarm symptom in patients using methadone. In Western countries, former or current intravenous drug use (IVDU) is a major mode of transmission of the hepatitis C virus (HCV). The diagnosis and work-up of patients with chronic HCV infection includes US to examine presence of severe liver fibrosis, cirrhosis or hepatocellular carcinoma (HCC). If methadone use is indeed associated with CBDD but without clinical consequences, a substantial part of newly diagnosed HCV patients will unnecessarily be exposed to the risks of endoscopic retrograde cholangiopancreatography (ERCP) (bleeding 0.02%, perforation 0.3%) or endoscopic US (complication rate 0,6%[10]) and excessive medical costs will be made[11]. Furthermore, an increase in prevalence of CBDD without clinical consequences could lower the awareness of the investigator. To be able to interpret the alarming CBDD correctly, knowledge on the normal distribution of the CBD diameter and prevalence and clinical implications of CBDD in patients on methadone prescription are of great clinical importance[12].

We aimed to determine the diameter of the CBD in patients without biliary disease referred for abdominal US at our outpatient clinic. Furthermore, we assessed the role of methadone and other factors of influence on the prevalence of CBDD and investigated the clinical implications of CBDD[2].

PATIENTS AND METHODS

From July 2010 until May 2011, we prospectively studied the diameter of the CBD in all patients that underwent abdominal US at the liver imaging unit of our gastroenterology and hepatology outpatient clinic. Three hepatologists with expertise on US performed the investigations. As usual, every patient was instructed to be sober up to four hours before US. The CBD diameter was measured at least two centimeters distal to the bifurcation and was restricted to the intraluminal space. Patients were included in our analysis if aged over 18 years and we applied the

following exclusion criteria; a history or the presence of liver transplantation, primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), pancreatic and / or cholangiocarcinoma, cholecystectomy, cholecystitis, focal liver lesions and traumatic and/or surgical procedures that might influence the anatomy of the biliary tract and/or gallbladder. Patient characteristics (gender, age at time of imaging, medical history, concomitant medication and substance use) were obtained from medical records. Medication was classified into methadone, other opioids, antipsychotics, buprenorphin, antidepressants, benzodiazepin agonists and other hypnotics. The first three subgroups have been applied because these types of medication have been described to influence the diameter of the CBD. The remaining subgroups were made because these types of medication are often prescribed together with the first three subgroups[7, 9, 13]. According to standard clinical care, if CBDD was present, patients were offered further investigation with endoscopic US, magnetic resonance cholangiopancreaticography (MRCP), computed tomography (CT) of the abdomen or contrast-enhanced US. Whether further investigation was initiated and which type of investigation was chosen, was kept at the discretion of the treating physician. If, for other clinical reason(s), US was repeated in a patient with CBDD before further investigation was performed, further investigation was not performed if CBDD was absent the second time. In case of CBDD, clinical follow-up was performed according to standard clinical care including laboratory investigation (signs of cholestasis or inflammation). If patients with CBDD were in regular care of the local addiction treatment center and unable to attend their regular visits in hospital, the physician of the addiction treatment center performed clinical follow-up.

Dichotomous baseline characteristics of patients with and without CBDD were tested for an association with CBDD with Pearson chi-square. With the independent samples t-test, mean age with and without CBDD was compared between groups. Univariate logistic regression analysis was performed in continuous baseline characteristics. Covariates with a statistical significance at the p-level < 0.20 were analyzed in a multivariate model. However, within the event CBDD, all patients with one ore more of the identified factors were on use of methadone. Collinearity of two factors invalidates multivariate analysis of these variables in the same model. Hence, multivariate analysis with a model of methadone and (one of) the other factor(s) was not performed. We selected methadone users (MU) and compared baseline factors in patients with and without CBDD using Pearson Chi-square and the independent samples T-test. Because none of the previously univariately identified factors appeared to be independently associated, we created a multivariate model with methadone. The following covariates were entered into the model because of clinical relevance: age as continuous variable, and use of opioids other than methadone[7, 9, 13]. Antipsychotics have been described in relation to CBDD, but because of collinearity this variable could not be studied in a multivariate model with methadone. Therefore, use of antipsychotics was investigated within MU as described above.

RESULTS

Of 1183 patients that underwent US during the study period, 530 patients were included in our analysis; the remaining 653 patients fulfilled one or more exclusion criteria of which liver transplantation, PBC, PSC or a combination of these were the most frequently reported. Data on use of medication and substance use were available in 529 of 530 and 489 of 530 patients, respectively. In 28 patients, the CBD was too small to visualize and in 227 patients, the CBD was visualized but too small to measure. In the remaining 275 of 530 included patients, CBD was measurable with a median CBD diameter of 3.5 mm (range 1.4 -16.0 mm).

CBDD was seen in 34 of 530 (6.4%) patients. Baseline characteristics are presented in table 1, according to the presence of CBDD. The following factors were associated with CBDD; male gender (p=0.02), HCV as indication for imaging (p<0.001); use of methadone (p<0.001), benzodiazepine agonists (p<0.001), antidepressants (p=0.02), and antipsychotics (p<0.001). In 46 MU, none of these factors were associated with CBDD (table 2). In multivariate analysis, adjusting for age as continuous variable and opioids other than methadone, methadone use was the only covariate independently associated with CBDD (Odds ratio (OR) 95.46, 95% confidence interval (CI) 36.80 – 247.66; p-value <0.001).

	Common bile duct dilatation N=34	No common bile duct dilatation N=496	p-value
Gender, n (%)			
Male	26 (74)	306 (63)	0.02*
Age, years			
Median (25^{th} – 75^{th} percentile)	48 (43 - 54)	50 (39 - 59)	1.00
Older than 50 years of age, n (%)			
Yes	16 (47)	248 (50)	0.74
Indication for imaging, n (%)			
Screening HCC	4 (12)	109 (22)	0.16
HCV	15 (44)	87 (18)	<0.01*
HBV	2 (6)	98 (20)	0.04*
Other	13 (38)	202 (41)	0.88
Concomitant medication, n (%)			
Methadon	27 (79)	19 (4)	<0.01*
Other types of opioids	2 (6)	13 (3)	0.48
Benzodiazepin agonists	10 (29)	26 (5)	<0.01*
Other hypnotics	0	5 (1)	0.81
Antidepressants	4 (12)	18 (4)	0.02*
Antipsychotics	6 (18)	13 (3)	<0.01*

Table 1. Baseline characteristics
*Statistically significant difference between group with and without common bile duct dilatation (Pearson Chi-square, p<0.05)

	CBDD	No CBDD	p-value*
	N=26	N=20	
Male gender	22 (85)	14 (74)	0.37
Older than 50 years of age	12 (46)	8 (42)	0.78
HCV as indication for imaging	14 (54)	14 (74)	0.18
HBV as indication for imaging	0 (0)	0 (0)	not applicable
Antipsychotics	6 (23)	2 (11)	0.28
Benzodiazepin agonists	10 (39)	6 (32)	0.63
Antidepressants	4 (15)	4 (21)	0.62

Table 2. Correlation of previously identified covariates with common bile duct dilatation within methadone users
* statistically significant difference following Pearson Chi-Square, p<0.05

CBDD was present in 26 of 46 (57%) MU and in eight of 479 (2%) non-MU. The intrahepatic biliary tract was dilated in five of 34 patients with CBDD and all these five patients used methadone. The pancreatic duct was not dilated in any of the patients with CBDD. CBD diameter was measurable in 42 of 46 MU and in 229 of 484 non-MU. In the remaining patients the CBD was too small to visualize or too small to measure. The CBD diameter distribution, according to the use of methadone, is presented in figure 1a. Both with and without methadone, the vast majority of measurements were closely distributed with a 95% distribution ranging from 3.3 to 3.7 mm and from 6.3 to 7.9 mm, respectively (figure 1b). Methadone dose was reported in 42 of 46 patients with a median of 73 mg (range 10 – 200 mg) and a median of 70 mg (10 – 120 mg) in patients with and without CBDD (p=0.88). Duration of methadone use was only seldom reported. Three patients used methadone in the past, but not at time of imaging; for these patients the last dose was at least one year before imaging and in these patients CBDD was not observed.

US was repeated during the study period in four non-MU with CBDD in the study period; CBDD was not seen. The remaining 30 patients with CBDD were indicated to undergo further investigation. Further investigation, besides the repeated abdominal US, was performed in two of four non-MU and in 12 of 26 MU. In the first non-MU, EUS revealed a normal, undilated CBD diameter; in the second non-MU, Contrast-enhanced US demonstrated HCC in segment IV. It should be noted that one of the remaining two non-MU with CBDD used morphine, which has been described to be able to induce CBDD as well. Among MU, subsequent investigation showed persistent dilatation without obstructive moment in 10 of 12 patients and a normal CBD without dilatation in the other two patients. Methadone dose did not differ statistically significant between patients with persistent CBDD and patients without CBDD at time of further investigation (p=0.375). Both in MU and non-MU, patients with CBDD did not show clinical or laboratory signs of cholestasis at time of imaging. Twenty-four of 26 MU with CBDD and three of four non-MU with CBDD did not show any signs of clinical worsening and in particular no signs of cholestasis. Obviously, the non-MU that was diagnosed with HCC was treated accordingly instead of follow-up.

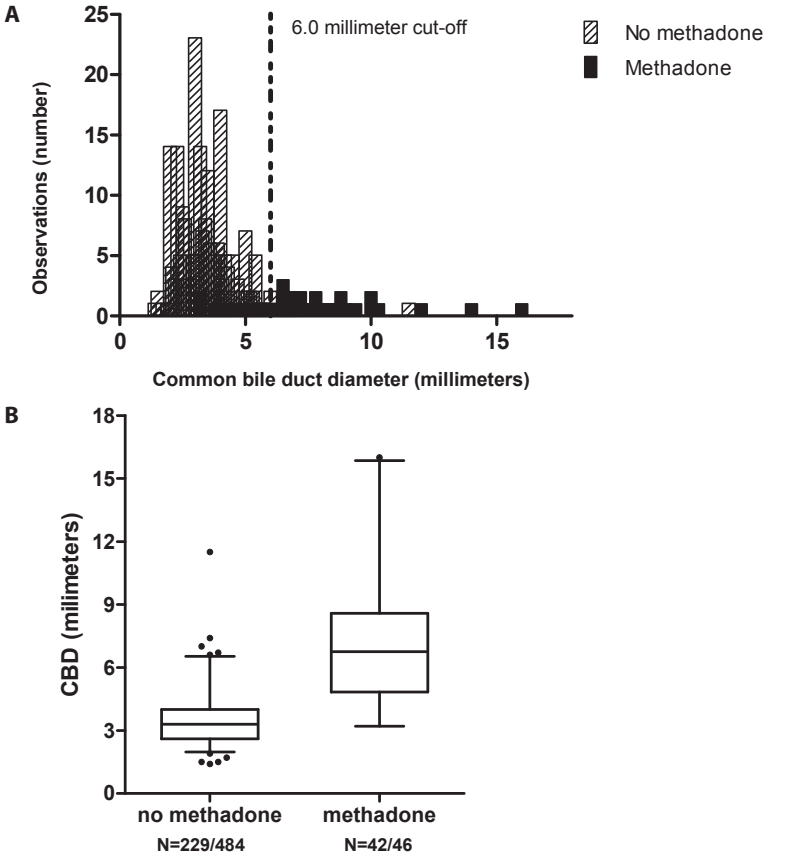

Figure 1. Common bile duct diameter measurements according to the use of methadone.
a. Histogram according to the cut-off of 6.0 millimeters.
With methadone use, median diameter 3.5 millimeters, range: 1.4 – 11.5 millimeters.
Without methadone use, median diameter 7.0 millimeters , range: 3.2 – 16.0 millimeters.
b. Box-plots
The solid line indicates the median value, the box shows the range where 25-75% of the data fall (called the inter-quartile range IQR). The "whiskers" show points that are within the 2.5-97.5th percentile. The dots indicate outliers.
Means are statistically significantly different following independent samples T-test, $p<0.001$.

DISCUSSION

In the present prospective observational study, we demonstrated that CBDD was found in 6.6% of patients referred for abdominal US at the gastroenterology and hepatology department of a tertiary referral center and that CBDD was independently associated with use of methadone ($p<0.001$). Furthermore, 95% of CBD diameter measurements were distributed closely together with (6.3 – 7.9 mm, median 6.8 mm) and without methadone use (3.3 – 3.7 mm, median 3.3 mm). Therefore, our results suggest a different upper limit of normal (ULN) than the widely used ULN of 6 mm according to the use of methadone.

Other previously described factors that could enlarge the CBD diameter are cholecystectomy and age[14, 15]. Bachar et al demonstrated an increase in CBD diameter with age over 50 years and Chawla et al found the largest CBD diameter in the seventh decade of life[3, 4]. Our study was designed to study factors of influence on the occurrence of CBDD in the general population without known biliary disease referred for abdominal US to our outpatient clinic. This resulted in a median age of 50 years (25^{th} – 75^{th} percentile: 39 – 58 years). Thus, we could not demonstrate an age-related increase in CBD diameter and this might be due to the relatively close age distribution of our middle-aged study population.

Our study is of clinical importance because it demonstrates the independent association of methadone use with CBDD. Previous reports were not prospective, did not include a control population, excluded patients with signs of cholestasis or concerned small numbers[16-18]. Moreover, the clinical implications of CBDD with methadone use have not been described or only in very small numbers[16]. To our knowledge, the largest prospective study in humans has been performed by Farahmand et al[17] who studied the CBD diameter of 110 chronic opioid users, and reported the distribution of CBD diameter classified by age. They found an increase in CBD diameter with increasing age and duration of opioid use. However, the type of use of opioids was not specified. In many countries, methadone is registered as maintenance therapy for heroin addiction. Different types of morphine-like narcotics have been described with a varying influence on the internal pressure of biliary ducts. Therefore, the prevalence of CBDD with chronic opioid use of 65.5%[17] cannot be applied to methadone users. Moreover, other types of medication or substance use that have been described with CBDD and are often used with opioids, e.g. antipsychotics, were not taken into account. Finally, patients with signs of cholestasis were excluded. Therefore, a comparison of patients with methadone use and CBDD with and without severe underlying illness and cholestatic expressions cannot be made. Because we aimed to study the clinical implications of CBDD seen with abdominal US, we did not exclude patients with (subclinical) cholestasis or other possible (early) signs of sever underlying illness.

By means of the present study design, we have controlled maximally for time-, patient- and investigator-related biases. Our study was prospective and exclusion criteria were based on the possibility of the presence of an altered anatomy of the biliary tract. Hence, our patient population was not selected by indication for imaging. Different investigators of the same center performed the measurements with abdominal US. Moreover, our study was not hampered by sample size as data were large enough to study and control for types of medication and/or substance use often used together with methadone. Unfortunately, we have not succeeded to obtain further examination in all of patients with CBDD. Especially MU were difficult to trace and, if able to reach, often unwilling to undergo further investigation. On the other hand, median follow-up was long with 9 months (range: 1-18) and 16 months (range: 1-17) in MU and non-MU, respectively. Except for one non-MU in whom liver malignancy was diagnosed, no patient showed signs of clinical worsening during follow-up, in particular no signs of cholestasis. Thus, severe malignancy is highly unlikely to underlie CBDD in the remaining patients

affected. Nevertheless, long-term outcome of patients with CBDD on methadone is unknown and future research should focus on natural course of CBDD. Secondly, we should note that we have not elucidated the pathophysiologic mechanism of sphincter of Oddi dysfunction in our patients with CBDD using methadone. The golden standard would be measurement of CBD internal pressure in patients with and without methadone use and with and without CBDD. This should be performed with sfincter of Oddi manometry, which is a highly specialized procedure associated with an increased risk of procedural complications with reported rates of pancreatitis as high as 19%[19]. We did not embark on this procedure in our patients, because the effects of morphine and morphine-like narcotics have been studied with, to our opinion, conclusive and solid arguments for sphincter of Oddi dysfunction in patients on opioid prescription. Proof of concept of morphine as a cause of sphincter of Oddi dysfunction in humans originates from a study by Helm et al in 1988[20]. Hereafter several research groups addressed the pathophysiology of the biliary effects of morphine[7,20,21]. Using manometry, Wang et al demonstrated that morphine administration leads to increased s pressure[7]. In 2002, Barlas et al demonstrated that opioids given before nuclear hepatobiliary imaging are associated with delayed CBD visualization (28.6% with versus 12% without opioid administration)[11]. In a narrative review of initial studies on biliary pressure after narcotic administration in animals, and postoperative and intraoperative cholecystetomy patients, in 2002, Thompson concluded that narcotics induced increases in phasic wave frequency interfering with sphincter of Oddi filling. Furthermore, increases in phasic wave frequency were considered responsible for the often observed increase in bile duct pressure[21]. In summary, these studies are consistent with our findings, given that in some patients with methadone use and CBDD, CBD diameter was normal with subsequent imaging. We consider current literature consistent and reliable in the finding that morphine (-derived break-down products) can cause sphincter of Oddi dysfunction.

The clinical relevance of our findings is that they enable to prevent excessive medical risk and costs in methadone users. Chronic HCV in Western countries is increasingly diagnosed in (former) substance users which often use methadone. Still, as many of these patients are middle-aged, CBDD should be considered as a sign necessating further work-up until proven otherwise. Because we studied prevalence and clinical implications of CBDD, our study did not allow pharmacological intervention. Future research should focus on long-term follow-up of methadone users with CBDD. More importantly, the exact pathophysiological mechanism responsible for the association of methadone use with CBDD may be studied.

To conclude, we demonstrated an independent association of methadone use with CBDD. In MU with CBDD without signs of cholestatis, CBDD was not associated with severe underlying pathology and patients did not suffer biliary obstruction. If CBDD is observed at abdominal ultrasonography, drug use, in particular methadone use, should be questioned. Furthermore, non-MU, the ULN CBD diameter is much smaller than previously described. Finally, our observations regarding CBD diameter in both MU and non-MU need validation in other cohorts.

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CHAPTER 2

NATURAL KILLER CELL ACTIVITY AND FUNCTION IN CHRONIC HCV-INFECTED PATIENTS DURING PEGINTERFERON AND RIBAVIRIN: EARLY EFFECTS OF ACTIVIE SUBSTANCE USE

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Antiviral Res. 2013 Jan 2;97(3):347-355.

ABSTRACT

In Western countries, chronic hepatitis C virus (HCV)-infection mostly affects former and active substance users. The effect of active substance use on interferon (IFN)-responsiveness and therapy efficacy is not well understood. In this study, we compared natural killer (NK) cell activity and function in healthy controls and chronic HCV-infected patients with and without active substance use, as well as the early effects of antiviral therapy with peg-IFN and ribavirin. No differences were observed between chronic HCV patients and healthy individuals in the number and frequencies of $CD56^{dim}$ and $CD56^{bright}$ NK cells. Also, IL-12/18-induced IFN-gamma production by NK cells was comparable between all groups, whereas the cytotoxic ability of NK cells (granzyme and CD107a levels) was more potent in HCV-infected patients as compared to healthy controls, and highest in non-substance users. Moreover, at baseline, the activation of NK cells was significantly lower in HCV-infected patients who used substances, when compared to healthy individuals. Therapy-induced viral load reduction assessed early at day 7 showed a similar decline in substance users and non-substance use HCV patients, with 25% substance users and 17% non substance users testing HCV-RNA negative at day 7. Furthermore, early during IFN-based therapy, NK cells from HCV patients remained responsive to IFN, and only a minor decline in the degree of STAT-1 phosphorylation was observed irrespective of substance use. These findings were further supported by comparable *in vitro* p-STAT-1 induction in all 3 experimental groups. Despite subtle differences at baseline between healthy individuals and chronic HCV patients, we observed that active substance use in chronic HCV-infected patients did not affect the immune responsiveness to IFN early after start of treatment, and thus, we found no evidence -from an immunological point of view- that antiviral therapy of our cohort of HCV-infected patients with active substance use is less efficient.

1. INTRODUCTION

Hepatitis C is a blood-borne virus infection with an estimated 170 million infected individuals worldwide and a prevalence of 3%. Chronic hepatitis C virus (HCV) infection carries an increased risk of developing liver cirrhosis and hepatocellular carcinoma and is currently the leading cause of end-stage liver disease [1, 2]. In chronic HCV infection, viral clearance can be achieved by antiviral therapy consisting of pegylated interferon-alpha (peg-IFN) and ribavirin for 24-48 weeks in 41-86% of individuals [3-6]. The HCV genotype and host gene polymorphisms are important factors determining the success of treatment. In combination with the recently introduced protease inhibitors telaprevir or boceprevir, which inhibit specific steps in viral replication, peg-IFN and ribavirin remain the backbone of chronic HCV treatment [7, 8].

In the developed world, injection drug use (IDU) is the most important risk factor for acquiring HCV [9]. Recent studies suggested that the burden of HCV would be lowered if active substance users would be offered antiviral therapy more often [10]. Substance users have been excluded from antiviral therapy for a long time because of the side effects of peg-IFN and the supposed increased risk of re-infection [11-13]. However, several research groups have demonstrated feasibility of antiviral therapy in former substance users with comparable rates of SVR and low rates of re-infection [14, 15]. Unfortunately, some studies excluded active substance users at the initiation of antiviral therapy or included only small numbers [14, 16]. As IDU constitutes the major mode of transmission, it is of clinical importance to know if active substance use affects the outcome of antiviral therapy. Dore et al. found that drug dependency was independently associated with a lower rate of SVR [15]. Grebely et al studied the outcome of antiviral treatment in former and current drug users and observed a significantly lower rate of SVR in a small subset of frequent injectors [17]. To treat active substance users effectively, more knowledge is needed on the potential effects of concomitant substance use on the effects of peg-IFN and ribavirin.

Peg-IFN exerts its antiviral effects through the induction of numerous products encoded by IFN-stimulated genes (ISG), which possess direct antiviral activity. Apart from the direct effects, the impact of IFN-based treatment on immune cells may also be important in determining the treatment response [18], and it was only recently reported that the induction of cytotoxic natural killer (NK) cell function by IFN-α correlates with virologic response to therapy [19]. This is highly relevant since NK cells can recognize virus-infected cells, and eliminate them via cytolytic (e.g. via perforins and granzymes) and non-cytolytic (e.g. via IFN-γ or TNF) pathways [20-22].

It is generally accepted that heroin deregulates the function of T-cells, B-cells and NK cells *in vitro* and *in vivo* [23-25], as well as *in vitro* HCV replication in cell culture [26-28]. However, little is known on the effects of substance use during IFN-based therapy on responsiveness of immune cells, such as NK cells. Cocaine appears to negatively affect human $CD4^+$ T-cell activation [29], and inhibitory effects have been observed on the functions of all subsets of lymphocytes and monocytes/macrophages in mice [30]. In contrast, in HCV-infected patients, the use of the combination

of both cocaine and heroin led to augmented levels of both Th1 and Th2-associated cytokines, while lymphocyte proliferation was reduced [31].

In several industrialized countries, heroin is prescribed as maintenance therapy. When treating patients who receive maintenance therapy, it is important to understand the clinical and biological outcome of the drug interactions. In addition, heroin use under supervision provides a controlled setting to study the effects of ongoing heroin use during antiviral therapy. Therefore, we aimed to study the effect of heroin as well as cocaine use on NK cell frequency and function before and during antiviral therapy with peg-IFN and ribavirin.

2. METHODS

2.1. Patients and antiviral therapy

Twenty-three patients with chronic HCV infections and 12 healthy individuals were included in the study (Table 1). Patients were seen at our outpatient clinic and at the local addiction treatment center. For the exact type, way and frequency of substance use, we refer to Supplementary Table 1, which also presents information on the use of nicotine, alcohol and medication. Data on illicit substance use as well as nicotine and alcohol use were reported by the patient to the physician and/or nurse of the addiction care unit, and is considered reliable since they receive maintenance therapy and are being seen at the addiction treatment center for many years. Also, the absence of substance use was reported by the HCV-infected patients themselves at the outpatient clinic. HCV-infected patients without substance use and healthy individuals were included only when they were not treated by the addiction treatment center and when there was no suspicion of any recent substance use. The physician questioned all patients for their habits related to substance use prior to inclusion and during antiviral therapy. According to standard clinical care and the guidelines for antiviral treatment of chronic HCV patients of the AASLD, (chronic) infection with the hepatitis B virus and/or human immunodeficiency virus was a contra-indication and hence tested for before initiation of therapy. Antiviral therapy consisted of peg-IFN-2b and ribavirin for 24-48 weeks. Peg-IFN-2b was given subcutaneously at 1.5 mg/kg/week; ribavirin was dosed 800-1200 mg (for all genotypes, and depending on weight). Blood was collected before the injection of peg-IFN-2b at baseline and at day 7 of antiviral therapy. Use of peg-IFN-2b and heroin were well controlled; at both treatment sites peg-IFN-2b was administered by a nurse, and heroin was prescribed and used at the addiction treatment center. HCV-RNA levels were monitored using the Ampliprep/Cobas Taqman HCV/HPS assay (Roche Molecular Systems; detection limit: 15 IU/mL). Genotyping of the IL28B-associated SNPs rs12979860 was performed using competitive allele-specific PCR in blood (KASP; kBioscience). The institutional review board of the Erasmus MC approved the protocols, and informed consent was obtained from all individuals.

Table 1. Baseline characteristics

	HCV-infected patients without substance use N=11	HCV-infected patients with substance use N=12	Healthy controls N=12
Gender, n (%)			
Male	9 (82)	9 (75)	7 (58)
Age, years			
Median (range)	51 (26-64)	53 (45-64)	50 (38-61)
Viral load, baseline IU/mL			
Median (range)	291000 (1420 - 9460000)	925000 (106000 - 3570000)	
Alanine aminotransferase (ALT), baseline, U/L			
Median (range)	84 (31-472)	58 (18-123)	
Cirrhosis			
n (%)	2 (18)	2 (17)	
IL28 rs12979860 genotype, n (%)			
CC	3 (27)	7 (58)	
CT	5 (45)	4 (33)	
TT	3 (27)	1 (8)	
Genotype, n (%)			
1	3 (27)	4 (33)	
2	1 (9)	0	
3	6 (55)	6 (50)	
4	1 (9)	1 (8)	
6	0	1 (8)	

2.2. Enumeration of leukocyte populations in whole blood, and quantitation of lymphocyte subpopulations

Absolute numbers of leukocytes, lymphocytes, monocytes and granulocytes in whole blood were measured by an automated impedance hematology analyzer (ABX Micros-60, Horiba Medical). To determine the frequency of distinct leukocyte subpopulations, PBMC were stained for CD3, CD56, and CD14. NK cells were defined as CD3-negative lymphocytes that expressed CD56, including $CD56^{dim}$ and $CD56^{bright}$ NK cells. All flow cytometric measurements were evaluated using a FACS Canto-II, and analyzed using Diva software (BD). Peripheral blood mononuclear cells (PBMC) were isolated from venous blood by ficoll separation (Ficoll-Paque™ plus, Amersham).

2.3. Flow cytometric analysis of pSTAT-1 staining

For determination of the phosphorylation of STAT-1, frozen PBMC were thawed and rested for 1h by incubation at 37°C in culture medium (RPMI1640 supplemented with L-glutamin, penicillin,

streptomycin, HEPES, and 5% human serum (Lonza). $2x10^6$ PBMC were stimulated in 200 µl with 10,000 U/ml IFNα-2b (Intron A, Schering-Plough). After 30 min, the cells were washed with PBS, incubated for 20 min with 2% formaldehyde and stained with CD3-Pacific Blue (UCHT1, BD Pharmingen), and CD56-APC (N901, Beckman). Stimulated cells were incubated with BD Phosflow Perm III buffer (BD) for 12 min on ice. After washing, cells were stained for 15 min with pSTAT-1-Alexa-Fluor-488 (4a, BD), and the phosphorylation state of STAT-1 was measured by flow cytometry. The medium condition was used to set the threshold for the IFNα condition.

2.4. Expression of intracellular and cell surface molecules by flow cytometry

Cytokine production by NK cells in PBMC was determined upon stimulation with 100 ng/mL IL-18 (MBL International Corporation) plus 10 ng/mL IL-12 (Miltenyi Biotec) for 24h in 48-well plates ($2x10^6$ cells / 250 µl). Brefeldin A (10 µg/mL; Sigma Aldrich) was added for the last 3h, before fixation with 2% formaldehyde. After washing, cells were permeabilised with 0.5% saponin (VWR), and stained with CD56-PE (MY31), CD3-PerCp-Cy5.5 (UCHT1), CD69-APC (L78) and IFNy-FITC (25723.11, all BD) for 15 min. The medium condition was used to set the threshold for the IL-12/IL-18 condition. Results are expressed as percentage of cytokine producing cells within the NK cell population.

For perforin and granzyme B staining, frozen PBMC were thawed, rested and fixed with 2% formaldehyde. After washing, the cells were permeabilized with 0.5% saponin for 10 min and then incubated with perforin-PerCp-Cy5.5 (dG9, eBioscience), granzyme B-PE (GB11, eBioscience), CD56-APC (N901, Beckman), CD3-Pacific Blue (UCHT1, BD Pharmingen), and CD69-PECy7 (TP1.55.3, eBioscience). Stained cells were analysed by flow cytometry.

2.5. CD107a degranulation assay

To measure degranulation of NK cells upon stimulation with K562 target cells, frozen PBMC were thawed and rested overnight in culture medium at 37°C. PBMC (250,000/200 µl) were then seeded with K562 cells in a 96 wells plate in a 10:1 ratio. CD107a-PE (H4A3, BD Pharmingen) was added to the culture. After 90 minutes, monensin (BD) was added, and the cells were incubated for an additional 3.5h under the same conditions. Samples were further stained using CD3-PerCp-Cy5.5 (UCHT1, BD) and CD56-APC (N901, Beckman). The medium condition was used to set the threshold for the K562 condition.

2.6. Statistical analysis

Among the three groups of participants (patients with and with substance use and healthy controls) mean values were compared and tested for statistically relevant differences using the Kruskal-Wallis test. Among patients with and without substance use, mean values were compared and tested for statistically relevant differences using Mann-Whitney.

3. RESULTS

3.1. Circulating leukocyte numbers are not affected in chronic HCV patients regardless of active substance use.

We first examined whether the absolute numbers of leukocyte populations in peripheral blood of chronic HCV patients differed from healthy controls and whether substance use affected their numbers. As shown in Figure 1, we observed that the absolute number of monocytes and lymphocytes, but not leukocytes and granulocytes, differed among healthy individuals and both groups of chronic HCV patients.

Recently, the induction of NK cell function by IFN-α has been shown to correlate with virologic response to therapy [19]. To examine possible differences between healthy individuals and chronic HCV patients with and without substance use, we focussed on NK cells, and observed no differences between the 3 groups in the absolute numbers of circulating $CD3^{-}CD56^{+}$ NK cells in blood (Figure 2). Within the NK cell compartment, also no significant differences were observed in the ratio between $CD56^{dim}$ and $CD56^{bright}$ NK cells between healthy controls and chronic HCV patients, irrespective of their substance use.

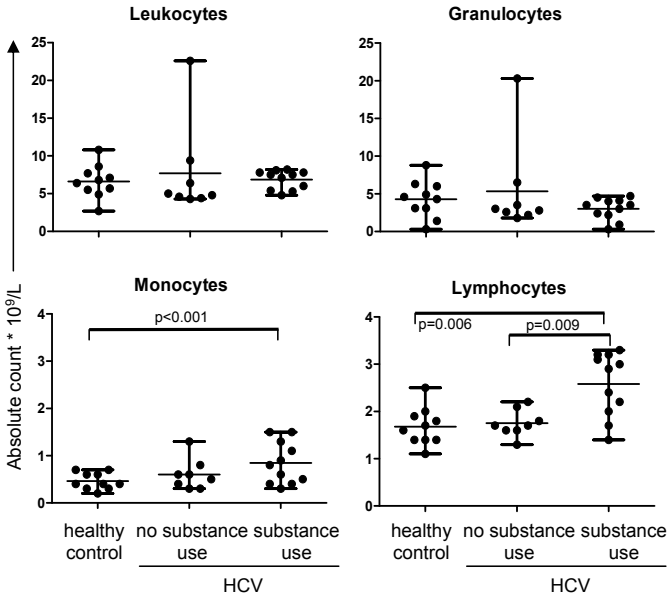

Figure 1. Circulating leukocyte numbers are not affected in chronic HCV patients who are active substance users. The individual absolute cell counts ($x10^9$ cells/liter) in blood of healthy individuals and chronic HCV patients are depicted with median and range. Some data points are not presented since the leukocount was not determined. The comparison of 3 groups and 2 groups have been performed and tested for statistical significance using Kruskall-Wallis test and Mann-Whitney test, respectively.

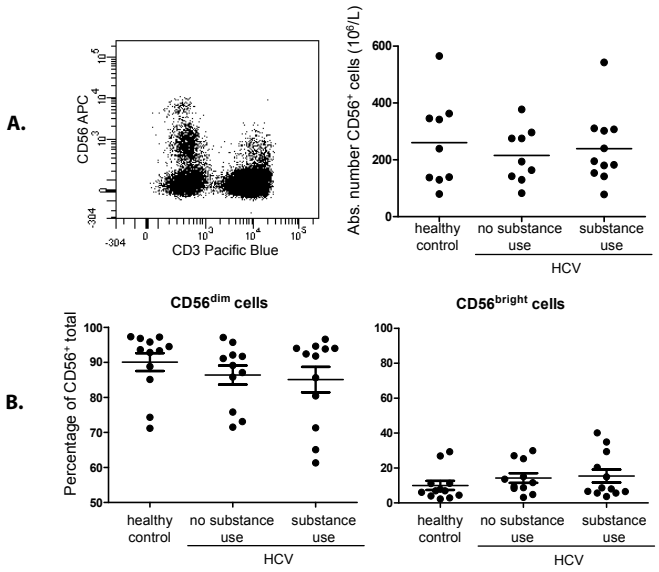

Figure 2. The numbers and composition of NK cells in blood were not affected by chronic HCV infection, or substance use. (A) The absolute number of $CD3^{-}CD56^{+}$ NK cells in in peripheral blood of healthy individuals and chronic HCV patients, with and without substance use, is shown. Some data points are not presented since the leukocount was not determined. (B) Lymphocytes were identified on the basis of their FSC/SSC profile, and further characterized by flow cytometry using CD56 and CD3 specific antibodies. The contribution of the specific subpopulation within the total NK cell pool is shown.

3.2. At baseline, NK cells from chronic HCV patients who are substance users show reduced expression of activation and cytotoxicity markers compared to non-substance users.

Next, we examined the functionality of circulating NK cells in more detail by evaluating if their activation status and function was affected as a consequence of chronic HCV infection and substance use. As shown in Figure 3A, flowcytometric analysis showed that the activation status of $CD56^{dim}$ NK cells, as demonstrated by CD69 expression, was lower only in patients who used substances, but not in HCV-infected non-users, as compared to healthy individuals. No differences in the frequency of CD69-expression $CD56^{bright}$ NK cells were observed between the 3 groups. NK cells are known to produce high levels of IFN-γ upon viral infection, which may lead to priming or activation of innate cells, and to modulate adaptive immune responses [21]. We now show that upon exposure to IL-12 and IL-18, similar frequencies of IFN-γ producing NK cells were observed when comparing patients and healthy individuals (Figure 3A). Also, substance use did not affect the frequencies of NK cells that produced IFN-γ.

NK cells are capable of direct cytolysis of virus-infected cells, which is dependent on release of granules containing perforins and granzymes [32]. Degranulation of these granules, as an indication of cytotoxic potential, can be determined by assessing CD107a expression upon co-culture with K562 target cells. As shown in Figure 3B, the percentage of NK cells in PBMC

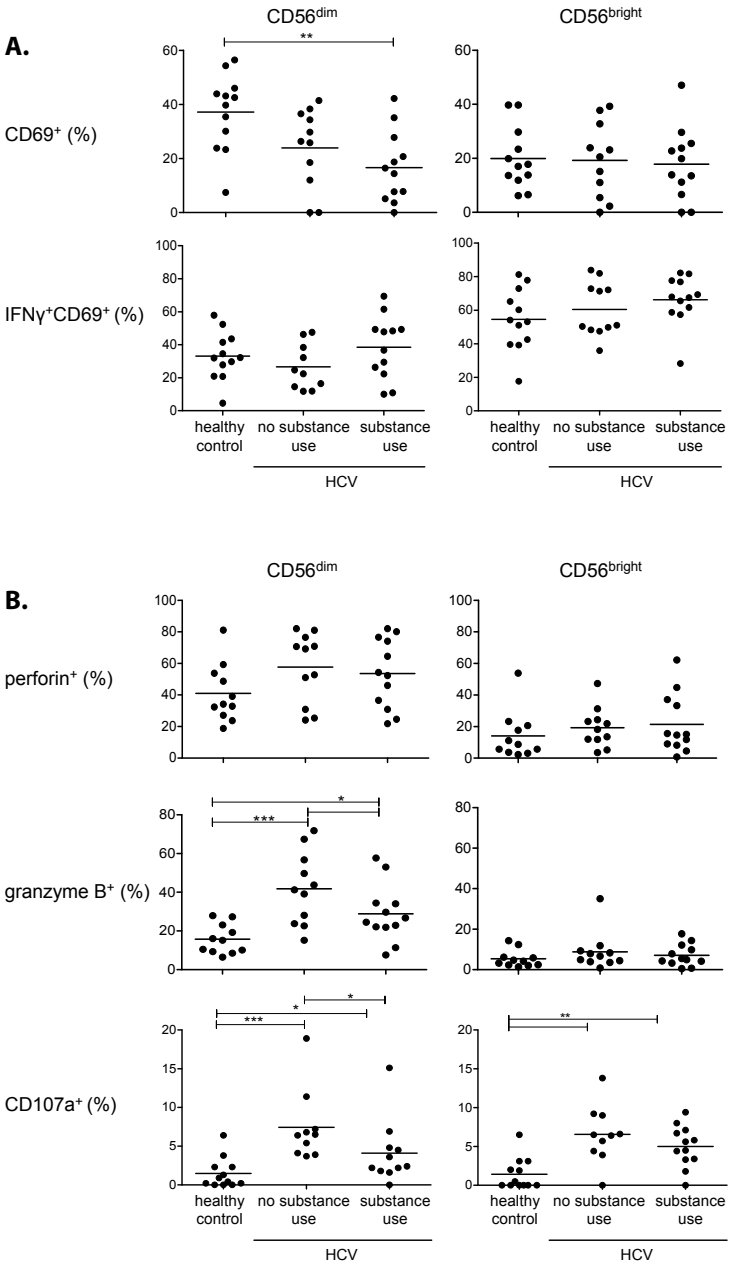

Figure 3. At baseline, CD56^{dim} NK cells from chronic HCV patients who are substance users show reduced expression of activation and cytotoxicity markers compared to non-substance users. (A) CD69 expression and intracellular IFNy upon stimulation with IL-12 and IL-18 for 24h were detected in PBMC of healthy controls, HCV patients who are non-users and patients with substance use by flowcytometric analysis. (B) Intracellular perforin, intracellular granzyme B, and CD107a expression were determined in CD56^{dim} and CD56^{bright} NK cells. Some data points are not presented since these samples did not show distinctive CD56^{dim} and CD56^{bright} NK cell populations. Individual data and the mean are depicted.

that stained positive for perforin was similar between all 3 groups. In contrast, the frequency of granzyme B-positive CD56^{dim} NK cells, as well as the frequency of CD107a-expressing CD56^{dim} and CD56^{bright} NK cells was significantly increased in chronic HCV patients as compared to healthy individuals. Interestingly, patients who did not use substances had higher levels of CD56^{dim} cells expressing granzyme B and CD107 as compared to chronic HCV patients who were substance users (Figure 3B).

3.3. In vitro, IFN-α induced pSTAT-1 levels are similar between substance user and non-user patients.

Next, we examined, *in vitro*, the responsiveness of NK cells to IFN-α in PBMC from chronic HCV patients to determine whether this was affected by ongoing heroin use. To study this, phosphorylation of STAT-1 was measured by flowcytometry upon exposure to IFN-α *in vitro*. As shown in Figure 4, no significant differences in pSTAT-1 levels were detected in the 3 experimental groups studied (Kruskall-Wallis test). These findings suggest that in our cohort of heroin and cocaine users the responsiveness of NK cells to IFN-α *in vitro* did not differ from NK cells obtained from non-user chronic HCV patients prior to therapy.

3.4. Therapy-induced modulation of pSTAT-1 levels by NK cells of substance user and non-user chronic HCV patients.

To study the consequences of heroin and cocaine use in more detail, we assessed if the efficacy of antiviral IFN-based therapy of chronic HCV patients with no substance use differed from patients with substance use. At the early stages after start of antiviral treatment no differences were observed in the decline of serum HCV-RNA levels at day 7, and in fact both groups showed non-detectable levels in the majority of patients (Figure 5). These findings suggest that in our patient cohort, substance use did not adversely affect the viral load decline in patients early after start of treatment (Mann-Whitney).

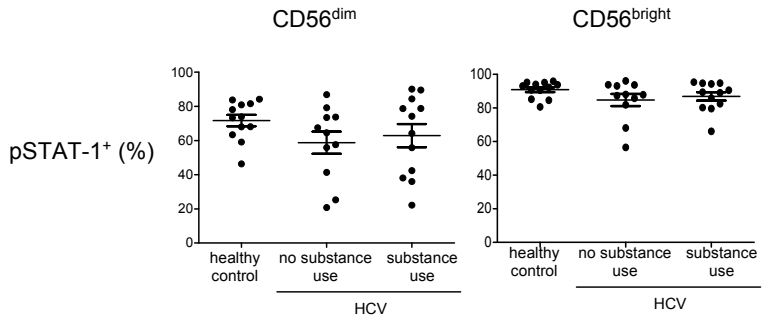

Figure 4. ***In vitro*, IFN-α induced pSTAT-1 levels are similar between substance user and non-user patients**. PBMC from healthy controls, HCV patients who are non-users and patients with substance use were stimulated with IFNα for 30 minutes. The pSTAT-1 levels were measured by flow cytometry. Data were analyzed by Kruskall-Wallis testing and no significant differences were observed.

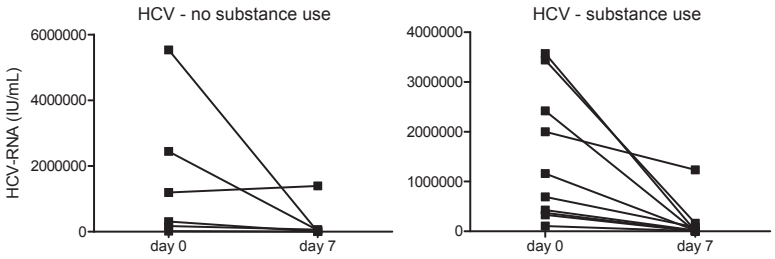

Figure 5. Therapy-induced viral decline in chronic HCV patients who are substance users is similar as in non-users (p=0.233). HCV RNA levels were determined in serum before the start of antiviral therapy (peg-IFN and ribavirin) and after 7 days. Individual data of HCV patients who are non-users and patients with substance use are presented. Data were analyzed by non-parametric Mann-Whitney testing and no significant differences were observed.

Additionally, little is known on the effects of substance use during therapy on immune cells, including NK cells, and we therefore also investigated the modulation of IFN-responsiveness by NK cells during IFN-based therapy in chronic HCV patients. Comparison of STAT-1 phosphorylation before and 7 days after start of therapy with peg-IFN-2b and ribavirin resulted in a reduction of pSTAT-1 levels as a consequence of therapy (Figure 6). Lower pSTAT-1 levels in $CD56^{dim}$ NK cells *on therapy* as compared to *before therapy* were observed for chronic HCV patients, and this decline was more pronounced in non users as compared to substance users. In contrast, reduction of the frequency of pSTAT-1-expressing $CD56^{bright}$ NK cells as a consequence of therapy was only observed in patients who did not use substances, but not in chronic HCV patients with ongoing substance use. The early effects of therapy, determined at day 7 after start of treatment, on the frequency of IFN-γ producing NK cells, and on NK cells expressing CD69, granzyme or perforin were modest in both experimental groups. Besides the effect on pSTAT-1 induction, the only significant difference observed was a mildly lower frequency of IFN-γ producing $CD56^{bright}$ cells in substance users versus non users.

4. DISCUSSION

In this study we examined whether use of morphine-derived products or cocaine affected NK cell function in chronic HCV patients both at baseline and at the early stages of peg-IFN/ribavirin therapy. Evaluation of the early effects of IFN-based treatment demonstrated that there is no indication for weaker responses to IFN-α in chronic HCV patients who are substance users as compared to non-users. Therapy-induced decline in viral load as well as responsiveness to IFN-α *in vitro* were similar between both groups at day 7 of antiviral therapy.

In our study we chose to examine the early response of chronic HCV patients to therapy in order to simultaneously examine the clinical effects (HCV RNA decline) as well as the sensitivity of NK cells to IFN-α. Our findings show that in both groups, at baseline, similar frequencies

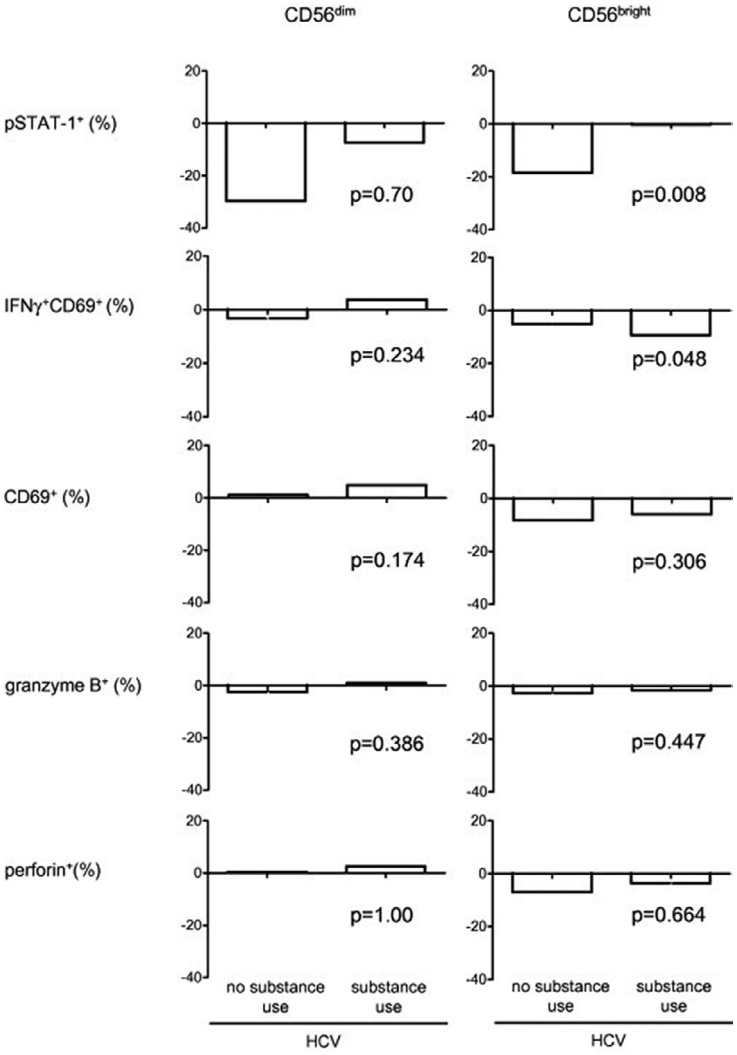

Figure 6. Therapy-induced modulation of the activity of NK cells of substance user and non-user chronic HCV patients. IFNα induced pSTAT-1 levels, CD69 expression and intracellular IFNy upon stimulation with IL-12 and IL-18 for 24h, and intracellular perforin and granzyme B are determined in $CD56^{dim}$ and $CD56^{bright}$ NK cells from HCV patients who are non-users and patients with substance use. The mean change in frequency of the parameters is depicted by comparing the frequencies before treatment and 7 days after start of antiviral therapy. Data were analyzed by non-parametric Mann-Whitney testing.

of pSTAT-1 expressing NK cells were seen. This finding indicates that use of heroin or cocaine does not affect responsiveness of NK cells at the level of IFN-α receptor expression or the direct downstream signalling events (Figure 4). Further, in both groups of HCV patients, irrespective of the use of heroin or cocaine, HCV RNA load declined strongly immediately after start of therapy, already within the first week. These findings are in agreement with clinical reports showing the high and comparable rates of SVR irrespective of substance use [33-35].

A general feature of cytokine exposure of cells is the initiation of a negative feedback mechanism that prevents long-lasting and excessive activation of cells. Also, negative feedback mechanisms have been described following IFN-α exposure [36]. Comparison of STAT-1 phosphorylation before start of therapy and on day 7 during treatment, showed that in chronic HCV patients who did not use substances, the pSTAT-1 levels were reduced as a consequence of exposure to peg-IFN and ribavirin for 1 week in both the $CD56^{dim}$ and $CD56^{bright}$ NK cells. Chronic HCV patients with substance use showed a milder reduction of pSTAT-1 expressing $CD56^{dim}$ NK cells, and no modulation of the frequency of pSTAT-1-positive $CD56^{bright}$ was observed by comparing day 0 and day 7. These findings demonstrate that the phenomenon of negative regulation to repeated IFN-α exposure does not lead to stronger impairment of responsiveness of NK cells from substance users than NK cells from chronic HCV patients who do not use substances. In fact, the lack of down-regulation of pSTAT-1 frequencies in $CD56^{bright}$ NK cells may even suggest that cells from substance users are weakly more sensitive to IFN as compared to the control patients. Therefore, also on treatment, there are no indications for impaired sensitivity or responsiveness of NK cells, and therefore our immunological study supported the clinical observations that substance use did not adversely affect the efficacy of IFN-based therapy. In line with our findings on pSTAT-1, we also found that therapy-induced modulation of the expression of intracellular perforin and granzyme B by NK cells was similar between substance user and non-user chronic HCV patients (data not shown).

In addition to the examination of their IFN-responsiveness, we also examined the NK cell compartment at baseline of both patient groups to determine if the immune status of substance users differed from non users. At baseline, $CD56^{dim}$ NK cells from chronic HCV patients who are substance users show lower expression of activation and cytotoxicity markers than non-substance users. The reason for the reduced activation state of NK cells in substance users, as shown by their CD69 expression, is currently unknown, although possible explanations may include a direct effect of the drug metabolites on NK cells as well as indirectly via altered serum cytokine profiles. Functionally, we found no indications that in patients who use heroin or cocaine the frequency of IFN-γ producing NK cells is affected, suggesting that this non-lytic mechanism to control infected cells is intact. In contrast, the frequencies of granzyme B- as well as CD107a positive $CD56^{dim}$ NK cells in substance users were lower as compared to non users, indicative of a weaker cytotoxic potential of the abundant subpopulation of $CD56^{dim}$ NK cells in substance users. The clinical consequences of these findings are difficult to assess. However, defective NK cells may be relevant for the control of viral load, but also highly relevant for disease progression, since it was reported recently that NK cells play an important role in the development of fibrosis [37, 38]. Several limitations should be noted. First of all, the number of subjects in this study is quite small. We chose the design of a pilot study because, to our knowledge, no comparable study has been published so far. Therefore, power calculation based on estimated clinically relevant differences could not be made. Secondly, within our group of substance users, we included both heroin and cocaine users. The effects on NK cell function of

both of these compounds may differ, as has been described in the introduction section, since the breakdown products of these illicit drugs are distinct. The patients are therefore a heterogeneous group of individuals, which resembles the actual group of substance users. Thirdly, we can not exclude that compounds other than heroin or cocaine, such as cannabis, alcohol or nicotine may influence the parameters determined in our study, and that these uncontrolled cofactors in the group of drug users contribute to the observed differences. Finally, it is important to mention that the size of the experimental groups as well as the controlled environment of the addiction treatment center may affect the clinical and immune parameters measured, which may influence the predictive value to all heroin or cocaine users.

In summary, although our study was performed on a relatively small number of patients, our study shows that despite subtle difference in NK cell parameters, we could not provide evidence that the IFN responsiveness of chronic HCV patients, upon *in vitro* stimulation as well as during the early stages of IFN-based therapy, was affected by use of heroin or cocaine.

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Supplementary Table 1. Individual substance use at day 0

Gender	Age	Methadone use (mg/day)	Heroin	Way of use	Frequency	Cocaine use	Route	Frequency	Alcohol use	Cannabis (joints)	Nicotin use	Amount per week	Other medication
Female	54	no	Illicit	Smoking	5 days 1/4 gr/day	Yes	Smoking	5 days 1/4 gram a day	Abuse in history, clean on alcohol for years.	None	shag	3 packages/ week	none
Male	49	80	On prescription	Smoking	400mg OD	None	NA	NA	once/2weeks one beer	One /day	shag	2 packages/ week	promethazine 25 mg OD
Male	45	70	On prescription	Injecting	120mg OD	None	NA	NA	once/month a few units	None	None		promethazine 50mg BID
Male	59	130	None	NA	NA	Yes	Smoking/ snorting	thrice weekly 0.5 gr	None	Sporadically	shag	2 packages/ week	once weekly diazepam, illicit use.
Female	41	120	Illicit	Smoking	once weekly 0.5 gr	None	NA	NA	Yes, 3-4 half litters of beer/ day	None	shag	1 package/ week	none
Male	53	110	Illicit	smoking	once weekly 0.10 gr	yes	smoking	once weekly 0.10 gr	Yes, 2 glasses of wine per day	None	shag	2 packages/ week	bromethazine 20mg TID diazepam 5mg TID movicolon if needed
Male	63	60	Illicit	snorting	daily 0.5gr	None	NA	NA	none	none	none	NA	NSAID because of ellbow pain
Male	49	120	On prescription	Smoking	400mg BID	None	NA	NA	Once in a month a few beers	none	shag	1.5 packages/ week	none
Male	46	120	Illicit	Smoking	twice weekly 0.5 gr	yes	Smoking	twice weekly 0.5 gr	none	one /week	shag	1 package/ week	omeprazol 20mg OD

Supplementary Table 1. Individual substance use at day 0 (*continued*)

Gender	Age	Methadone use (mg/day)	Heroin	Way of use	Frequency	Cocaine use	Route	Frequency	Alcohol use	Cannabis (joints)	Nicotin use	Amount per week	Other medication
Female	51	90	None	NA	NA	Yes	Smoking/ snorting	twice weekly 0.5 gr	Alcohol abuse in history, now once weekly 0.5L beer	None	yes	2 packages/ week	metformin 1000mg TID glibenclamide 10mg OD and 5 mg OD enalapril 5mg OD
Male	52	20	Illicit	Smoking	Once weekly 0.5 gr	Yes	Smoking	once weekly 0.10 gr	Once weekly two beers	None	cigarettes	1 - 3 cigarettes/ day	none
Male	41	75	illicit	smoking	Once weekly 0.5 gr	Yes	Smoking	Daily 1/4 gr	None	None	cigarettes	10 cigarettes/ day	sporadically diuretics in case of edema, now: none.

CHAPTER 3

Effects of selective serotonin reuptake inhibitor prophylaxis during antiviral treatment for chronic hepatitis C in patients with a history of injecting drug use and depression

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Resubmitted to the Journal of Clinical Psychiatry.

ABSTRACT

Objective. Psychiatric side effects of interferon(IFN)-based therapy are the most important cause of early treatment discontinuation in chronic hepatitis C virus (HCV)infected patients. We aimed to identify those HCV patients who benefit most from prophylactic treatment with selective serotonin reuptake inhibitors (SSRIs) during antiviral therapy. **Method**. We performed post hoc analyses on a prospective randomized controlled trial of escitalopram versus placebo during antiviral therapy with PEG-IFN and ribavirin, conducted between August 2005 and June 2008.and including 79 patients. We analyzed risk factors for depression and studied effects of prophylactic escitalopram on depressive symptoms. Depression was diagnosed using Mini International Neuropsychiatric Interview (M.I.N.I), a short structured interview used to diagnose DSM-IV-TR and ICD-10 disorders. Depressive symptoms were monitored on-treatment using the depression scale of Symptom Check List-90 (SCL-90), Montgomery-Asberg Depression Rating Scale (MADRS) and Beck Depression Inventory (BDI at baseline, week 4,12 and 24 of antiviral therapy. **Results.** Depression occurred in 14 patients receiving placebo and in 5 patients receiving escitalopram(Pearson χ^2, p=0.01). Combination of history of depression and injecting drug use (IDU) was associated with depression (odds ratio 12.60; 95% confidence interval 2.47–64.34,p<0.01). Moreover, SSRI treatment was associated with a significant reduction in estimated mean depressive symptoms measured by SCL-90(p=0.03) and BDI(p=0.048), but not with MADRS(p=0.64). **Conclusion.** HCV-infected patients with a history of depression and IDU carry the highest risk to develop IFN-induced depression. In this subset of patients, prophylaxis with escitalopram results in the most substantial decrease of IFN-induced depressive symptoms on the SCL-90 depression scale and the BDI.

Introduction

Chronic hepatitis C virus (HCV) infection is a major health problem with 170 million people being infected worldwide (World Health Organization. Hepatitis C—global prevalence (update). Weekly Epidemiological Record. 1999;74:425-7.World Health Organization, Hepatitis C—global prevalence (update). Weekly Epidemiological Record. 1999;74425-7). Antiviral therapy with pegylated interferon (PEG-IFN) and ribavirin (RBV) leads to sustained virologic response (SVR) in 41-84% of patients after 24 to 48 weeks of therapy. However, this antiviral therapy is associated with many side effects and these side effects are an important reason for dose reductions and treatment discontinuation in clinical practice [1]. In fact, psychiatric side effects of antiviral therapy have shown to be the most important cause of early treatment discontinuation. Irritability and depression are the most frequently reported psychiatric side effects with an incidence of 24% and 37% respectively[2-4]. Notably, sustained virological response rates may be similar in patients with psychiatric disorders to the response rates in other HCV patients provided that interdisciplinary care and antidepressant treatment are available [5]. In fact one study even suggested that interferon-induced depression might be positively correlated with sustained virological response, but a prospective study addressing this question directly found no effect of depression on SVR [6 7].

In our previously described retrospective cohort of HCV-infected patients treated with PEG-IFN/RBV[8], we found a similarly high incidence of depression (30%) and association of the presence of a history of injecting drug use (IDU) with new onset depression during antiviral therapy. Despite this high incidence of depression, depressive symptoms may be overlooked by routine clinical interviews, which are usually focused on physical rather than psychiatric complaints[4]. Although new direct-acting antiviral regimens are being developed, these agents still require PEG-IFN as a backbone of therapy and it can therefore be anticipated that interferon (IFN)-induced depression will remain a major challenge in the treatment of HCV.

Several baseline characteristics have been identified as predictors of depression of which higher depression scores at baseline and depression with previous IFN-based therapy are the most consistent ones in literature. A recent study suggested that antidepressant prophylaxis for each patient might prevent depressive symptoms and a major depressive episode[9], although other research groups could not demonstrate a significant advantage for SSRI prophylaxis in reducing the likelihood of developing major depression[10, 11]. A disadvantage of prophylactic SSRI treatment is that a significant amount of patients who would never have developed depressive symptoms would receive antidepressant treatment. Since IFN-induced depression affects only a subgroup of patients and has shown to be highly responsive to SSRI treatment, we hypothesized that the efficacy of SSRI prophylaxis in the prevention of IFN-induced depression would vary among HCV-infected patients. Therefore, we aimed to identify those HCV patients who would benefit the most from prophylactic SSRI treatment during PEG-IFN/RBV and to quantify the effect of prophylactic treatment in these patients.

Patients and methods

We performed post hoc analyses of a recent randomized, double-blind, placebo-controlled trial that investigated the effect of prophylactic treatment with escitalopram versus placebo[9]. The study was conducted between August 2005 and June 2008. We defined 'benefit' of SSRI prophylaxis as absence of a major depressive episode according to current DSM-IV classification[12] and/or significant reduction in estimated mean depression score during antiviral therapy when dosed escitalopram instead of placebo. Hence, our primary outcome measure was the association of baseline parameters with the development of depression during antiviral therapy; our second outcome measure was the score on the depression scales of the Symptom Check List-90 (SCL-90), Montgomery-Asberg Depression Rating Scale (MADRS) and Beck Depression Inventory (BDI)during antiviral therapy. Further, relation between success of antiviral therapy, SVR and risk factors for depression were investigated.

The original trial pursued to obtain a study population representative of the HCV-infected patient population in Western countries and therefore permitted use of psychotropic medication such as methadone and benzodiazepines. The existence of a major depressive episode at screening was an exclusion criterion. Both treating hepatologist and psychiatrist were blinded with regard to type of study drug, which was initiated simultaneously with antiviral therapy[9]. Psychiatric evaluation was performed at baseline, week 4, 12 and 24 of antiviral therapy and at 24 weeks of follow-up after cessation of antiviral therapy and included conduct of the relevant module of the Mini International Neuropsychiatric Interview (M.I.N.I) and the SCL-90. One of the investigators (GB) performed the psychiatric ratings after extensive training. The M.I.N.I. is a short structured diagnostic interview for DSM-IV-TR and ICD-10 psychiatric disorders and was used to diagnose the event 'depressive episode', or, briefly, 'depression'. The SCL-90 is a well-validated, multidimensional self-report symptom inventory, designed to assess various dimensions of psychopathology, including depressive symptoms. The SCL-90 was used to study the degree and course of depressive symptoms over time. The score of the depression scale is constructed with the results of 16 questions and interpreted according to its classification in one of the 7 groups (very low, low, below average, average, above average, high, very high). In the general Dutch population, the observed range is 16 – 76 with 16 – 19 corresponding to the classifications very low until below average, 20 – 23 to average, 24 to above average, 25 – 35 to high and ≥ 36 to very high[13]. Depressive symptoms were assessed using the MADRS, a clinician-rated depression scale and one of the most commonly used symptom severity scales in depression [14]. consisting of 10 items each of which is scored from 0 to 6. The BDI is a well validated and reliable 21-item self-report questionnaire designed to measure depressive symptoms [15]. Five somatic items (e.g. measuring fatigue and loss of appetite) of the BDI were also recorded.

First, we studied baseline covariates in relation to the development of depression. The following baseline covariates were collected: patient characteristics (gender, age, genotype 1 or other, race, living situation), data on psychiatric history (history of depression, history of

psychosis, or previous suicide attempt; admission to a psychiatric hospital or admission to a center for addiction treatment; IDU as route of transmission, former or current use of alcohol, tobacco, drug use and type of drug used, or use of psychotropic medication), routine laboratory examinations (sodium, albumin, bilirubin, prothrombin time, gammaglutaminyl transferase (GGT), alanine transaminase (ALT), thyroid function, creatinin) and two specific laboratory values hypothesized to underlie psychiatric side effects of IFN-based antiviral therapy (serotonin, tryptophane) prior to initiation of antiviral therapy. After identification of baseline risk factors for the development of IFN-induced depression, we compared depression scores during antiviral therapy to identify which patients would benefit from antidepressant prophylaxis.

Statistics

Univariate logistic regression analysis was used to identify baseline factors associated with the development of depression in the placebo-group. In a multivariate logistic model, we investigated the same factors with the addition of the prophylactic therapy as to determine which factors were associated with depression in the total group with correction for study treatment (escitalopram / placebo). Determinants with statistical significance at the 0.20 level in the univariate analysis were included in the multivariate analysis with backward selection based on the likelihood ratio. Data were analyzed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). The covariates 'admission to psychiatric hospital/ward', 'tentamen suicide in history', 'having ever been treated with psychoactive drugs' were mostly covered by the variable 'depression in history'. Therefore, we used 'depression in history' in our multivariate analysis.

In general, strong co-linearity between two factors hampers the interpretation of the results of a multivariate logistic model. The baseline characteristic 'presence of a history of IDU' almost fully covered patients who reported a past depressive episode. However, previous depression and psychiatric co-morbidity have been described in relation to IFN-induced depression. Therefore, we investigated these two covariates in the same multivariate model, and separately.

To study the development and severity of depressive symptoms over time, we investigated the scores on the SCL-90 subscale depression, the BDI and the MADRS sum score as continuous variable. We corrected for baseline values and estimated the mean scores of the SCL-90, the BDI and the MADRS using SAS mixed procedures with an autoregressive covariance structure taking the repeated measurement structure of the data into account. Covariates that had been identified as risk factors for depression with the above described logistic regression analysis were selected; these covariates (depression in history, presence of a history of IDU) and the interaction with study treatment were studied in the mixed procedure as fixed effects.

Results

Forty patients were randomized to escitalopram and 39 patients were randomized to placebo. Table 1a summarizes the baseline characteristics of these patients. In the escitalopram group, more female patients participated (p=0.04, Pearson Chi-square) and fewer patients had ever used hard drugs (p=0.03, Pearson Chi-square); otherwise baseline characteristics were comparable. Overall, 23 (29%) patients had a history positive for depression, 34 patients (44%) had been hospitalized in mental health centers or addiction centers and 22 patients (28%) were in contact with outpatient mental health service or addiction service at the time of intake. Forty-seven patients (60%) reported to have ever used heroin or cocaine of which the majority

Table 1a. Baseline characteristics of participants of the study on prevention of depression

		Escitalopram N=40	Placebo N=39	Total N=79
Male gender, n (%)		27 (68)*	34 (87)*	61 (77)
Age, years, mean (range)		48 (22-68)	45 (30-61)	47 (22-68)
Born in the Netherlands, n (%)		22 (55)	25 (66)	47 (60)
Living situation, n (%)	with partner	31 (78)	23 (59)	54 (68)
	other	9 (23)	16 (42)	25 (32)
Paid job, n (%)		20 (50)	15 (39)	35 (44)
Route of transmission, n (%)	intravenous drug use	19 (48)	21 (54)	40 (51)
	other	21 (53)	18 (47)	39 (49)
Race, n (%)	Caucasian	34 (85)	34 (87)	68 (86)
	other	6 (15)	5 (11)	11 (13)
Genotype, n (%)	1	16 (40)	15 (38)	28 (35)
	2	7 (18)	5 (13)	12 (15)
	3	14 (35)	16 (41)	30 (38)
	4	2 (3)	3 (8)	5 (6)
	Unknown	1 (3)	0 (0)	1 (1)
Psychiatric medical history, n (%)	One or more episodes of depression	8 (20)	16 (41)	24 (30)
	Suicides attempts/overdose	5 (13)	6 (16)	11 (14)
	Psychosis	15 (38)	20 (51)	35 (44)
	Ever use of heroin and/or cocaine	19 (48)*	28 (72)*	47 (60)
	Intravenous use of heroin and/or cocaine in past 3 months	1 (3)	0 (0)	1 (1)
	Non-intravenous use of heroin and/or cocainein past 3 months	2 (5)	3 (8)	5 (6)
	Admission to addiction facility	11 (28)	17 (44)	28 (35)
	Current contact with Mental Health or Addiction Service	9 (23)	13 (33)	22 (28)

(46 of 47 patients, 98%) reported IDU; these 47 patients will be referred to as patients with presence of a history of IDU from here. At screening, 6 of these 46 patients reported use of heroin/cocaine within the past 3 months. Baseline scores of SCL-90 were comparable between the escitalopram and placebo groups. SVR was achieved in 23 (68%) and 19 patients (61%) of the escitalopram arm and the placebo arm, respectively (p=0.59).

Depression occurred in 14 of 39 (36%) patients randomized to placebo and in 5 of 40 (13%) patients randomized to escitalopram (Pearson Chi-square, p=0.01). In the univariate logistic analysis in placebo-dosed patients, history of depression, past IDU and IDU as route of transmission were associated with the occurrence of depression during antiviral therapy. More importantly, the combination of history of depression and IDU was strongly associated with the occurrence of depression. No association was found between the occurrence of depression and baseline scores of the psychiatric questionnaires or baseline laboratory results (table 1b).

Multivariate logistic regression analysis was hampered by collinearity of history of depression and presence of a history of IDU; 18 of the 24 patients (75%) with a history of depression had injected drugs. Thirty-eight percent of patients who had ever injected drugs reported to have a history of depression. This co-linearity was even stronger with the presence of the event depression; within this group 85% of patients with a history of depression reported to have ever injected drugs and 73% of patients who ever injected hard drugs had a history of depression. Multivariate analysis including the covariates "history of depression", "the presence of a history of IDU" and "gender", resulted in "history of depression" as covariate associated with the event

Table 1b. Results of multivariate logistic regression analyses on incidence of depression with antiviral therapy for chronic HCV

New onset depression with antiviral therapy	**Placebo arm**			**All patients***		
Multivariate models	**OR**	**95% C.I.**	**p-value**	**OR**	**95% C.I.**	**p-value**
history of depression and IVDU * living without partner						
history of depression and IVDU	12.46	2.35 – 65.94	0.003	10.52	2.80 – 39.57	<0.001
living without partner	2.43	0.46 – 12.77	0.290	3.33	0.75 – 14.75	0.113
history of depression and IVDU * baseline serotonin level						
history of depression and IVDU	13.16	1.56 – 109.18	0.017	14.01	2.48 – 79.20	0.003
baseline serotonin level	0.93	0.84 – 1.04	0.203	0.91	0.84 – 0.99	0.105
history of depression and IVDU * gender						
history of depression and IVDU	13.53	2.55 – 71.75	0.002	10.45	2.90 – 37.70	<0.001
Gender	1.92	0.21 – 17.74	0.567	1.52	0.34 – 6.91	0.58
history of depression and IVDU * age						
history of depression and IVDU	12.48	2.37 – 65.66	0.003	10.23	2.86 – 36.59	<0.001
age	1.00	0.90 – 1.12	0.950	0.99	0.92 – 1.06	0.72

OR, odds ratio; 95% C.I., 95 percent confidence interval; BMI, body mass index; IVDU, intravenous drug use; AVT, antiviral therapy.

* After correction for randomization escitalopram or placebo.

Table 1c. Univariate logistic regression analysis.

Event depression							
Placebo only				**All patients, after correction for study drug**			
	OR	95% C.I.	p-value	OR	95% C.I.	p-value	p-value*
General characteristics							
Sex	0.857	0.125–5.874	0.875	0.768	0.197 – 3.002	0.705	
Age	1.011	0.922–1.109	0.811	0.963	0.900 – 1.031	0.282	0.017
Non-caucasian race	1.833	0.229-14.709	0.568	1.679	0.355-7.947	0.513	
IDU as transmission route	3.50	0.854–14.412	0.083	2.78	0.890 – 8.702	0.078	
No paid job	1.286	0.330–5.017	0.718	0.152	0.018 – 1.304	0.086	0.024
Living alone	0.400	0.098-1.636	0.202	0.314	0.084-1.166	0.083	
Rs12979860			0.417			0.828	
TT with TC as default	5.000	0.419-59.659	0.203	1.833	0.150-22.366	0.635	0.472
CC with TC as default	2.500	0.361-17.315	0.353	0.786	0.127-4.873	0.796	0.198
Rs12980275			0.145			0.772	0.292
AA with GA as default	5.500	0.545–55.494	0.148	0.489	0.069-3.440	0.472	0.117
GG with GA as default	22.000	0.938–515.872	0.055	0.000	0.000-NA	0.999	0.999
Rs4803217			0.610			0.801	0.789
CC with CA as default	2.500	0.389-16.049	0.334	1.000	0.123-8.128	1.000	0.521
AA with CA as default	2.500	0.146-42.800	0.527	2.333	0.156-34.894	0.539	0.972
Rs8099917			0.646			0.970	0.653
TT with TG as default	0.500	0.067-3.745	0.500	0.947	0.138-6.525	0.956	0.653
GG with TG as default	1.500	0.055-40.633	0.810	1.500	0.055-40.633	0.810	
Rs8103142			0.610			0.530	
TT with TC as default	1.400	0.199-9.869	0.736	1.115	0.268-4.631	0.881	
CC with TC as default	3.500	0.284-43.161	0.328	2.686	0.432-16.691	0.289	
Genotype 1	1.250	0.326–4.788	0.745	0.444	0.042 – 4.708	0.501	0.455
Sodium	1.060	0.770–1.458	0 721	0.985	0.379–2.559	0.975	1.037
Creatinin	0.985	0.928–1.044	0.604	0.961	0.913–1.011	0.128	0.012
Thyroid stimulating hormone	1.106	0.705-1.735	0.662	0.898	0.348–2.319	0.824	0.698
bilirubin	0.896	0.724–1.109	0.314	0.760	0.610–0.948	0.015	
Alanine transaminase	1.002	0.993–1.011	0.632	1.001	0.988–1.014	0.897	0.872
Gammaglutanyl transaminase	1.002	0.996–1.008	0.483	0.985	0.968–1.003	0.112	0.052
Albumin	0.897	0.703–1.146	0.387	1.019	0.813–1.276	0.873	0.456
Platelets	0.994	0.982–1.007	0.377	1.022	1.002–1.042	0.028	0.020
Pro-trombine time	1.000	1.000–1.000	0.812	1.000	1.000-1.001	0.183	
Psychiatric co-morbidity and substance use							
Depression in history	9.50	2.075 – 43.502	0.04	9.317	2.763 – 31.420	<0.001*	
Admission to psychiatric hospital/ward	6.111	0.996–37.490	0.050	4.558	1.126 – 18.448	0.033	
Tentamen suicide in history	12.778	1.305–125.067	0.029	9.468	2.094–42.814	0.04	
Ever having been treated with psychoactive drugs	3.103	0.687 – 14.018	0.141	4.980	1.258 – 19.721	0.022	

Table 1c. (continued)

	OR	95% C.I.	p-value	OR	95% C.I.	p-value	p-value*
Psychosis in history	2.127	0.548-8.259	0.275	0.375	0.038-3.715	0.402	0.202
Admission to addiction treatment center	2.222	0.580 – 8.511	0.244	0.625	0.062 – 6.301	0.690	0.352
Currently contact with Mental Health Service	0.667	0.161 – 2.769	0.577	0.844	0.082 – 8.663	0.886	0.866
Ever use of cocain/heroin	3.600	0.651–19.902	0.142	2.677	0.761–9.414	0.125	
Ever non-injecting use of cocain/heroin	2.955	0.721– 12.107	0.132	2.485	0.798 – 7.736	0.116	
History of depression and use of cocaine/ heroin	12.600	2.467 – 64.341	0.002	10.259	2.862 – 36.777	<0.001*	
Current alcohol abuse	0.778	0.199-3.035	0.718	2.875	0.421 – 19.620	0.281	0.276
Ever use of cannabis/marihuana	3.00	0.537 – 16.767	0.211	3.00	0.758 – 11.877	0.118	
Currently treated with psychoactive drug	0.000	0.000-infinite	0.999	7.250	0.988–53.222	0.051	0.999
Current use of nicotine	0.333	0.060 – 1.863	0.211	0.348	0.088–1.380	0.133	
Current use of cannabis/marihuana	1.200	0.272–5.285	0.810	1.915	0.557–6.590	0.303	
Ever use of nicotine	0.000	0.000-NA	0.999	1.938	0.171-21.923	0.593	
Psychiatric questionnaires, scores, baseline							
Event hostility	1.363	0.702 – 2.644	0.360	1.398	0.826 –2.368	0.212	
Event concentration	1.458	0.882 – 2.409	0.142	1.513	0.993–2.305	0.054	
Event mentioned sadness	1.209	0.561-2.604	0.629	1.366	0.754-2.743	0.303	
BDI, sum	0.994	0.90 – 1.097	0.909	1.193	1.005-1.417	0.044	
SCL-90							
Depression scale	1.004	0.924 – 1.091	0.923	1.026	0.960 – 1.097	0.448	
Hostility	0.947	0.698 – 1.285	0.727	1.102	0.894 – 1.359	0.361	
Phobic anxiety	1.035	0.795 – 1.348	0.797	1.035	0.824-1.302	0.765	
Anxiety	1.027	0.902 – 1.170	0.687	1.049	0.940-1.171	0.391	
Insufficiency in thinking and acting	1.055	0.914 – 1.218	0.462	1.109	0.981-1.254	0.097	
Sleeping	1.048	0.852 – 1.289	0.658	1.126	0.929-1.364	0.228	
Total	1.001	0.984– 1.019	0.887	1.006	0.990 – 1.021	0.477	
MADRS, sum	1.054	1.07 – 1.226	0.495	1.080	0.942-1.237	0.270	
Psychiatric lab results, baseline							
Neopterin	1.010	0.938 - 1.086	0.800	0.949	0.831-1.083	0.438	
Tryptophane	1.029	0.941 – 1.125	0.536	0.984	0.903-1.071	0.704	0.016
Hydroxytryptophane	1.005	0.979 – 1.032	0.708	1.013	0.989-1.038	0.301	
Serotonine	0.942	0.861 – 1.031	0.193	0.865	0.771-0.971	0.014	0.011
Totalbiopterine	0.997	0.832 – 1.195	0.973	0.798	0.602-1.058	0.117	0.043
Tetrahydrobiopterine	0.857	0.613 – 1.197	0.365	0.867	0.657-1.144	0.313	
Kynurenine	1.558	0.506 – 4.800	0.440	0.487	0.147-1.610	0.238	0.011
Kynurenine/tryptophane ratio	1.025	0.965 – 1.089	0.417	0.959	0.895-1.027	0.231	0.010

*p-value if interaction study drug with variable stays in the model.
OR, odds ratio; C.I., confidence interval; IDU, injecting drug use; NA, not applicable;
SCL90, Symptom Checklist-90; MADRS, Montgomery-Asberg Depression Rating Scale;
BDI, Beck Depression Inventory.

depression: odds ratio (OR): 9.50, 95% confidence interval (C.I.) 2.08 – 43.50, p=0.004 in the placebo-arm and OR: 10.61, 95% C.I. 3.25 – 34.64, p<0.001 in the overall study group, corrected for study medication. A subsequent multivariate analysis was performed to overcome the described collinearity, combining history of depression and IDU as single covariate with other baseline covariates in a model. We included the following clinically relevant covariates in our multivariate analysis: female gender, younger age and living situation (alone or with partner) as a measure for lack of social support (table 1b)[16-19].

Table 2. Baseline characteristics categorized by history of depression and/or substance use.

		No history of depression nor IDU N=26	History of both depression and IDU N=18	History of IDU, no history of depression N=29	No history of IDU, history of depression N=6	p-value
Male gender, n (%)		18 (69)	15 (83)	26 (90)	2 (33)	0.015*
Age, years, mean (range)		48 (34-68)	45 (22-58)	45 (30-62)	51 (35-67)	0.305
Born in the Netherlands, n (%)		13 (50)	13 (72)	19 (66)	2 (33)	0.229
Living situation with partner, n (%)		21 (81)	13 (72)	15 (52)	5 (83)	0.098
Paid job, n (%)		13 (50)	8 (44)	11 (38)	3 (50)	0.826
Caucasian race, n (%)		21 (81)	16 (89)	26 (90)	5 (83)	0.783
Genotype						0.002*
	1, n (%)	13 (50)	5 (29)	12 ()41	1 (17)	
	2, n (%)	6 (23)	1 (6)	1 (3)	4 (67)	
	3, n (%)	5 (19)	10 (59)	15 (52)	0 (0)	
	4, n (%)	2 (8)	1 (6)	1 (3)	1 (17)	
	Unknown, n (%)	0 (0)	1 (6)	0 (0)	0 (0)	
Psychiatric medical history	One or more episodes of depression, n (%)	0 (0)	18 (100)	0 (0)	6 (100)	<0.001*
	Suicides attempts/overdose, n (%)	0 (0)	7 (39)	3 (10)	1 (17)	0.003*
	Psychosis, n (%)	1 (4)	15 (83)	17 (58)	2 (33)	<0.001*
	Ever use of heroin and/or cocaine, n (%)	0 (0)	18 (100)	29 (100)	0 (0)	
	Intravenous use of heroin and/or cocaine in past 3 months, n (%)	0 (0)	0 (0)	1 (3)	0 (0)	0.627
	Non-intravenous use of heroin and/or cocaine in past 3 months, n (%)	0 (0)	1 (6)	4 (14)	0 (0)	0.179
	Admission to addiction facility, n (%)	0 (0)	13 (72)	15 (51)	0 (0)	<0.001*
	Current contact with Mental Health or Addiction Service, n (%)	1 (4)	7 (39)	14 (48)	0 (0)	0.001*

N, number; SVR, sustained virologic response; IDU, injecting drug use.
* statistically significant following Pearson Chi-Square.

Because we identified the characteristic 'history of both IDU and depression' as risk factor for IFN-induced depression, we provided baseline characteristics according to this covariate in table 2. SVR rate was not significantly different in patients with (n=8, 57%) and without (n=34, 67%) a history of depression and IDU (p=0.509).

Treatment effect of escitalopram

We studied the treatment effect of study drug regarding depressive symptoms. We took the presence of history of depression and IDU into account because these characteristics were associated with depression during antiviral therapy following logistic regression analysis. One patient did not complete the SCL-90 questionnaire at week 0, therefore, this patients was not included in this analyses of depression score over time. We selected patients with the presence of one of these two risk factors in both placebo arm and escitalopram arm. Subsequently, we compared the estimated mean scores of depressive symptoms over time using the SLC-90 depression scale, MADRS and BDI. Within the group of patients with a history of depression and IDU escitalopram-dosed patients showed lower estimated mean scores on the SCL-90 during IFN treatment than placebo-dosed patients (p=0.03) (Figure 1A). With respect to the BDI score, escitalopram-dosed patients with a history of depression and IDU demonstrated a benefit when compared to placebo in the prevention of development of depressive symptoms during IFN treatment p=0.0479) (Figure 1b).

Regarding the MADRS score, escitalopram did not show a benefit regarding the mean score during IFN-treatment in patients with a history of depression or IDU p=0.64) (Figure 1c).

A.

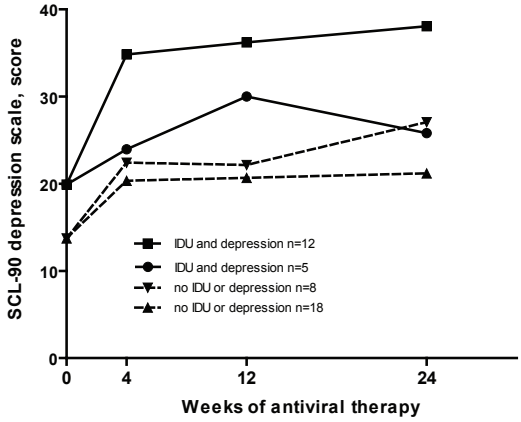

Figure 1a. Estimated mean scores of the SCL-90 depression scale in patients on AVT with treatment effect of study drug according to the presence of a history of depression and/or previous IDU during AVT. With a history of depression and IDU, escitalopram prevents the development of depressive symptoms and shows a reduction of 9.81 points (95% C.I. 0.95-18.67) on the depression score (p-value 0.03). Without a history of depression and/or IDU, escitalopram prevents the development of depressive symptoms and shows a reduction of 3.96 points (95% C.I. 0.12-7.80) on the depression score (p-value 0.04). SCL, Symptom Checklist; AVT, antiviral therapy; IDU, intravenous drug use; C.I., confidence interval.

B.

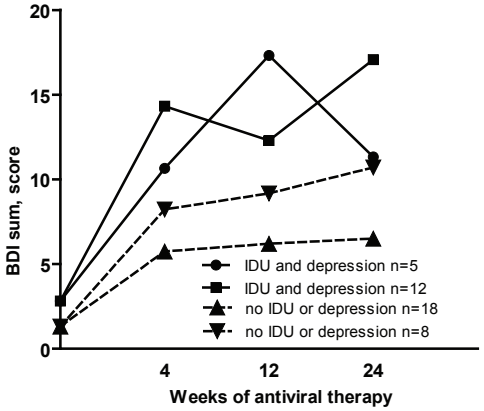

Figure 1b. Estimated mean scores of the BDI sum in patients on AVT with treatment effect of study drug according to the presence of a history of depression and/or previous IDU.With a history of depression and IDU, escitalopram prevents the development of depressive symptoms and shows a reduction of 7.58 points (95% C.I. 0.07-15.10) on the depression score (p-value 0.0479). Without a history of depression and/or IDU, escitalopram prevents the development of depressive symptoms and shows a reduction of 4.22 points (95% C.I. 1.03-7.42) on the depression score (p-value 0.01). BDI, Beck Depression Inventory; AVT, antiviral therapy; IDU, intravenous drug use.

C.

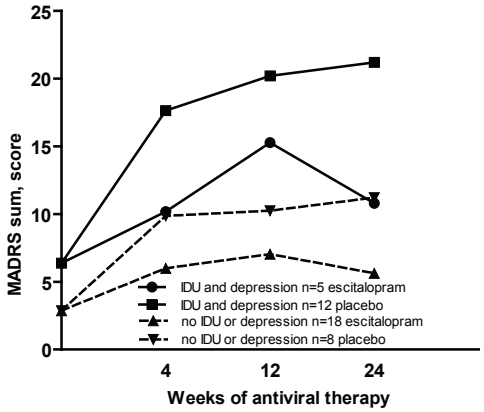

Figure 1c. Estimated mean scores of the MADRS sum in patients on AVT with treatment effect of study drug according to the presence of a history of depression and/or previous IDU. With a history of depression and IDU, escitalopram does not prevent the development of depressive symptoms (p-value 0.64). Without a history of depression and/or IDU, escitalopram prevents the development of depressive symptoms and shows reduction of 3.22 points (95% C.I. 0.55-5.89) on the depression score (p-value 0.02). MADRS, Montgomery Asberg Depression Scale (MADRS); AVT, antiviral therapy; IDU, intravenous drug use.

Thus, placebo-treated patients with a history of IDU and depression showed significantly higher scores on the estimated mean SCL-90 depression scale and significantly higher scores on the estimated mean MADRS scores, but not on the estimated mean BDI score during IFN-treatment, compared with patients receiving escitalopram.

Discussion

Our study shows that chronic hepatitis C patients with a history of depression and IDU are most vulnerable for the development of overt depression during treatment with PEG-IFN and RBV. Furthermore, it shows that the development of depressive symptoms in these patients might be prevented with prophylactic treatment with the SSRI escitalopram.

Our study is important for clinical practice because it identifies patients at baseline who are prone for IFN-induced depression irrespective of prior IFN-treatment, and who are most likely to benefit from primary prevention with SSRI prophylaxis. Previously, several baseline covariates have been identified as risk factors for IFN-induced depression with elevation of depression scores just prior to initiation of antiviral therapy being the most consistent one[20, 21]. In our study we found no correlation between mood, anxiety and depression scores at baseline and depression, probably because patients with depression at baseline were excluded from the study. Other previously described risk factors for IFN-induced depression are depression with a previous IFN-based regimen for chronic HCV, younger age, lower social support, the personal trait "low self-directedness" and reporting depressed feelings[16, 17, 20, 22, 23]. With respect to age, we could not demonstrate an association between age as continuous variable and the event depression in our study population.

The serotonin pathway is expected to be of great clinical importance in the development of depressive symptoms with antiviral therapy. Although the use of baseline and on-treatment 5-HT concentrations could not be linked to IFN-induced depression in a study by Raisin et al. [20], a more recent study by Loftis et al. did show a significant correlation between somatic symptoms of depression and lower serotonin levels at baseline [24].

Some reports suggest that a family history of depression may be a factor associated with interferon-induced depression (Nickel T, Sonntag A, Backmund M, Pollmächer T. Depression during therapy with interferon alpha--how long should an antidepressant treatment last? Pharmacopsychiatry. 2005 Mar;38(2):102-4). This might point towards an underlying genetic vulnerability. Promising results have been reported in genetic studies where traits have been investigated and identified as related to IFN-induced depression. The recent results of Kraus et al demonstrating the specific polymorphism in the serotonin receptor gene (C-1019G of the HTR1A gene: homozygosity for the G allele) to predispose to IFN-induced depression may be very important[18]. Pierucci-Lagha et al suggested a role of polymofisms in the serotonin transporter as a risk factor for the development of IFN-induced depression as well [26]. More specific, Smith et al demonstrated the importance of a polymorphism in the IDO1-2,3 gene in Caucasian patients without a history of clinically relevant depression [24]. If these genetic risk factor can be confirmed in other studies they may provide additional tools to predict the development of depression in HCV patients treated with antiviral therapy. [18, 24]. In fact, previous depression and for example a family history of depression suggest a vulnerability to depression which might have a genetic basis.

Interestingly, interleukin-28b polymorphisms which have been shown to predict treatment response to interferon in chronic hepatitis C patients also predict interferon-induced neurovegetative symptoms such as appetite, energy, and sleep complaints [28]. Indeed, Raison et al showed that chronic activation of the immune system and exposure to an innate immune cytokine affects sleep continuity and depth of sleep [28]. However, no effect of interleukin-28b polymorphism was seen on development of major depressive disorder [28].

Regarding prevention, previous research on prophylactic antidepressant treatment of the general HCV population has been inconsistent[9, 10, 30, 31]. In high-dose IFN treatment for malignant melanoma, prophylactic treatment with paroxetine effectively prevented IFN-induced depression. In HCV patients, Morasco et al[10] did demonstrate a reduction in depression of almost 50% with their double-blind placebo-controlled trial, but the study was underpowered to detect a clinically relevant significant difference. Hence, the authors concluded that prophylaxis was not beneficial. Diez-Quevedo et al[11] studied the effects of 14 weeks escitalopram vs. placebo on new onset depression in 129 HCV patients. Rates of depression were remarkably low (5.4%) and did not differ between placebo (3.2%) and escitalopram (7.6%). The results are difficult to translate to clinical practice as patients with baseline mental disorders and/or recent or concomitant drug use were excluded. This selection of patients might explain the low depression rate in the total group of patients. Schafer et al[30] demonstrated the beneficial effect of antidepressant prophylaxis in psychiatric HCV-infected patients on antiviral therapy, resulting in even lower rates of depression in HCV patients with psychiatric disorders than a control group of HCV patients without psychiatric risk factors. A recently conducted double-blind randomized placebo-controlled trial with escitalopram proved the efficacy of prophylactic treatment with a significant decrease in the incidence of depression in patients randomized to escitalopram[9]. Taken these studies together, HCV-infected patients constitute a heterogenic group of patients with varying degrees of psychiatric co-morbidity and results of such trials might be difficult to compare. In combination with the fact that about 70% of HCV-infected patients will not suffer depression with IFN-based antiviral therapy, this asks for refinement of the indication for prophylactic SSRI treatment. The results of the current study suggest that it is possible to select these patients who benefit the most from SSRI prophylaxis.

An alternative strategy to prophylactic SSRI treatment is to detect and treat psychiatric symptoms as soon as they develop during antiviral therapy. Kraus et al[32] have demonstrated the high responsiveness of IFN-induced depression for serotonergic antidepressant medication, when initiated after early detection with a psychometric instrument[32]. Nevertheless, pre-emptive treatment of patients at high risk for depression may be favoured since these symptoms may be underreported and are known to develop early in the course of antiviral therapy. We offer a practical strategy to prevent depressive symptoms in vulnerable patients with a minimal risk of excessive treatment. Although we could not demonstrate a difference in SVR with SSRI prophylaxis in patients who would experience less depressive symptoms during

antiviral therapy, previous studies[4, 33] have shown the presence of depression to be associated with a poorer rate of SVR. Therefore, prevention of IFN-induced side effects would mean an important step forward in the treatment of chronic hepatitis C. Further, our analysis is based on a randomized-controlled trial. Both clinician and patient could be expected to be at least or even more motivated to avoid early drop-out. Therefore, it is unclear whether SSRI prophylaxis in patients prone for the development of depression and depressive symptoms would positively affect drop-out rate in clinical practice and, as a consequence, increase rate of SVR.

There are several limitations to our study. Since the size of our study population was limited, less strong associations between baseline characteristics and depression may have remained undetected in our analysis. Nevertheless, the study population contained sufficient power to detect a strong association between history of depression and IDU with PEG-IFN/RBV therapy in the placebo-dosed patients and the total group of participants, when corrected for the use of SSRI.

More patients in the escitalopram group withdrew before completing the trial. We do not believe that this may have led to underreporting of depressive symptoms, since these patients remainded in follow-up after withdrawal. However, in theory, early withdrawal might have led to lower incidence of depressive symptoms due to shorter exposure to peginterferon.

In our study no lead-in period with escitalopram was used. The treatment effect might have been more pronounced using a lead-in period, so that the SRI would be fully active in the first weeks of anti-viral therapy.

Finally, our study population includes a large percentage of patients with previous psychiatric and substance use disorders. It is well possible that studies in other populations with less psychiatric comorbidity may show different results. Nevertheless, we think that our study represents clinical practice, where psychiatric comorbidity is frequent among HCV patients and these patients might actually be underrepresented in many randomized trials evaluating treatment for HCV [8, 34, 35].

In conclusion, prophylactic treatment with escitalopram significantly reduces depressive symptoms in HCV-infected patients with a history of both depression and IDU during antiviral therapy and should therefore be considered in this subset of patients.

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CHAPTER 4

PHARMACOKINETICS AND ANTIVIRAL ACTIVITY OF PHX1766, A NOVEL HEPATITIS C PROTEASE INHIBITOR USING AN ACCELERATED PHASE 1 STUDY DESIGN

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Antivir Ther. 2012;17(4):657-67.

ABSTRACT

Background. PHX1766 is a novel hepatitis C virus NS3/4 protease inhibitor with robust potency and high selectivity in replicon studies (EC_{50} 8nM). Two clinical trials investigated safety, tolerability, pharmacokinetics and antiviral activity of PHX1766 in healthy volunteers (HV) and chronic hepatitis C patients, by use of a dose adaptive overlapping clinical trial design. **Methods.** Two randomized, double-blind, placebo-controlled clinical trials were conducted. Single doses of PHX1766 or placebo were administered to 25 HV and 6 HCV genotype 1–infected patients (50 mg QD-1000 mg QD, 250 mg BID and 100 mg of a new formulation of PHX1766 QD). Multiple doses of PHX1766 or placebo were administered to 32 HV and 7 HCV genotype 1–infected patients (50 mg QD-800 mg BID). **Results.** Oral administration of PHX1766 was safe and well tolerated at all dose levels with rapid absorption (T_{max} of 1–4 hours) with mean terminal half-lives of 4-23 hours. Multiple doses of PHX1766 800 mg BID in HCV patients produced an AUC0-last accumulation ratio of 2.3. The mean maximal observed HCV-RNA decline was 0.6 log10 IU/mL in the first 24 hr in the single-dose protocol and 1.5 log10 IU/mL after 6 days of PHX1766 dosing. **Conclusion**. An overlapping, dose adaptive single- and multiple-dose escalating design in HV and HCV infected patients proved to be highly efficient in identifying a therapeutic dose. Although in-vitro replicon studies indicated a robust HCV-RNA viral decline of PHX1766, the study in HCV patients demonstrated only modest viral load reduction.

INTRODUCTION

Hepatitis C is a blood-borne virus infection with an estimated 170 million infected individuals worldwide and a prevalence of 3% [1]. Chronic HCV infection carries an increased risk of developing liver cirrhosis and hepatocellular carcinoma and is presently the leading cause of end-stage liver disease. The current standard of care consists of combination therapy with pegylated interferon alfa-2a/b plus ribavirin [2-5]. The sustained virologic response (SVR) rates vary from 41-84% after 24-48 weeks of therapy, mainly depending on HCV genotype [2, 3, 6, 7]. Current antiviral treatment is associated with frequent and severe side effects and has a negative impact on patients' quality of life [8]. Therefore, adherence can be a challenge and physician-directed dose reductions (pegylated IFN and / or ribavirin) are not uncommon, both of which have shown to result in lower SVR rates [4, 9-12]. Thus, there is an urgent need for a more effective, better tolerable and shorter regimen of antiviral therapy.

Current developments in HCV therapy focus at direct acting antivirals (DAAs). These drugs inhibit viral replication in a direct manner by blocking or modulating specific steps in viral replication. The HCV-encoded serine protease NS3/4A is essential for viral replication and is therefore one of the targets of DAA research. The small molecule BILN-2061 was the first selective inhibitor of the NS3/4A protease that demonstrated antiviral activity in reducing HCV-RNA in human [13-15]. This proof of concept in human led to further development of NS3/4A protease inhibitors such as telaprevir (VX-950) and boceprevir (SCH 503034). These compounds have shown to induce impressive HCV-RNA declines, and are at present investigated in Phase 3 studies [16, 17]. However, it should be anticipated that DAAs still require combination therapy with peginterferon and ribavirin as monotherapy is associated with frequent selection of resistant HCV variants [18-20]. Triple therapy has shown to improve SVR rates but also induced additional side effects to standard of care; rash is seen more often with the addition of telaprevir and anemia is more severe when peginterferon and ribavirin are combined with boceprevir [21-24].

PHX1766 is a novel NS3/4A HCV protease inhibitor discovered by Phenomix Corporation (Figure 1). In the replicon system and biochemical assays, PHX1766 showed to be a potent, tight-binding, reversible, and highly selective HCV NS3/4A protease inhibitor (EC_{50} 8nM, Ki 0.05 nM). In vitro synergy was observed when PHX1766 was administered in combination with interferon- α (IFN-α) and NS5B inhibitors in decreasing HCV replication in the replicon system [25]. In vitro studies also showed that PHX1766 was active against genotypes 1a, 1b, 2a, 3a, and common HCV mutants selected by telaprevir and boceprevir therapy (A156, R155, V36, T54, D168, and V170) [25]. These preclinical data indicated that certain clinically relevant mutations which have reduced susceptibility against telaprevir and boceprevir would be expected to remain sensitive to PHX1766. The IC_{50}-values of PHX1766 against these mutants and relative fold shift compared to wild type HCV 1b were generally several fold lower than the IC_{50}-values of telaprevir and boceprevir, although with PHX1766 they were not lower than one nM [25]. In the HCV 1b replicon, analysis for mutations selected for in the presence of PHX1766 demonstrated

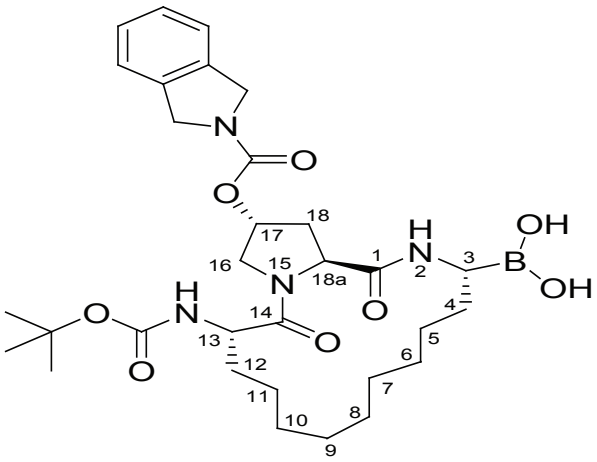

Figure 1. Structure of PHX1766.

the NS3 protease mutations I18V/T, P67T/R/S/Q, I71V/T, Q80R, P89S/T/L, R109K/G, A156S/V/T, D168T/A/V/E/G/N/H/Y/P/C and M179T/I. Replicon cells with NS3 mutations conferring reduced susceptibility to PHX1766 all remained sensitive to IFN-α treatment.

We report the safety and tolerability of two Phase 1 studies conducted with PHX1766 in healthy volunteers and HCV infected patients using an accelerated trial design. We also present the antiviral activity and pharmacokinetic profile of PHX1766. A dose adaptive and overlapping design was applied to accelerate therapeutic dose finding as the results of the single-dose study guided dose selection for the multiple-dose study and vice versa. This study design seemed highly informative in a short period of time and the most cost-effective approach without compromising the safety of the participants.

METHODS AND MATERIALS

Study design

Preclinical studies indicated that PHX1766 has a low clearance and a high volume of distribution in humans with a predicted terminal half-live between 12 and 24 hours, which supported the potential of once daily dosing. Animal studies suggested a high human liver-to-plasma concentration ratio and a low risk of drug-drug interactions as PHX1766 did inhibit the CYP3/4A at high concentrations (IC_{50} of 13.7 μM) but did not show inhibition of other major human CYP isoenzymes. Regarding serum binding, preclinical studies have shown a high plasma or serum protein binding in human, rat, monkey and dog (99.3%, >99.6%, 99.3% and 97.6%, respectively). The human starting dose of 50 mg/day was calculated based on 1/10th of the level of no observed adverse effect (NOAEL) in animals.

A single- and a multiple-dose clinical protocol was designed to provide initial safety and tolerability information of PHX1766 in healthy volunteers (HV) and chronic hepatitis C patients. Additionally, viral load decline data from chronic hepatitis C patients were aimed to assist in identifying an anticipated therapeutic dose range. Therefore, the starting dose was defined at the initiation of the protocols, with the subsequent dosages to be guided by the viral load decline results of the previously dosed hepatitis C patients.

The single ascending dose protocol was a randomized, double blind, placebo-controlled study, in HV, alternated by an open label single-dose trial in chronic hepatitis C patients infected with genotype 1. The multiple ascending dose study was a randomized and placebo-controlled study, in which HV cohorts and cohorts of chronic hepatitis C patients were alternately dosed. (See Fig 2A and Fig 2B) A safety review by the Ethics Committee (EC) was conducted and approval of the EC was received prior to each dose escalation step. The trials were carried out in accordance with the Declaration of Helsinki version 2008 and in compliance with the current regulations and standards of Good Clinical Practice (26,27). The single-dose and multiple-dose clinical trials were conducted from October 2008 to April 2009 and January 2009 to May 2009, respectively. Both studies were conducted at three sites in The Netherlands. All volunteers signed the Informed Consent Form before participating in any study-related activity. The studies were conducted as in-house studies, with all patients admitted to the clinical facility one day prior to first dosing and discharged 48 hours (single-dose protocol) or 24 hours (multiple-dose protocol) after the last dose of the study drug.

Participants were dosed with oral capsules of PHX1766 or, not discernable of the active study drug, oral capsules of placebo. The capsules of PHX1766 were produced in a 75:25 ratio of the pharmacologically inactive substances to PHX1766 (Formulation A). To investigate if a different formulation would lead to a higher gastrointestinal absorption of PHX1766, Formulation B was developed with a 90:10 ratio of the excipients to PHX1766.

Dosing regimen

The single-dose study was carried out in three cohorts of HV (cohort 1, 2, 6) and three cohorts of hepatitis C patients (cohort 3, 4, 5). The multiple-dose study was carried out in four cohorts of HV (cohort 7, 8, 9, 10) and two cohorts of hepatitis C patients (cohort 11, 12). The design was adaptive regarding dose selection; the first cohort of HCV infected patients was enrolled at 100 mg once daily (QD) and the subsequent single and multiple dose levels were based on HCV-RNA decline data of the previous cohort.

Inclusion and exclusion criteria of healthy volunteers and chronic hepatitis C patients

The single-dose study was carried out in three cohorts of HV (cohort 1, 2, 6) and three cohorts of hepatitis C patients (cohort 3, 4, 5). The multiple-dose study was carried out in four cohorts of HV (cohort 7, 8, 9, 10) and two cohorts of hepatitis C patients (cohort 11, 12). The design

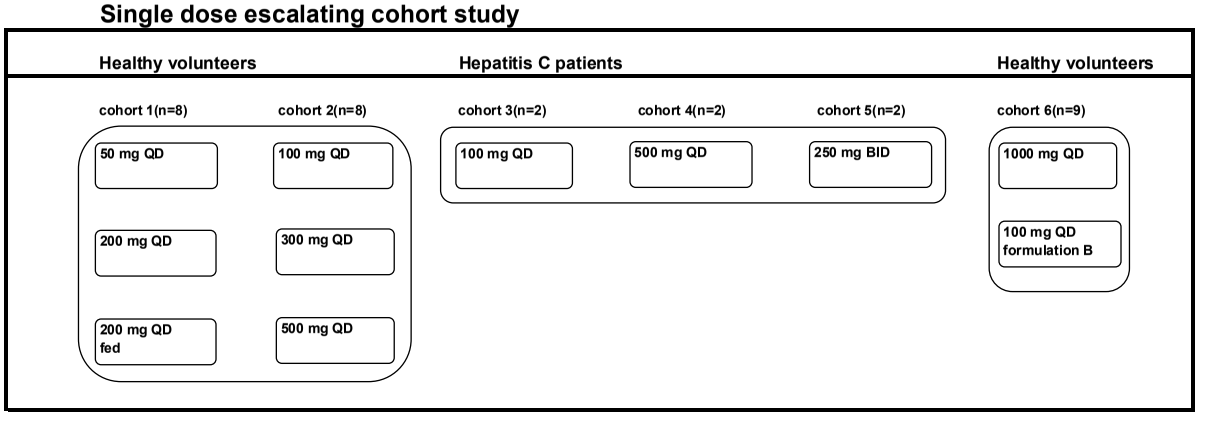

Figure 2a. Single-dose protocol.
All participants dosed PHX1766 received formulation A, unless otherwise denoted. Cohort 1 and 2 were dosed three rising doses in an alternating manner with at least 7 days between dose escalations. Cohort 6 was dosed two different dosages with at least 7 days between the doses. The last dosing period of cohort 1 was no dose escalation, but exploration of food effect (fed state). Ratio of dosing PHX1766 and placebo: 6 to 2 for cohorts 1, 2; 7 to 2 for cohort 6. Cohorts 3, 4, and 5 were dosed PHX1766, no placebo. QD, once daily; BID, twice daily.

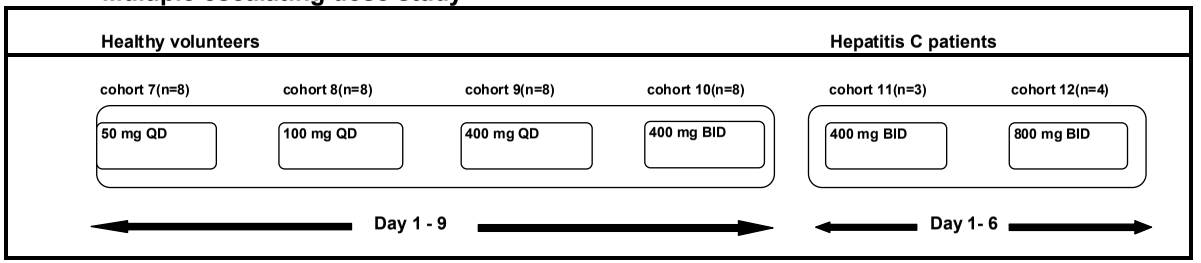

Figure 2b. Multiple-dose protocol.
All participants dosed PHX1766 received formulation A, unless otherwise denoted. Ratio of dosing PHX1766 and placebo: 6 to 2 for cohorts 7, 8, 9, 10; 2 to 1 for cohort 11, 3 to 1 for cohort 12. Cohorts 3, 4, and 5 were dosed PHX1766, no placebo. QD, once daily; BID, twice daily.

was adaptive regarding dose selection; the first cohort of HCV infected patients was enrolled at 100 mg once daily (QD) and the subsequent single and multiple dose levels were based on HCV-RNA decline data of the previous cohort.

Safety assessments

HV and patients were monitored for safety and tolerability at regular intervals from the start of dosing throughout the study and follow-up. Safety parameters included, vital signs, physical examination, laboratory parameters (biochemistry, hematology, urinalysis), electrocardiograms and the recording of all adverse events (AEs). In the single-dose study, the assessments were made at least four times on the dosing days. Afterwards, they were performed on day 2 and day 3 and at the day of follow-up which was day 7-9, counted from the last dose. In the multiple-dose study, safety assessments were made at screening, admission to the clinic and, in the dosing period on a daily basis. After dosing, safety assessments were made at the day of discharge in healthy volunteers and 17 ± 3 days or 20 ± 3 days after the first dose of study drug in healthy volunteers and HCV-infected patients, respectively.

Pharmacokinetic assessments

Plasma samples for measurement of PHX1766 levels were collected in each treatment group of the single- and multiple-dose study just before dosing, at various time points of each dosing day and at follow-up (day 7,8 or 9 from the last dose in the single-dose study; day 7-10 from the last dose in multiple-dose study with addition of day 17 and 20 for the HCV-infected subjects). Plasma samples were analyzed by Tandem Labs (Salt Lake City, Utah) using a validated high performance liquid chromatography tandem mass spectrometry method.

The pharmacokinetic parameters were estimated using a non-compartmental model with the WinNonlin Pro software. PHX1766 C_{max} and T_{max} were obtained by calculation of the plasma concentration data.

Pharmacodynamic assessments

In the single-dose study, samples for HCV-RNA measurement were collected between 3 hours until 30 minutes before the morning dose and at various time points on the dosing day, 48 and 72 hours post-dose and during follow-up. In the multiple-dose study, HCV-RNA measurements were performed 3 hours until 30 minutes pre-dose, post dose throughout day 1 and day 6, on days 2-5, and during follow-up. Plasma HCV-RNA levels were determined using the Roche Ampliprep/Cobas Taqman HCV/HPS assay (Roche Molecular Systems Inc., Branchburg, NJ). The lower limit of detection of this assay was 15 IU/mL and the linear range was 43 – 6.90 x 107 IU/mL. The Truegene Assay was used to determine the genotype of all patients [26].

RESULTS

Baseline demographics of healthy volunteers and hepatitis C patients

Twenty-five HV (cohort 1, 2, 6) and 6 chronic hepatitis C patients (cohort 3, 4, 5) were enrolled in the single-dose protocol. One HV (cohort 2) was discontinued after the second dose due to an intercurrent febrile illness. One HV was only available for one of the two dose groups, so cohort 6 required another HV for the second dose group and resulted in 9 HV.

In the multiple-dose protocol 32 HV (cohort, 7, 8, 9, 10) and 7 hepatitis C patients (cohort 11, 12) were enrolled. No subject was discontinued from the study. In both clinical protocols, the hepatitis C patients were infected with genotype 1a or 1b. Of the 13 hepatitis C patients

Table 1. Baseline characteristics of healthy volunteers and HCV infected patients that participated in the single-dose and multiple-dose protocol.

Parameters	Healthy volunteers	HCV infected patients
	Cohort 1, 2, 6, 7, 8, 9, 10	Cohort 3, 4, 5, 11, 12
	50 mg QD – 400 mg BID	100 mg QD – 800 mg BID
	N=57	N=13
Sex, n (%)		
Male	29 (51)	12 (92)
Race, n (%)		
White / Caucasian	56 (98)	11 (85)
Asian	1 (2)	1 (8)
Black	(0)	1 (8)
Age, years		
Median (range)	55 (18-65)	49 (22-60)
BMI, kg/m2		
Median (range)	26 (19-32)	28 (21-32)
ALT, U/L		
Median (range)		74 (26-149)
HCV subtype, n (%)		
1a		9 (69)
1b		4 (31)
Baseline HCV-RNA IU/mL log_{10}		
Median (range)		6.4 (5.3-7.0)
Prior IFN treatment, n (%)		
Experienced		9 (69)

QD, once daily; BID, twice daily.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; SD, standard deviation; BMI, body mass index.

enrolled, 9 were experienced and four naïve to an IFN-based regimen. Baseline demographics are summarized in Table 1. There were no significant differences in baseline characteristics between the HV in the different dose groups and HCV-infected patients.

Safety and tolerability in healthy volunteers and hepatitis C patients

Thirty-three AEs were reported by 16 HV and 7 AEs by 3 hepatitis C patients who received PHX1766 or placebo during the single-dose protocol. Only 2 AEs were considered related to PHX1766. Both AEs concerned myalgia which were considered mild in intensity and resolved spontaneously during follow-up (Table 2). There was one serious AE during the study that led to the death of one of the HV. The subject was administered 100 mg of PHX1766 once daily and 300 mg of PHX1766 once daily (cohort 2) consistent with the protocol. During follow-up,

Tabel 2. Adverse events among healthy volunteers and HCV infected patients that were considered related to the active study drug PHX1766.

Trial	Subject	Dose PHX1766	Adverse event	Intensity	Intervention	Outcome
Single-dose protocol	Healthy volunteer	100 mg QD	Myalgia	Mild	Paracetamol 1000 mg QD	Resolved
	Healthy volunteer	100 mg QD	Myalgia	Mild	Paracetamol 500 mg QD	Resolved
Multiple-dose protocol	Healthy volunteer	50 mg QD	Feeling cold	Mild	No	Resolved
	Healthy volunteer	400 mg QD	Erythema	Mild	No	Resolved
	Healthy volunteer	400 mg QD	Myalgia	Mild	Paracetamol 500 mg QD	Resolved
			Myalgia	Mild	No	Resolved
			Myalgia	Mild	No	Resolved
	Healthy volunteer	400 mg BID	Headache	Mild	No	Resolved
	Healthy volunteer	400 mg BID	Headache	Mild	No	Resolved
	HCV infected patient	400 mg BID	Dizziness	Mild	No	Resolved
	HCV infected patient	800 mg BID	Fatigue	Mild	No	Resolved
			Nausea	Mild	No	Resolved
			Upper abdominal pain	Mild	No	Resolved 2 weeks after end of study
	HCV infected patient	800 mg BID	Fatigue	Mild	No	Resolved

QD, once daily; BID, twice daily; HCV, hepatitis C virus.

two weeks after the second dose, the subject presented with a febrile illness for which study treatment was discontinued. One week later, all safety assessments were normal. Two days after that follow-up visit, the HV underwent an elective eyelid and abdominal wall correction as an outpatient and died suddenly one day after surgery. Autopsy determined the cause of death as postoperative multiple pulmonary emboli and the event was considered not related to the study treatment. Among the remaining 24 HV and 6 hepatitis C patients, no clinically important changes were seen concerning vital signs, laboratory assessments, physical examination and ECG from the start of the study to the end of the study.

In the multiple-dose protocol, 37 AEs were reported by 17 HV and twenty AEs by 5 HCV patients who received PHX1766 or placebo. Twelve AEs were considered to be related to PHX1766; they were all considered mild in intensity resolved after treatment cessation (Table

Table 3a. Summary Statistics of PHX1766 plasma pharmacokinetic parameters single-dose study

Treatment PHX1766	Cmax (ng/mL) (N = 29)	tmax (h) (N = 29)	AUC(0-last) (ng.h/mL) (N = 29)	T1/2 (h) (N = 29)
Healthy Subjects (n = 6)				
50 mg	10.57	3.00	40.38	7.45
	(3.28 – 26.50)	(1.00 – 4.00)	(15.1 – 72.1)	(7.29 – 10.52)
100 mg	18.44	2.00	63.00	9.63
	(4.77 – 35.30)	(1.00 – 3.00)	(11.7 – 114.3)	(6.06 – 17.52)
100 mg B*	76.99	2.52	189.29	10.61
	(42.30 – 203.0)	(1.00 – 4.02)	(91.0 – 388.9)	(5.20 – 14.05)
200 mg fasted	59.80	3.00	187.08	17.7
	(15.30 – 129.0)	(2.00 – 4.00)	(71.6 – 354.7)	(10.25 – 32.01)
200 mg fed	97.99	3.00	300.9	15.82
	(28.80 – 216.0)	(2.00 – 4.03)	(111.6 – 505.7)	(10.11 – 21.94)
300 mg	90.39	2.01	271.19	8.92
	(62.0 – 159.0)	(2.00 – 6.00)	(170.1 – 381.9)	(5.84 – 14.32)
500 mg	262.881	3.00	872.36	12.10
	(70.10 – 441.0)	(2.00 – 3.09)	(358.5 – 1341.6)	(8.16 – 19.13)
1000 mg	699.71	3.00	2443.5	14.98
	(375.0 – 1500.0)	(3.00 – 4.00)	(1656.2 – 4388.7)	(11.63 – 17.67)
HCV Patients (n = 2) (n=2)				
100 mg A	--	--	--	--
	(32.90 – 37.20)	(2.00 – 4.00)	(119.4 – 151.3)	(16.36 – 17.81)
500 mg	--	--	--	--
	(275.0 – 2170.0)	(3.00 – 4.00)	(977.3 – 8548.5)	(13.9 – 32.80)
250 mg BID	--	--	--	--
	(177.0– 227.0)	(20.0 – 20.0)	(1828.4 – 2802.2)	(7.13 – 26.86)

For Cmax, AUC(0-last) and $t_{1/2}$, the geometric mean (range) is presented; for t_{max} the median (range) is presented.

2). There were no clinically significant changes in the vital signs, ECG, or physical examination from the start until the end of study.

Pharmacokinetics

Following single-doses with PHX1766, plasma concentrations were detectable within 30 minutes after dosing and the median T_{max} ranged from 1-3 hours. In HV, the systemic exposure of PHX1766 in Formulation A increased dose-dependently from 50 to 1000 mg, with the mean C_{max} increasing from 10.6 to 699.7 ng/mL and the mean $AUC_{0\text{-last}}$ from 40.4 hr*ng/mL to 2443.5 hr*ng/mL, indicating that the increase was more than dose-proportional in the tested dose range. PHX1766 exhibited a moderate terminal half-life, with the means ranging from 7 – 16 hours in HV dosed formulation A and 18 hours in HV dosed formulation B. HCV infected

Table 3b. Summary Statistics of PHX1766 plasma pharmacokinetic parameters in multiple-dose study.

Treatment PHX1766	C_{max} (ng/mL) (N = 29)	t_{max} (h) (N = 29)	$AUC_{(0\text{-last})}$ (ng.h/mL) (N = 29)	$t_{1/2}$ (h) (N = 29)
Healthy Subjects (n = 6)				
50 mg OD				
Day 1	8.69 (5.06-12.4)	2.00 (1.00-3.0)	19.86 (9.5-27.2)	11.95 (7.32-23.0)
Day 5	7.31 (4.11-14.5)	2.00 (2.00-8.00)	22.06 (15.5-31.6)	13.10 (6.68-22.2)
Day 9	8.09 (2.39-11.3)	2.50 (2.00-8.00)	21.56 (7.9-39.7)	9.36 (7.26-14.4)
100 mg OD				
Day 1	20.3 (2.32-94.6)	2.53 (2.00-10.0)	55.4 (20.0-146.5)	6.92 (5.56-9.57)
Day 5	8.67 (2.57-22.2)	2.00 (2.00-8.00)	31.1 (14.5-58.2)	8.46 (4.04-16.7)
Day 9	11.0 (2.36-43.6)	2.50 (1.00-3.00)	51.7 (20.3-144.3)	13.0 (6.44-21.3)
400 mg OD				
Day 1	211.0 (117.0-311.0)	2.00 (1.00-3.00)	525.5 (283.1-816.7)	6.21 (4.76-9.96)
Day 5	229.9 (177.0-337.0)	2.00 (2.00-3.00)	570.2 (469.7-725.3)	6.06 (5.05-7.65)
Day 9	166.5 (106.0- 265.0)	2.00 (1.00-3.00)	513.4 (319.8-685.3)	17.5 (12.0-23.0)
400 mg BID				
Day 1	183.2 (95.8-373.0)	2.00 (0.50-3.02)	556.9 (296-1022)	3.53 (2.42-4.99)
Day 5	208.1 (125.0-535.0)	3.00 (1.00-3.00)	779.2 (395.0-1485.0)	5.55 (3.53-8.66)
Day 9	228.7 (86.4-561.0)	2.00 (1.00-3.00)	828.0 (382.2-1844)	4.30 (3.01-5.66)
HCV Patients (n=2)				
400 mg BID (n=2)				
Day 1	- (105.0, 698.0)	- (1.00, 3.00)	- (394.5, 2071)	- (3.86, 4.5)
Day 6	- (139.0-952.0)	- (1.00, 1.00)	- (560.0, 3105.1)	- (4.38, 4.41)
800 mg BID (n=3)				
Day 1	1048.3 (762.0-1260.0)	1.01 (1.00-3.00)	2833.7 (1927-4088)	3.61 (2.50-4.77)
Day 6	1844.1 (1750-1980)	1.00 (1.00-4.00)	6413.7 (6242-6723)	7.25 (4.86-13.0)

For Cmax, $AUC_{(0\text{-last})}$ and $t_{1/2}$, the geometric mean (range) is presented; for t_{max} the median (range) is presented.

patients showed a somewhat longer terminal half-life (means 17-23 hours). Fed state increased the systemic exposure but was not statistically significant. Formulation B enhanced the systemic exposure in terms of AUC0-last by 3.7-fold as compared to Formulation A. The individual maximal C_{min} at 24 hours after dosing was 0.255 ng/mL with formulation A 100 mg QD and 0.847 ng/mL with formulation B 100 mg QD. The systemic exposure of PHX1766 appeared to be higher in HCV-infected patients than in HV (Table 3a and table 3b).

With the multiple dose protocol, steady state was reached within three days of dosing. There was no apparent accumulation in systemic exposure at steady state versus the first dose in HV (Figure 3a). In contrast, there was approximately 2-fold accumulation in systemic exposure at steady state versus the first dose in chronic hepatitis C patients (Figure3b), suggesting a difference in uptake. The average accumulation ratio with respect to AUC0-last was 1.5 in HV (400 mg

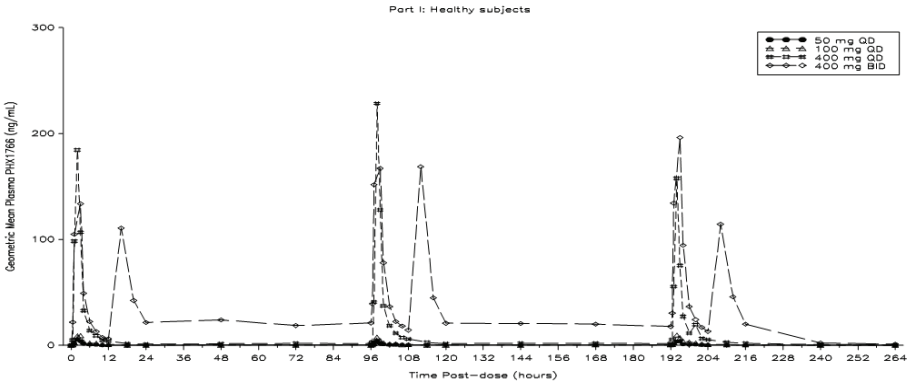

Figure 3a. Geometric Mean Plasma Concentration Time Profile of PHX1766 (linear scale) in ng/mL. The four dose groups of healthy volunteers of the multiple-dose protocol are shown. PHX1766 was administered for 9 days; each dose cohort consisted of 6 healthy volunteers. QD, once daily; BID, twice daily.

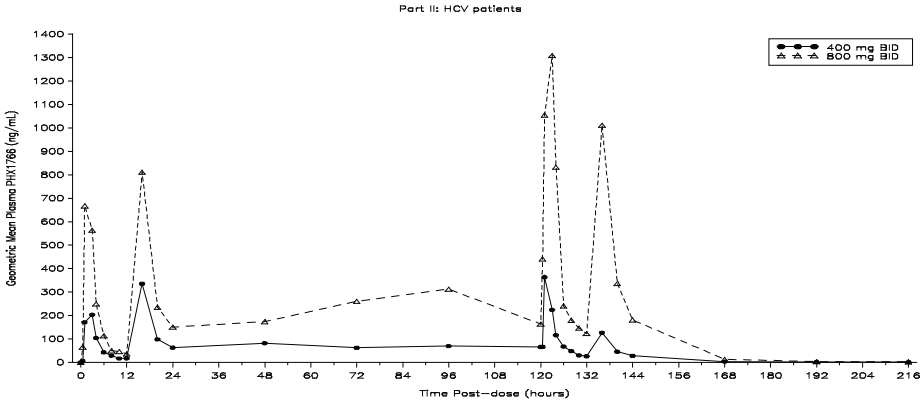

Figure 3b. Geometric Mean Plasma Concentration Time Profile of PHX1766 (linear scale) in ng/mL.
The two dose groups of HCV infected patients of the multiple-dose protocol are shown.
PHX1766 was administered for 6 days; 2 patients were dosed 400 mg BID, 3 patients were dosed 800 mg BID. BID, twice daily; HCV, hepatitis C virus.

BID) and 2.3 in HCV infected patients who were dosed 800mg BID. The highest average trough plasma concentration of PHX1766 in HV was 21.0 ng/mL in the 400 mg BID dose group at 96 hours post-dose (Figure 3a) and was lower than the EC_{50} of 8 nM (~49 ng/mL) in the replicon system. Among the HCV infected patients, the highest average trough-level was measured at 120 hours post-dose in the 800 mg BID dose group (163.5 ng/mL) and was >3 times higher than the EC_{50} (Figure 3b).

Viral response

In the single-dose study, there was a transient decrease in plasma viral load with a mean maximal HCV-RNA decline of 0.6 log10 IU/mL (± 0.50) which was achieved during the first 24 hours post PHX1766 administration (100 mg QD – 500 mg QD; 250 mg BID). The individual maximal HCV-RNA decline was 1.5 log10 IU/mL and was observed in the 500 mg QD dose group (Cohort 4). In 3 out of 6 hepatitis C patients, the maximal viral load decline occurred during the first 12 hours (including the individual with the highest viral load drop observed) with return to baseline values at 16 hours post-dose already. In the multiple-dose study, all HCV patients treated with PHX1766 showed a transient decrease in plasma viral load with, after 6 days of dosing, a mean maximal HCV-RNA decline of 1.5 log10 IU/mL (±0.49) (Figure 4). The individual maximal HCV-RNA decline was 1.5 log10 IU/mL within the first 24 hours and 2.2 log10 IU/mL after 6 days dosing, both in the 800 mg BID dosing group (Cohort 12). All patients demonstrated a similar

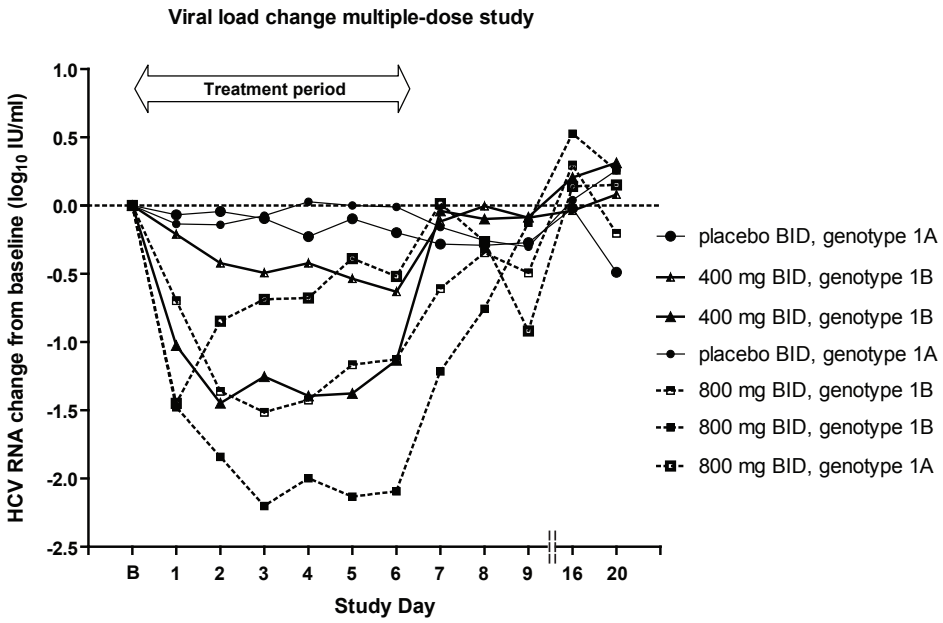

Figure 4. Individual viral load decline of the 7 dosed HCV infected patients during the multiple PHX1766 dose study. The baseline value is determined just before dosing. The dose levels are on the right side of the graphs. Patients received PHX1766 400 mg twice daily (BID), 800 mg BID or placebo for 6 days. HCV, hepatitis C virus.

return of viral load to baseline at the follow-up visits. No significant changes in HCV-RNA levels were observed in patients who received placebo (Figure 4).

DISCUSSION

This study is the first to describe the in vitro and in vivo profile of the novel HCV NS3/4A protease inhibitor PHX1766. A trial design, adaptive to dose selection, was used to explore the safety and pharmacokinetic and -dynamic profile of PHX1766 in a short period of time in both HV and hepatitis C patients.

The primary objectives of these clinical trials were to investigate the safety and tolerability of oral doses of PHX1766 in healthy volunteers and chronic hepatitis C patients. Treatment with PHX1766 was generally well tolerated. Regarding both single- and multiple-dose protocol, one HV was discontinued from the study and this subject developed a serious AE. The cause of death was investigated by autopsy and considered unrelated to PHX1766 dosing. There were no other study discontinuations or serious AEs. The most frequently reported AEs that were considered related to PHX1766 concerned flu-like symptoms which were all mild in intensity and resolved spontaneously. Overall, administration of PHX1766 in single doses up to 1000 mg and multiple doses up to 800 mg BID in HV and HCV infected patients was safe and well tolerated.

The secondary objectives were to investigate the pharmacokinetic profile and antiviral activity of PHX1766. First of all, there was a clear discrepancy in terminal half-life and accumulation between HV and HCV infected patients in favour of the HCV infected subjects, suggesting a difference in uptake of PHX1766. Liver functions such as drug metabolism and biliary excretion may be impaired in chronic hepatitis C patients. PHX1766 is cleared partially via biliary excretion, which explaines the longer terminal half-life and accumulation ratio of PHX1766 in hepatitis C virus infected patients when compared to the HV. Viral load suppression with single oral doses of PHX1766 did not last for 24 hours and this suggested a twice daily or thrice daily dosing regimen. However, with the current standard of care, the aim was to develop a highly effective antiviral agent with the potential of once daily dosing. Thereby, twice daily dosing for 6 days resulted in an only modest average viral load drop in HCV infected patients. Nevertheless, in vitro, PHX1766 was a selective protease inhibitor with an EC_{50} of 8 nM, low human clearance and a high volume of distribution with a predicted human terminal half-live between 12 and 24 hours, which supported the potential of once daily dosing. In the multiple-dose protocol, the highest dose group (800 mg BID) produced a trough-level 120 hours post-dose of only 3 times the EC_{50}. This is suboptimal when compared to other new DAAs like TMC435 but would be sufficient if viral inhibition was strong [27]. Mutations selected for in the presence of PHX1766 have been demonstrated in the HCV 1b replicon. As HCV-RNA suppression showed to be incomplete in the clinical trials, it can be anticipated that viral mutations would have occurred.

. With the suboptimal viral suppression achieved with PHX1766, sequencing of possible viral mutations did not seem worthwhile and has not been performed. Regarding kinetics, there was accumulation in the HCV infected patients, a moderate human terminal half-life, but an, apparently, insufficient trough-level to produce vigorous and lasting viral inhibition with QD or BID dosing. As replicon studies showed PHX1766 to be a potent protease inhibitor (EC50 8 nM) and animal studies suggested a high human-to-plasma concentration ratio, there is a clear discrepancy between the results of the clinical trials and the earlier described preclinical results concerning the pharmacokinetic profile and antiviral effect.

New compounds that are introduced to clinical research often follow the different phases of clinical trials chronologically. Our design accelerated dose finding as it implemented an adaptive dose regimen with the investigation of safety and tolerability of PHX1766 in HV and HCV infected patients. The overlapping design of the single and multiple dose protocols has proven to be a time efficient and safe way to investigate the new protease inhibitor PHX1766. Thereby, with this design, fewer subjects are exposed to the experimental protease inhibitor which is of great importance in two ways. Firstly, it minimizes possible health risks as fewer participants are dosed. The second reason is that less exposure reduces the occurrence of mutations and, subsequently, resistance, as this phenomenon is inevitable with the administration of a protease inhibitor as monotherapy. To conclude, time efficiency and the requirement of fewer subjects and fewer study drug, results in less costs. Taken these arguments together, this design should be considered in studies of antiviral compounds in order to rapidly assess initial safety, tolerability as well as antiviral effect.

Several limitations of both Phase 1 studies should be noted. First, the number of hepatitis C patients that were dosed was small. These patients were randomized into different dose groups making a solid statistical analysis of the pharmacokinetic and –dynamic results difficult. Second, the conducted trials did not investigate combination therapy of PHX1766 with peg-IFN (and ribavirin). The in vitro studies showed a synergistic effect of PHX1766 in combination with IFN-α, which encourages research of the antiviral effect of combination therapy.

In conclusion, single and multiple doses of PHX1766 in HV and HCV infected patients were safe and well tolerated. The overlapping and dose adaptive clinical trial design proved to be a safe and highly efficient way to investigate the molecule PHX1766 in healthy volunteers and HCV infected patients and should be considered for future Phase 1 clinical trials. PHX1766 dosing in humans resulted in only a modest HCV-RNA decrease, in contrast to the in-vitro replicon results, which indicated a robust viral load decline. Consequently further development of PHX1766 was not pursued.

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CHAPTER 5

SUSTAINED VIROLOGIC RESPONSE AFTER THERAPY WITH THE HCV PROTEASE INHIBITOR NARLAPREVIR IN COMBINATION WITH PEGINTERFERON AND RIBAVIRIN IS DURABLE THROUGH LONG-TERM FOLLOW-UP

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J Viral Hepat. 2013 Apr;20(4):e78-e81

ABSTRACT

Background. Achievement of a sustained virologic response (SVR) after peginterferon (PEG-IFN) and ribavirin (RBV) treatment is considered to be a marker for cure of chronic hepatitis C virus (HCV) infection. Long-term follow-up of patients with SVR after treatment with a direct acting antiviral has not yet been described. **Methods.** We used a randomized placebo-controlled, double-blind, two-period phase 1b trial that was conducted in 40 HCV genotype 1 (treatment-naïve and treatment-experienced)-infected patients. Nineteen patients achieved SVR after treatment with the HCV protease inhibitor narlaprevir followed by PEG-IFN / RBV. In these patients, HCV-RNA tests were scheduled at three, six, 12 and 24 months after end of treatment. **Results.** Patients were followed for a median of 27 months (range 15-32) after end of treatment with a median number of follow-up visits of 4 (range 3-8). All patients remained HCV-RNA negative over time. **Conclusions**. SVR achieved following narlaprevir and PEG-IFN / RBV therapy was durable up to 32 months end of treatment.

INTRODUCTION

Recently, the standard of care antiviral treatment (AVT) for chronic hepatitis C virus (HCV) infection, peginterferon (PEG-IFN) and ribavirin (RBV) has been modified to include a direct acting antiviral (DAA), either telaprevir or boceprevir [1].

Successful HCV treatment has been defined as achievement of a sustained virologic response (SVR), undetectable HCV-RNA 24 weeks after end of treatment (EoT). After treatment with PEG-IFN / RBV, SVR has been shown to be durable up to 18 years post-treatment and is therefore considered a marker for successful HCV antiviral therapy [2]. Accordingly, clinical studies investigating triple therapy regimens including DAAs have evaluated antiviral efficacy with SVR rates. However, to date, undetectability of HCV-RNA 24 weeks after DAA-based therapy has not yet been described to be a marker of long-term sustained viral eradication. It is important to note that, in contrast to PEG-IFN/RBV, DAAs have the ability to select for resistant HCV strains and their immune modifying capacities are limited [3-6]. Trace amounts of HCV-RNA have been detected in different compartments of the human body years after SVR following PEG-IFN / RBV [7, 8]. Veerapu et al. showed that, with long-term follow-up after achieving SVR, HCV-RNA reappearances in plasma induced HCV-specific T cell responses [7]. It is not known if traces of HCV-RNA also persist after successful DAA-based therapy, and whether these traces are subject to immune control or could result in a late virologic relapse.

We studied durability of SVR after treatment with the protease inhibitor narlaprevir (with or without ritonavir), PEG-IFN with narlaprevir, followed by PEG-IFN / RBV for 24-48 weeks [9].

METHODS AND MATERIALS

For our analysis, we used a randomized, placebo-controlled, double-blind, two-period phase 1b trial that was conducted in 40 HCV genotype (GT) 1-infected (naïve and treatment-experienced) patients in the Netherlands [9]. In period 1, narlaprevir was administered for seven days as 800 mg three times daily without ritonavir or 400 mg twice daily with 200 mg ritonavir twice daily. In period 2, after a 4-week washout, the same dose and regimen of narlaprevir was administered in combination with PEG-IFN-α-2b for 14 days. Upon completion of period 2, all patients initiated PEG-IFN-α-2b and RBV treatment which lasted 24 weeks if a rapid virologic response was achieved, otherwise 48 weeks.

With this regimen, six of 20 (30%) treatment-experienced and 13 of 20 (65%) treatment-naïve patients achieved SVR and these were all randomized narlaprevir [9]. These patients were followed up after EoT as in regular clinical practice. Follow-up visits were scheduled at three months, six months, 12 and 24 months after EoT. At each follow-up visit, blood was collected and tested for HCV-RNA using Roche Ampliprep/Cobas Taqman HCV/HPS assay (Roche Molecular Systems Inc., Branchburg, NJ) with lower limit of detection (LLOD) of 15 IU)/ mL or

the VERSANT® TMA HCV RNA qualitative assay (Siemens Medical Solutions Diagnostics, Eindhoven, the Netherlands) with LLOD of 5.3 IU/mL. At the last follow-up visit, which was at least 24 months after EoT, the VERSANT® TMA HCV RNA qualitative assay was used.

Additionally, at the final follow-up visit, blood was collected to study *in vitro* HCV-specific T cell proliferation in blood. Reappearances of HCV-RNA during follow-up after achievement of SVR with PEG-IFN/RBV have been described to induce HCV-specific T cell responses. Therefore, this immunologic analysis was performed to support the presence or absence of circulating traces of HCV-RNA. Peripheral blood mononuclear cells (PBMC) were collected to test for HCV-RNA. PBMC can be considered as a different compartment than the peripheral circulation and, in patients with long-term follow-up of SVR after PEG-IFN/RBV, PBMC tested positive for HCV-RNA previously [10]. Informed consent was obtained before blood samples were collected at follow-up visits. PBMC were isolated from venous blood by ficoll separation (Ficoll-Paque^{TM} plus, Amersham, Buckinghamshire, UK) and immediately cultured in quadruplets in 96-well round-bottom plates ($2x10^5$ cells in 200 microliter (µL)). Cells were stimulated with overlapping peptide pools (1 microgram (µg)/milliliter (mL) per individual peptide; spanning the core, NS3, NS4, NS5a and NS5b HCV genome; clone J4, genotype 1b; BEI Resources, Manassas, USA), cytomegalovirus (CMV) antigens (34 µg/mL; AD-169; Microbix, Toronto, Canada) or no stimulus. Culture medium was RPMI 1640 supplemented with L-glutamin, Penicillin-Streptomycin, HEPES, and 5% human serum (all from Lonza, Verviers, Belgium), anti-CD28 and anti-CD49d antibodies (both 1 µg/mL; eBioscience, San Diego, USA) and anti-CD49d antibody (1 µg/mL; 9F10; eBioscience). After stimulation for 5 days, cells were pulsed for 16h with [^{3}H]-thymidine (0.5 µCi/well; Amersham, Little Chalfont, UK). Proliferation was determined as counts per minute by liquid scintillation. HCV-specific T cell proliferation was compared with medium, consisting of unstimulated cultures of blood lymphocytes from the same patients, and CMV T cell proliferation; the first as a negative control, the latter as a positive control for T cell proliferation. PBMC were isolated from venous blood collected at the last follow-up visit by ficoll separation (Ficoll-PaqueTM plus, Amersham, Buckinghamshire, UK) and stored at -150°C. Cells were lysed by repeated freeze-thaw cycles and tested for HCV-RNA using the VERSANT® TMA HCV RNA qualitative assay.

In 2008 / 2009, in 22 treatment-naïve chronic HCV-infected patients immune response to HCV during AVT was prospectively studied. These patients were treated with PEG-IFN-alfa2b and RBV for 24 weeks (genotype 2 and 3) or 48 weeks (genotype 1 and 4) and, as part of study protocol, underwent the same immunologic assays at time of SVR as the narlaprevir treated patients did at the final follow-up visit [11]. Six of these patients treated with PEG-IFN-alfa2b and RBV were GT-1-infected and achieved SVR. The results of the immunologic assays in these six patients were used as a comparison to the patients that received narlaprevir treatment.

RESULTS

Of the 19 patients that achieved SVR with the narlaprevir-based regimen, 14 (75%) were male, 15 (79%) of Caucasian race, median age was 56 years (range 33-65 years) and six patients (32%) were previously treated with an IFN-based regimen. At follow-up, three patients (16%) had signs of cirrhosis which was already present before narlaprevir treatment, and median alanine transaminase (ALT) was 23 (range: 11-89). Only the three cirrhotic patients had ALT levels above the upper limit of normal. After EoT, the median number of follow-up visits was 4 (range 3-8) and the median follow-up time in months was 27 (range 15-32) months. To be more specific regarding patients with a higher chance of relapse; number of follow-up visits and median follow-up time in patients with cirrhosis was 3 and 27 (range 24-30) months, respectively. One patient did not respond to the call for his final visit; his final follow-up visit was 12 months after EoT. All patients had undetectable HCV-RNA at all follow-up visits. In addition, no clinically relevant abnormalities were observed for any patient at their follow-up visits, in particular no signs of worsening of liver disease.

In vitro quantification of HCV-specific T cell proliferation in blood was performed in six patients (one patient with cirrhosis) with SVR after narlaprevir-based therapy at the final follow-up visit, which was at least 24 months after EoT (median 25 months, range 23-30 months). HCV-specific T cell proliferation was low when compared to the positive control CMV and comparable to the negative control of culture medium alone. Also, the 6 patients who achieved SVR after PEG-IFN / RBV- therapy showed no HCV-specific T cell proliferation at follow-up when compared to medium (figure 1a/1b). Regarding PBMC, five out of six patients tested HCV-RNA negative; the remaining patient tested positive for HCV-RNA. This patient did not suffer cirrhosis and/or changes in ALT.

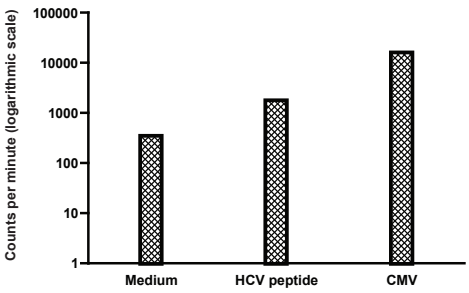

Figure 1a.

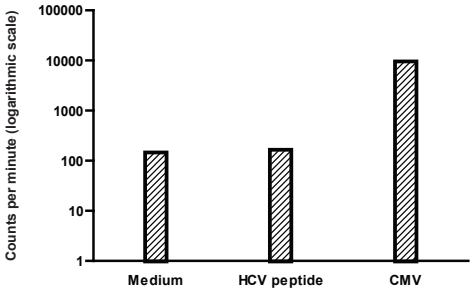

Figure 1b.

DISCUSSION

In the present long-term follow-up study in HCV genotype-1-infected patients, we demonstrated durability of SVR achieved following antiviral treatment with the protease inhibitor

narlaprevir (+/- ritonavir) followed by narlaprevir/PEG-IFN-2b (+/- ritonavir) and 24-48 weeks of PEG-IFN/RBV. In addition, at long-term follow-up, no detectable HCV-specific T cell responses were measured in blood, as expected in the absence of HCV-RNA in the circulation.

SVR, defined as undetectable HCV-RNA 24 weeks after cessation of treatment, is considered a marker for cure because of its durability and associated improved clinical outcomes [2, 12, 13]. Therefore, SVR is a major clinical goal of HCV treatment and, consequently, the rate of SVR is used to describe efficacy in clinical trials with DAAs.

In our study, no reappearances of HCV RNA during the follow-up period were observed, and, at the final follow-up visit, T cell proliferation against HCV in blood was low. In a subset of patients who achieved SVR and were only treated with PEG-IFN/RBV, HCV-specific T cell proliferation was low as well. These T cell proliferation findings are in line with the inability to detect HCV-RNA at follow-up; both findings are indicative of viral eradication. These findings are important since some studies, in which HCV patients achieved SVR with PEG-IFN/RBV, minute amounts of HCV-RNA were detected in the circulation at long-term follow-up after SVR [7, 8]. Veerapu et al demonstrated HCV-specific T cell responses with detection of small amounts of HCV-RNA [7]. Consequently, the absence of HCV-specific T cell response supports the undetectable HCV-RNA test results over time, suggesting viral eradication. Because we used a highly sensitive test for the detection of HCV-RNA (LLOD 5.3 IU/mL), performed frequent follow-up visits and maintained patients for a long time in follow-up and because a virologic relapse would be expected to result in persistent viremia, we consider SVR durable in these narlaprevir-treated patients, even up to 32 months after EoT and even in cirrhotics. In 1 of 6 patients, PBMC tested positive for HCV-RNA, while other tests, HCV-RNA in the peripheral circulation, HCV-specific T cell responses and ALT, were not suggestive of viral relapse. With long-term follow-up of patients that achieved SVR after PEG-IFN/RBV, HCV-RNA has been demonstrated in PBMC as well, even in the absence of viral relapse [10]. Our study is limited by the small number of patients. Recently, similar results have been presented in a larger cohort of telaprevir-treated patients, although this has not been published yet [14]. With the introduction of DAAs to PEG-IFN/RBV as standard of care for HCV infection, patients should be monitored long-term until our important clinical finding is validated in larger cohorts and in cohort that have been treated with other DAA-based regimens.

In conclusion, SVR achieved with narlaprevir in combination with PEG-IFN and RBV was durable, suggesting SVR to be a valid surrogate marker for successful DAA-based treatment for chronic HCV infection.

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CHAPTER 6

HEPATITIS E INFECTION AMONG CHRONIC HEPATITIS C-INFECTED PATIENTS: RISK FACTORS AND CLINICAL OUTCOME

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ABSTRACT

Background and Aims. To study risk factors for hepatitis E virus (HEV) infection and clinical characteristics in chronic hepatitis C virus (HCV) infection. **Methods**. Cross-sectional retrospective cohort study, inclusion criterion: presence of pre antiviral treatment sample. Tests: anti-IgM-HEV, anti-IgG-HEV (Wantai, Bejing China); HEV RNA was detected by an internally controlled quantitative real-time polymerase chain reaction..

Results. 261 patients, anti-IgM-HEV: 0.4%; anti-IgG-HEV: 21%, HEV-RNA: 0%. Multivariate regression analysis: age (odds ratio (OR): 1.052, 95% Confidence Interval (C.I.): 1.014-1.091; p=0.005), born in a Southern-Mediterranean country (OR: 4.225, 95% C.I.: 1.591-11.216, p=0.004) or The Pacific (OR: 3.656, 95% C.I.: 1.381-9.678, p=0.009). Cirrhosis and alanine transferase, corrected for age, were not associated (p=0.627, p=0.440, respectively). **Conclusions.** HEV prevalence was high, associated with age and being born in a Southern Mediterranean country or The Pacific, but not with more severe disease.

INTRODUCTION

Hepatitis E virus (HEV) is known as a common cause of acute, self-limiting viral hepatitis in developing countries. The virus consists of non-enveloped single-stranded positive sense RNA and transmission takes place enterally. In mammals, HEV strains are classified into 4 major genotypes. HEV genotype 1 is responsible for most endemic and epidemic cases of hepatitis E in Asia [1], and genotype 2 is prevalent in Central America and Africa[1]. There is no known animal reservoir for HEV genotypes 1 and 2; transmission occurs mainly via the oral–faecal route due to faecal contamination of drinking water. In contrast, HEV genotype 3 can infect humans and other animal species [2, 3].

In western countries, HEV infection was seen only seldomly and mostly concerned acute infection in travellers. The past years, genotype 3 HEV infection has been demonstrated in otherwise unexplained non-traveller hepatitis [4]. In immunocompromised patients, such as solid organ recipients, genotype 3 HEV infection has been demonstrated as a cause of chronic hepatitis [5]. In the general population, increased anti-IgG-HEV seroprevalence has been noted as well [6]. However, besides epidemiological changes, improvements and differences in quality of serologic tests might have influenced these changes as well [7]. Genotype 3 HEV infection appears to be related to contact with wild boars and/or consumption of undercooked meat. Other identified transmission routes are transfusion of infected blood products and vertical transmission. However, the exact routes of transmission in both immunocompetent and in immunocompromised subjects need to be determined [8].

Acute HEV (super)infection has been demonstrated as a cause of decompensation of pre-existent chronic liver disease (CLD) [9]. The effect of HEV superinfection and/or HEV co-infection on natural course of HCV-related liver disease is unclear. For comparison, hepatitis A virus (HAV) infection in chronic hepatitis C (HCV)-infected patients carries an increased risk of fulminant hepatitis [10]. Since it has now become clear that autochthonous HEV is common in developed countries more knowledge on the effects of superinfection of HEV of pre-existent CLD is needed. Recently, injecting drug use (IDU) was suggested as risk factor for HEV infection [11]. IDU is the main route of transmission in chronic HCV-infected patients. Therefore, if IDU is a risk factor for HEV infection, many chronic HCV-infected patients are at increased risk of HEV infection. The clinical consequences of HEV superinfection are unclear. We aimed to identify prevalence of HEV infection in chronic HCV-infected patients, to determine risk factors for HEV infection and to describe the influence of HEV infection on natural course of HCV-related liver disease.

Methods

Patients and materials

For our analyses, we used a well-characterized consecutive series of 321 HCV patients treated with (peg)interferon (and ribavirin) between 2000 and 2009 [12]. Samples of these patients were collected during routine visits to our outpatient clinic for clinical assessments had been stored at -20°C (serum) and -80°C (EDTA-plasma). Each enrolled subject had consented in future testing of archived bio-samples. The study protocol confirms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the local medical ethical committee (MEC approval: MEC-2011-090). Regarding our cohort, cirrhosis was defined as the presence of a result of liver biopsy and/or liver elastography suggesting cirrhosis. HEV prevalence is subject to geographical differences. These differences might be due to dietary practices or other reasons. We included region of origin as variable to take into account these geographical differences. Because there are many countries of birth, we categorised countries of birth following; Northern Europe and Russia, Northern Mediterranean countries, Southern Mediterranean countries, Africa, Asia and New Zeeland, Northern, Middle and Southern America and unknown.

Peginterferon and, especially, ribavirin have been described as possible antiviral treatment for HEV infection. Hence, we included all patients of whom a pre-treatment sample was available and used this sample for HEV testing.

HEV specific antibody detection

For both HEV specific IgM and HEV specific IgG detection in serum or EDTA plasma samples the commercially available enzyme-linked immunosorbent assay (ELISA) (Wantai, Beijing, China) was used according to the manufacturer's instructions.

HEV-RNA detection

All samples, which were positive for HEV specific IgM or HEV specific IgG were screened for the presence of HEV RNA by an internally controlled quantitative real-time RT-PCR, described previously [5]. The RT-PCR had a lower limit of detection (95% hit rate) of 143 IU/ml as determined by the 1st WHO standard for HEV RNA NAT-Based assays (6329/10, Paul Ehrlich Institute, Germany).

Statistics

Baseline characteristics between patients were compared according to the IgG- anti-HEV test result using Pearson-Chi Square or the independent samples T-test in case of dichotomous or continuous variables, respectively. To identify independent risk factors for HEV infection, we first performed univariate logistic regression analysis in continuous variables. Pearson Chi-Square was already performed in dichotomous baseline variables according to the test result and provides the same p-value as univariate logistic regression analysis. Determinants with statistical significance at the 0.20 level following Pearson Chi-Square and/or the univariate analysis were

included in the multivariate analysis with backward selection based on the likelihood ratio. Both with univariate and multivariate regression analyses, IgG-anti-HEV was the dependent variable. Data were analyzed using SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS

Of 261 patients out of 321 subjects in the cohort, a pre-treatment sample was available; HEV specific IgG antibodies were found in 54 patients (21%) and HEV specific IgM antibodies in only 1 patient (0.4%). No HEV RNA was detected in any of the seropositive samples, which suggests that there was one subacute HEV infection in our cohort and that there were no chronic HEV infections. Baseline characteristics are presented in table 1 and compared according to the test result of IgG-anti-HEV serology. Univariate logistic regression analysis added the parameter 'age' as robust correlation (odds ratio (OR) 1.053, 95% confidence interval (C.I.) 1.019-1.088; p=0.002) to the variables which were associated with positive IgG-anti-HEV serology. Because 92% of patients with a positive test result for IgG anti-HEV and a history of IDU were born in Northern Europe or Russia, analysis of IDU and region of origin in relation to IgG-anti-HEV was invalidated

Table 1. Baseline characteristics

	IgG-anti-HEV positive	IgG-anti-HEV negative	p-value
	N=54	N=207	
Male gender, n (%)	38 (70)	137 (66)	0.560
Age, years, mean (SD)	49 (10)	44 (10)	0.002*
Severe liver damage, cirrhosis, n (%)	16 (30)	43 (21)	0.166
Alanine transaminase, mean (SD)	103 (49)	109 (76)	0.440
Past of injecting drug use, n (%)	14 (26)	113 (55)	0.001**
Country of birth, region, n (%)	54 (100)	207 (100)	0.001**
Northern Europe and Russia	23 (45)	142 (71)	0.001**
Northern Mediterranean countries	4 (8)	8 (4)	0.247
Southern Mediterranean countries	11 (22)	12 (6)	0.001**
Africa	1 (2)	4 (2)	0.999
The Pacific (Asia and New Zeeland)	9 (18)	14 (7)	0.018**
Northern, Middle and Southern America	3 (6)	21 (10)	0.321
Unknown	3 (6)	6 (3)	0.341
Sustained virologic response following PEG-IFN/RBV, n (%)	35 (65)	118 (77)	0.394
Chronic hepatitis C infection genotype, n (%)	54 (100)	207 (100)	0.181
1	22 (41)	102 (49)	0.263
2	11 (20)	27 (13)	0.174
3	17 (32)	73 (35)	0.602
4	4 (7)	5 (2)	0.073

Table 2. Independent risk factors for positive IgG-anti-HEV serology.

Multivariate model	Odds ratio	95% confidence interval	p-value
Age * cirrhosis * region of origin			
Age	1.052	1.013-1.092	0.006*
Cirrhosis	0.966	0.442-2.113	0.931
Region of origin			0.008*
Compared to Northern Europe and Russia:			
Northern Mediterranean countries	2.848	0.757-10.716	0.122
Southern Mediterranean countries	4.225	1.591-11.216	0.004*
Africa	1.114	0.115-10.766	0.926
The Pacific (Asia and New Zeeland)	3.656	1.381-9.678	0.009*
Northern, Southern and Middle America	0.638	0.169-2.409	0.507

by collinearity. Therefore, we performed multivariate logistic regression analysis within patients born in Northern Europe or Russia, corrected for age; no association of IDU could be made with the presence of IgG-anti-HEV (p=0.252). Multivariate regression analysis identified age (OR: 1.052, 95% C.I.: 1.014-1.091; p=0.005) and region of origin (p=0.008) as risk factors (table 2). When corrected for age, the presence of cirrhosis was not associated with positive IgG-anti-HEV serology at all (OR: 1.192, 95% C.I. 0.586-2.425; p=0.627).

DISCUSSION

With this retrospective cross-sectional study in chronic HCV-infected patients, we demonstrated that previous HEV infection is a common finding (21%) and is not associated with an increased prevalence of cirrhosis. Further, recent HEV infection was a seldom finding en chronic HEV was not diagnosed in these immunocompetent HCV-infected patients. Therefore, our study suggests that HEV infection is common among chronic HCV-infected patients and does not carry severe clinical consequences. This is of clinical relevance, because HEV appears to be more common in developed countries than previously thought[6].

Acute HEV has the potential to worsen pre-existent CLD [9]. However, a closer look at these studies shows that patients affected seldomly concern patients that suffer solely chronic HCV infection as pre-existent disease [9, 13, 14]. De Silva et al. retrospectively studied patients in whom HEV serology was requested for work-up of either unexplained abnormal liver function tests or decompensation in the presence of CLD. Acute HEV infection was present in five of 164 patients in whom serological testing was requested. Four out of five patients had a history positive for recent travel and the hepatitis was self-limiting. The fifth patient had well compensated cirrhosis from chronic HCV infection and alcohol, which however decompensated after acute HEV infection with slow clinical amelioration. This study does not proof that HEV leads to hepatic decompensation in chronic HCV infection, because of the presence of concomitant alcohol use.

Alcohol use is a well-known risk factor for hepatic decompensation from acute HEV infection. Radha Krishna et al. studied predictors of outcome of acute-on-chronic-liver failure due to HAV and/or HEV infection in patients suffering CLD of varying aetiology. Chronic HCV was present in five (4.1%) out of 121 patients included. Unfortunately, the article does not elucidate the type of superinfection (HEV, HAV or both) nor describe the clinical course of disease in this subset of patients [14]. Kumar Acharya followed patients with cirrhosis prospectively and studied the effect of HEV on natural course of the disease [13]. Thirty out of 107 patients (28%) with cirrhosis have detectable HEV RNA in their sera. Twenty-one of these 30 patients demonstrated hepatic decompensation while previously asymptomatic. Chronic HCV prevalence was low in the total group of patients (n=13, 12%) and even lower in patients with detectable HEV RNA infection (n=2, 7%). Unfortunately, from this paper, it is not clear whether these two HEV/HCV-coinfected patients demonstrated hepatic decompensation. Therefore, to our knowledge, the risk of hepatic decompensation due to HEV infection in pre-existent chronic HCV infection is not known from current literature. Our study shows that, within HCV-infected patients, HEV seroprevalence is high and not associated with hepatic decompensation or more severe liver disease. In contrast to previous reports [11], our study could not demonstrate IDU to be associated with positive IgG anti HEV serology. We identified age and being born in a Southern Mediterranean country or being borne in The Pacific associated with positive HEV serology. These findings have been described before [2]. We explain age as a risk factor for increase in exposure. In Asian countries HEV is endemic, which might explain being born in The Pacific as risk factor for positive HEV serology. In Mediterranean countries, dietary practices are different and have been suggested as risk factor. Our study, however, did not identify being born in Northern Mediterranean countries as risk factor. On the contrary, being born in a Southern Mediterranean country was associated with positive HEV serology. Other routes of transmission might be iatrogenic and enteral with a relatively lesser degree of hygiene.

Several weaknesses of the study should be mentioned. First of all, this is a retrospective cross-sectional study. Therefore, with this study, we were not able to define whether HCV infection or HEV infection occurred first. In the Netherlands, HCV incidence has been very low in the past two decades. Because HEV seroprevalence appears to be high, it might well be that at least some HCV-infected patients suffered HCV before they became infected with HEV. Future longitudinal studies should be performed to answer this question. Secondly, we used a well-characterized retrospectively collected database of HCV-infected patients. Regarding HEV infection, dietary practices have been suggested to affect risk of transmission, for example, eating uncooked porc. Data on this subject were not available. Therefore, we cannot estimate the time of possible HEV infection. Furthermore, with this study we tried to identify risk factors for HEV infection and could not control for this possible risk factor.

In conclusion, with our retrospective cross-sectional study on HEV infection in 261 chronic HCV-infected patients, we found a high seroprevalence of HEV (21%) without chronic HEV infection, only one case of acute HEV infection and no signs of more severe liver disease with

positive HEV serology. Risk factors for previous HEV infection were age and being born in Southern Mediterranean countries or The Pacific and not IDU. Our study suggests that HEV infection does not alter natural course of pre-existent chronic HCV-infection. Prospective studies are needed to confirm this.

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SUMMARY AND DISCUSSION

The hepatitis C virus (HCV) has been identified in 1989. Although this is quite recent, substantial changes in epidemiology and therapy have been accomplished already. HCV incidence reduced substantially with the implementation of harm reduction measures of which syringe exchange programs and screening of blood(-derived) products are foremost [1]. Sustained virologic response (SVR) rates increased from less than 20% with interferon monotherapy to 70-80% of untreated genotype 1 (GT-1) infected patients following peginterferon (PEG-IFN)/ribavirin (RBV) and telaprevir or boceprevir. Despite these optimistic developments, nowadays, chronic HCV infection is responsible for a substantial burden of disease worldwide and has become the leading indication for liver transplantation. Consequently, all efforts should be made to enable diagnosis and treatment before the development of end-stage liver disease (decompensated cirrhosis, hepatocellular carcinoma) irreversible has developed. Because (former) injecting drug users (IDUs) constitute the main risk group of HCV infection, diagnosis and treatment are offered increasingly in opiate substitution treatment centers [2, 3]. In **Chapter 1**, we describe our prospective observational study of the diameter of the common bile duct (CBD) in patients referred for abdominal ultrasonography at the gastroenterology and hepatology outpatient clinic. We demonstrate that methadone is associated with CBD dilatation (CBDD), a finding that is known as a first sign of biliary or pancreatic malignancy. Whereas CBDD was only seen in 7% of subjects referred for abdominal ultrasonography in general, asymptomatic CBDD was present in 56% of methadone users. Further, after a median follow-up of 16 months (range: 1-17 months), there were no signs of clinical worsening. Therefore, we concluded that CBDD is an asymptomatic finding in methadone users. This is of clinical relevance because cost and risk of unnecessary further investigation and unnecessary delay of HCV antiviral therapy can be prevented.

To decrease the burden of HCV, increased HCV treatment of injecting drug users (IDUs) is needed[4]. Although the feasibility of HCV-treatment has been well established in substance users, active is still quite often an exclusion criterion or not well defined in these studies. The antiviral effects of PEG-IFN/RBV are largely immunomodulatory and heroin or heroin-derivatives are considered to deregulate the function of T-cells, B-cells and NK cells *in vitro* and *in vivo. NK cells. It has recently been demonstrated that t*he induction of cytotoxic natural killer (NK) cell function by IFN-α correlates with virologic response to therapy[5]. The effects of active substance use on IFN-responsiveness are not well understood. In smaller groups of patients, IDUs that frequently inject drugs showed a lower rate of SVR [6]. In **Chapter 2**, we studied the natural killer (NK) cell population, activation and effects during PEG-IFN/RBV in chronic HCV-infected patients with and without active substance use at baseline and at day 7 of PEG-IFN/RBV compared with healthy controls. The most important findings are that, at baseline, the activation of NK cells was significantly lower in HCV-infected patients who used substances when compared to healthy controles. Furthermore, NK cells from HCV patients remained responsive to IFN and only a minor decline in degree of STAT-1 phosphorylation was observed, irrespective of substance use. Moreover, viral load decline at day 7 was similar between HCV patients

with and without substance use. Therefore, we concluded that active substance use in chronic HCV-infected patients did not affect the immune responsiveness to IFN early after initiation of treatment. We found no immunologic arguments against PEG-IFN/RBV in HCV-infected patients with active substance use. Our results suggest that the previously described lower rate of SVR in frequent injectors is not a result of the (frequency of) concomitant substance use [6]. Future studies should be performed to investigate whether substance use affects HCV therapy when combined with a direct acting antiviral agent.

Antiviral therapy with PEG-INF/RBV for chronic HCV infection is hampered by substantial and frequent side effects. Depression is a frequent side effect and has been demonstrated to be the main reason for early drop-out[7]. **Chapter 3** describes our post-hoc analyses on a prospective randomized controlled trial of escitalopram versus placebo during antiviral therapy with PEG-IFN/RBV. We identified risk factors for depression and studied depressive symptoms during antiviral therapy in patients with and without risk factors for IFN-induced depression. We found that patients with a history of IDU and depression are most vulnerable for the development of IFN-induced depression (odds ratio: 12.60; 95% confidence interval 2.47-64.34, $p<0.01$). Moreover, treatment with the selective serotonin reuptake inhibitor (SSRI) escitalopram was associated with a significant reduction in depressive symptoms measured with the Symptom Checklist-90 (SCL-90) ($p=0.03$), Becks Depression Inventory (BDI) ($p=0.0479$), but not with the Montgomery-Asberg Depression Rating Scale ($p=0.64$). We concluded that HCV-infected patients with history of depression and IDU carry the highest risk to develop IFN-induced depression. Further, prophylaxis with escitalopram results in a decrease of depressive symptoms on the SCL-90 and the BDI. This is of great clinical relevance, because evidence from current trials suggests that PEG-IFN/RBV will remain a central element in many future HCV regimens. The characteristics that we identified are easy to determine pre-treatment. Our study population included a large percentage of patients with previous psychiatric and substance use disorders. It might well be that studies in other populations with less psychiatric comorbidity show different results. Future research should be performed to confirm our findings. Further, it might be interesting to study the effect of SSRI prophylaxis on the frequency of early drop-out.

The most recent change of HCV standard of care antiviral therapy has been the addition of the protease inhibitors telaprevir/boceprevir. In contrast to PEG-IFN/RBV, the antiviral potential of direct acting antivirals (DAAs) does not rely on immunomodulatory effects. The infection and replication processes utilized by the HCV provide potential therapeutic targets. DAAs are classified according to their therapeutic target and mechanism of action. Nonstructural proteins targeted include the NS2/NS3 region, the NS3 serine protease RNA helicase, the NS4A peptide cofactor of NS3, the NS5A protein and the NS5B RNA-dependent polymerase. In **Chapter 4**, we studied the safety, tolerability and effectiveness of the protease inhibitor PHX1766. We used an overlapping, dose-adaptive single-dose and multiple-dose escalating design in healthy controls and HCV-infected patients. The main findings of our study are that PHX1766 was safe and well tolerated at all dose levels with rapid absorption (time at which concentration maximum is

reached 1-4 hours) and with mean terminal half-lives of 4-23 hours. Viral decline was only modest with a mean maximal observed HCV-RNA decline of 1.5 $\log_{10}$ IU/ml after 6 days of PHX1766 dosing. We concluded that our study design was highly efficient in identifying a therapeutic dose. Because the virologic results were disappointing, we concluded that in vitro effectivity is not always predictive for in vivo effectivity.

In contrast to PEG-IFN/RBV, DAAs select for resistant strains and their immune modifying capacities are limited [8-11]

Historically, success of antiviral therapy following PEG-IFN/RBV is assessed by rate of SVR. SVR is defined as the non-detectability of HCV-RNA at 24 weeks after end of IFN-based antiviral treatment. SVR has proven to be durable after long-term follow-up and is therefore considered a reliable surrogate marker for cure of HCV [12]. In **Chapter 5**, we describe the follow-up of nineteen patients that achieved SVR following treatment with the HCV protease inhibitor narlaprevir followed by PEG-IFN/RBV. Patients were followed for a median of 27 months (range 15-32) after end of treatment. The median number of follow-up visits was 4 (range 3-8), which is relevant because sporadic reappearrances of HCV-RNA have been described after PEG-IFN/RBV [13]. In our subset of patients, HCV-RNA was not detected. Further, at the final follow-up visit, we investigated HCV-specific T-cell proliferation which was low. In contrast, Veerapu et al did find HCV-specific T-cell proliferation at time of HCV-RNA reappearances[13]. Therefore, both these findings are indicative of viral clearance. We concluded that SVR achieved with narlaprevir in combination with PEG-IFN/RBV was durable, suggesting SVR to be a valid surrogate marker for cure also for DAA-based HCV treatment. Nevertheless, patients treated with DAAs should be followed-up long-term to validate our findings in larger groups of patients. **Chapter 6** describes the results of our retrospective, cross-sectional study investigating prevalence and clinical characteristics of hepatitis E virus (HEV) infection in chronic HCV-infected patients. The past few years, genotype 3 HEV infection has been demonstrated in otherwise unexplained non-traveller acute hepatitis[14]. In immunocompromised patients, such as solid organ recipients, genotype 3 HEV infection has been demonstrated as a cause of chronic hepatitis[15, 16]. We demonstrated that positive anti-IgG-HEV-serology (Wantai, Bejing China) is a frequent finding in chronic HCV-infected patients. More importantly, we found that positive anti-IgG-HEV-serology is not associated with signs of more severe HCV-related liver disease.

CONCLUSIONS

In the diagnostic work-up of HCV-related liver disease in methadone users, CBDD will be frequently seen and should be considered a side effect of methadone without underlying pathology. PEG-IFN/RBV therapy is not hampered by active substance use by itself. HCV-infected patients with a history of depression and IDU are most vulnerable for the development of IFN-induced depression and might benefit from SSRI prophylaxis. In the development of DAAs,

it should be noted that robust antiviral potency is not predictive of in vivo antiviral activity. SVR achieved following a DAA in a regimen containing PEG-IFN is durable. HEV is a common self-limiting infection that does not lead to clinical worsening in HCV-infected patients.

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NEDERLANDSE SAMENVATTING EN DISCUSSIE

Het hepatitis C virus (HCV) is in 1989 geïdentificeerd. Hoewel dit niet lang geleden is, zijn zowel epidemiologie als behandeling van de ziekte chronische HCV de afgelopen tijd enorm veranderd. Preventieve maatregelen zoals spuitomruil en screening van bloedproducten hebben geleid tot een afname van nieuwe HCV infecties [1]. De behandeling is een stuk succesvoller geworden. Met interferon monotherapie bereikte minder dan 20% van de patiënten een 'sustained virologic response' (SVR). Tegenwoordig bestaat de behandeling uit peginterferon (PEG-IFN)/ribavirine (RBV) met telaprevir of boceprevir en bereikt 70-80% van de niet eerder behandelde genotype 1-geïnfecteerde patiënten SVR. Ondanks deze optimistische resultaten is chronische HCV vandaag de dag verantwoordelijk voor een substantiële ziektelast wereldwijd. Tevens is chronische HCV de meest frequente indicatie voor levertransplantatie. Daarom is het van belang de diagnose te stellen en behandeling te laten plaatsvinden alvorens de patiënt eindstadium leverziekte (gedecompenseerde cirrose, hepatocellulair carcinoom) heeft bereikt. Injecterende druggebruikers (IDGs), ook voormalig IDGs, vormen de belangrijkste risicogroep voor chronische HCV. Diagnostiek naar en behandeling van chronische HCV wordt steeds vaker gecombineerd met opiaatverstrekking, omdat dit (voormalig) injecterend druggebruikers (IDG) betreffen [2, 3]. In **Hoofdstuk 1** beschrijven we de resultaten van onze prospectieve observationele studie naar de diameter van de ductus choledochus bij patiënten die verwezen zijn naar onze polikliniek Maag-, darm- en leverziekten voor het ondergaan van abdominale echografie. Uit onze studie blijkt dat methadon geassocieerd is met verwijding van de ductus choledochus. Verwijding van de ductus choledochus kan een eerste teken zijn van galweg- of alvleesklierkanker. Wij tonen aan dat deze verwijding met echografie gezien werd bij 7% van de totale onderzoeksgroep en bij 56% van de subgroep van methadongebruikers. Na mediane follow-up van 16 maanden (range: 1-17 maanden) was geen sprake van klinische verslechtering. Daarom hebben we geconcludeerd dat verwijding van de ductus choledochus een veel voorkomende en asymptomatische bevinding is bij methadongebruikers. Dit klinische relevantie van deze bevinding vertaalt zich in het feit dat onnodig verder onderzoek naar een onderliggend lijden wordt voorkómen en daarmee onnodige vertraging van antivirale therapie voor chronische HCV. Om de ziektelast van HCV te verlagen is behandeling van IDGs nodig [4]. Ondanks dat ruimschoots is aangetoond dat HCV behandeling haalbaar is in druggebruikers, de studies die dit aantonen excluderen actief druggebruk of omschrijven niet goed wat met actief druggebruik bedoeld wordt. De antivirale werking van PEG-IFN/RBV is voornamelijk immunomodulatoir. Heroïne en heroïne-afbraakproducten zouden van invloed zijn op de functie van T-cellen, B-cellen en natural killer (NK) cellen, zowel in vitro als in vivo. Recentelijk is aangetoond dat de inductie van cytotoxische NK cellen tijdens antivirale therapie met interferon-α correleeert met de virologische response op therapie [5]. Het effect van actief druggebruik op de respons op IFN is niet duidelijk. Een studie met een kleine subgroep van IDGs toonde een lagere percentage SVR in patiënten die frequent drugs injecteerden [6]. Wij bestudeerden de NK cel populatie, activatie en effecten tijdens PEG-IFN/RBV in chronische HCV patiënten met en zonder actief druggebruik bij begin en op dag zeven van PEG-IFN/RBV en we vergeleken met gezonde controles

(**hoofdstuk 2**). De belangrijkste bevindingen zijn dat, bij begin van therapie, de activatie van NK cellen significant lager is in HCV patiënten die drugs gebruiken vergeleken met gezonde controles. Bovendien bleven NK cellen van HCV patiënten gevoelig voor IFN en slechts een kleine afname in mate van STAT-1 fosforylatie werd gezien, onafhankelijk van druggebruik. Tevens werd een afname in virusconcentratie gezien op dag 7 welke vergelijkbaar was tussen HCV patiënten met en zonder druggebruik. Daarom concludeerden wij dat actief druggebruik in chronische HCV patiënten de immunologische gevoeligheid voor IFN niet veranderd vlak na start van therapie. Aldus vonden wij geen immunologische argument ten nadele van PEG-IFN/RBV bij HCV patiënten die actief drugs gebruiken. Onze resultaten suggereren dat de eerder gerapporteerde lagere SVR bij frequent IDG niet veroorzaakt werd door het actief druggebruik zelf [6]. Het is nog niet duidelijk of actief druggebruik van invloed is op HCV therapie met de toevoeging van een direct antiviraal middel en hier is verder onderzoek voor nodig.

PEG-IFN/RBV als antivirale behandeling van chronische HCV wordt bemoeilijkt door aanzienlijke en frequente bijwerkingen. Depressie is een frequente bijwerkingen en zou de belangrijkste oorzaak zijn van vroegtijdig stoppen met antivirale therapie [7]. **Hoofdstuk 3** betreft onze post-hoc analyses van een prospectieve gerandomiseerde studie welke escitalopram met placebo vergeleek tijdens PEG-IFN/RBV. Wij identificeerden risicofactoren voor depressie. Vervolgens bestudeerden we depressieve symptomen tijdens antivirale therapie in patiënten die belast waren met deze risicofactoren. Patiënten met een voorgeschiedenis van depressie en IDG bleken meest kwetsbaar voor het ontwikkelen van een IFN-geïnduceerde depressie (odds ratio: 12.60; 95% confidence interval 2.47-64.34, $p<0.01$). Bovendien was behandeling met de selectieve serotonine reuptake inhibitor (SSRI) escitalopram geassocieerd met een significante reductie in depressieve symptomen gemeten met de Symptom Checklist-90 (SCL-90) ($p=0.03$), Becks Depression Inventory (BDI) ($p=0.0479$), maar niet met de Montgomery-Asberg Depression Rating Scale ($p=0.64$). Aldus concludeerden wij dat HCV-geïnfecteerde patiënten met een voorgeschiedenis van depressie en IDG het hoogste risico hebben op IFN-geïnduceerde depressie en beschermd lijken tegen depressieve symptomen middels profylaxe met escitalopram. Deze bevinding is van enorm klinisch belang omdat het zeer waarschijnlijk is dat PEG-IFN/RBV de komende jaren een centraal onderdeel blijft van de antivirale behandeling van chronische HCV. De risicofactoren die wij hebben geïdentificeerd zijn makkelijk om vóór behandeling vast te stellen. Wat betreft onze onderzoekspopulatie dient te worden opgemerkt dat een groot deel van de patiënten belast is met psychiatrische co-morbiditeit waaronder verslaving. Het kan dus goed dat eenzelfde studie in een andere onderzoekspopulatie met minder psychiatrische co-morbiditeit tot andere uitkomsten leidt. Onze bevindingen moeten daarom worden bevestigd. Tevens moet worden onderzocht of profylaxe met escitalopram leidt tot minder uitval tijdens antivirale therapie.

De belangrijkste meest recente verandering van de antivirale behandeling van chronische HCV is de toevoeging van de proteaseremmers telaprevir/boceprevir. In tegenstelling tot PEG-IFN/RBV berust de werking van direct antivirale middelen niet op immunomodulatoire

effecten. De mechanismen die het virus gebruikt om te infecteren en te repliceren voorzien in therapeutische aangrijpingspunten. Direct antivirale middelen zijn geclassificeerd naar hun therapeutisch aangrijpingspunt en mechanisme van werking. Nonstructurele proteasen waar direct antivirale middelen op aangrijpen zijn de NS2/NS3 regio, het NS3 serine protease RNA helicase, de NS4A peptide cofactor van NS3, het NS5A eiwit en de NS5B RNA-afhankelijke polymerase. In **hoofdstuk 4** beschrijven we onze studie naar de veiligheid en het verdragen van PHX1766 en de antivirale werking ervan. Voor deze studie gebruikten we een overlappende, dosis-adaptieve enkelvoudige-dosis en meervoudige-dosis studie opzet in gezonde controles en HCV geïnfecteerde patiënten. De belangrijkste bevindingen van deze studie zin dat PHX1766 veilig was, goed verdragen werd in alle doses, snel geabsoerbeerd werd (tijd waarop de maximale concentratie bereikt werd was 1-4 uur) en een gemiddelde halfwaarde tijd had van 4-23 uur. De invloed op de virusconcentratie was slechts bescheiden met een gemiddelde maximale afname in HCV-RNA van 1.5 $\log_{10}$ IU/ml na 6 dagen inname van PHX1766. Wij concludeerden dat onze studie opzet zeer efficiënt was in het identificeren van een therapeutische dosis. Omdat de virologische resultaten tegenvielen concludeerden wij dat in vitro effectiviteit niet altijd voorspellend is voor in vivo effectiviteit.

In tegenstelling tot PEG-IFN/RBV leidt behandeling met direct antivirale middelen tot selectie van resistente stammen en zijn de immuunmodulerende eigenschappen beperkt [8-11]. Historisch geizen werd succes van antivirale therapie voor HCV afgemeten aan percentage SVR. SVR is gedefinieerd als niet detecteerbaar zijn van HCV-RNA 24 weken na staken van op IFN gebaseerde therapie. Het is gebleken dat SVR duurzaam is na langdurig vervolgen van patienten die SVR hebben bereikt. Daarom wordt het beschouwd als een betrouwbare surrogaat marker voor genezing van HCV [12]. In **hoofdstuk 5** beschrijven we onze follow-up studie in negentien patiënten die SVR hebben bereikt na behandeling met de FHCV proteaseremmer narlaprevir gevolgt door PEG-IFN/RBV. De mediane follow-up was 27 maanden (range: 15-32 maanden) na staken van antivirale therapie. Het mediane aantal follow-up bezoeken was 4 (range: 308 bezoeken). Dit is een belangrijk gegeven, omdat beschreven is dat SVR met PEG-IFN/RBV HCV-RNA soms spontaan opduikt in de bloedbaan [13]. In onze patiëntengroep werd geen HCV-RNA gemeten. Tevens hebben we bij het laatste follow-up bezoek gekeken naar de HCV-specifieke T-cel proliferatie, welke laag bleek. Veerapu et al beschreef de eerder genoemde spontane aanwezigheid van HCV-RNA in de bloedbaan. Ten tijde van deze detecteerbaarheid van HCV-RNA was sprake van HCV-specifieke T-cel proliferatie [13]. Daarom concluderen wij dat de afwezigheid van HCV-RNA tijdens follow-up en de afwezigheid van HCV-specifieke T-cel proliferatie suggestief is voor virale klaring. Aldus is SVR bereikt met narlaprevir gevolgd door PEG-IFN/RBV duurzaam. Dit suggereert dat SVR, ook bij antivirale therapie met een direct antiviraal middel, een valide surrogaat marker is voor genezing van HCV. Desalniettemin moeten onze resultaten bevestigd worden in grotere groepen en na langere follow-up. **Hoofdstuk 6** beschrijft de resultaten van onze retrospectieve, cross-sectionele studie naar het vóórkomen van en de klinische gevolgen van hepatitis E virus (HEV) infectie bij chronische HCV patiënten.

De afgelopen paar jaar is gebleken dat HEV verantwoordelijk is voor eerder onverklaarde, niet met reizen samenhangende acute hepatitis [14]. In immuungecompromitteerde patiënten, zoals patiënten die via donatie een organ hebben ontvangen, is gebleken dat HEV GT-3 infectie verantwoordelijk is voor de ontwikkeling van chronische hepatitis [15, 16]. Wij toonden aan dat antistoffen tegen HEV met gebruik van de Wantai test (Wantai, Bejing China) een frequent bevinding is in chronische HCV patiënten. Bovendien vonden we dat chronische HCV patiënten met antistoffen tegen HEV geen ernstigere schade aan de lever lijken te hebben.

CONCLUSIES

In de diagnostiek van HCV en de voorbereiding voor starten van antivirale therapie bij methadongebruikers is verwijding van de ductus choledochus een frequente echografische bevinding. Deze bevinding zou moeten worden beschouwd als een bijwerking van methadon zonder onderliggende ziekte. PEG-IFN/RBV therapie wordt niet gehinderd door het actief druggebruik zelf tijdens therapie. HCV patiënten met in de voorgeschiedenis depressie en IDG zijn meest kwetsbaar voor het ontwikkelen van IFN-geïnduceerde depressie en zouden baat kunnen hebben bij SSRI prophylaxis. Wat betreft de ontwikkeling van nieuwe direct antivirale middelen is het van belang dat krachtige antivirale activiteit niet voorspellend is voor in vivo antivirale activiteit. SVR na antivirale therapie met narlaprevir en PEG-IFN/RBV is duurzaam. HEV is een veel voorkomende zelf limiterende infectie die niet leidt tot verergering van leverschade bij HCV patiënten.

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LIST OF PUBLICATIONS

1. **Hotho DM**, de Bruijne J, Spaan M, Treitel MA, Boonstra A, de Knegt RJ, Janssen HL, Reesink HW. *Sustained virologic response after therapy with the HCV protease inhibitor narlaprevir in combination with peginterferon and ribavirin is durable through long-term follow-up*. J Viral Hepat. 2013 Apr;20(4):e78-e81.
2. **Hotho DM**, Kreefft K, Groothuismink ZM, Janssen HL, de Knegt RJ, Boonstra A. *Natural Killer cell activity and function in chronic HCV-infected patients during peginterferon and ribavirin: early effects of active substance use*. Antiviral Res. 2013 Jan 2;97(3):347-355.
3. **Hotho DM**, de Bruijne J, O'Farrell AM, Boyea T, Li J, Bracken M, Li X, Campbell D, Guler HP, Weegink CJ, Schinkel J, Molenkamp R, van de Wetering de Rooij J, van Vliet A, Janssen HL, de Knegt RJ, Reesink HW. *Pharmacokinetics and antiviral activity of PHX1766, a novel HCV protease inhibitor, using an accelerated Phase I study design*. Antivir Ther. 2012;17(4):657-67.
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7. **Hotho DM**, Schouten JN, Taimr P, Borst J, Hansen BE, Janssen HL, de Knegt RJ. *Common bile duct dilatation in methadone users: a common finding without underlying pathology.* Submitted.
8. **Hotho DM**, Pas SD, Hansen BE, Osterhaus AD, Janssen HL, de Knegt RJ, van der Eijk AA. *Hepatitis E infection among chronic hepatitis C-infected patients: risk factors and clinical outcome.* Submitted.
9. **Hotho DM**, Bezemer G, Hansen BE, Van Gool AR, de Knegt RJ, Janssen HL, Veldt BJ. *Effects of selective serotonin reuptake inhibitor prophylaxis during antiviral treatment for chronic hepatitis C in patients with a history of injecting drug use and depression.* Resubmitted to the Journal of Clinical Psychiatry.

PHD PORTFOLIO

Name PhD student: Daphne M. Hotho
PhD period: 2008-2013
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International conferences

	YEAR	WORKLOAD
63rd Annual Meeting of the American Association for the Study of Liver Diseases Boston, USA	2012	28 hours
47th Annual Meeting for the European Association for the Study of the Liver Barcelona, Spain	2012	28 hours
62nd Annual Meeting of the American Association for the Study of Liver Diseases San Francisco, USA	2011	28 hours
46th Annual Meeting for the European Association for the Study of the Liver Berlin, Germany	2011	28 hours
61st Annual Meeting of the American Association for the Study of Liver Diseases Boston, USA	2010	28 hours
45th Annual Meeting for the European Association for the Study of the Liver Vienna, Austria	2010	28 hours
2nd International symposium on hepatitis treatment in substance users, Brussels, Belgium	2009	24 hours
1st international symposium on hepatitis treatment in substance users, Zürich, Switserland	2008	24 hours

Oral presentations

	YEAR	WORKLOAD
Hepatitis E virus infection among chronic hepatitis C-infected patients: risk factors and clinical outcome. NVH, Zeist, the Netherlands	2012	12 hours
Natural killer cell activity and function in chronic HCV-ionfected patients during peginterferon and ribavirin: effects of active substance use. NVH, Zeist, the Netherlands	2012	12 hours
A prognostic model to select patients for prophylactic SSRI therapy during antiviral treatment for chronic hepatitis C infection. NVGE, Veldhoven, the Netherlands	2011	12 hours
Common bile duct dilatation, an erroneous and misleading sign in the diagnostic approach of methadone users at the hepatology outpatient clinic. NVGE, Veldhoven, the Netherlands	2011	12 hours
Project Actief Testen! Hepatitis C behandeling bij druggebruikers. Gezonder Rotterdam, Rotterdam, the Netherlands	2011	12 hours
High prevalence of common bile duct dilatation among chronic HCV-infected methadone users. NVGE, Veldhoven, the Netherlands	2010	12 hours

Prevalence of HCV among former and current drug users and marginally housed people: results of a multidisciplinary approach on screening and antiviral treatment. NVGE, Veldhoven, the Netherlands	2009	12 hours

Poster presentations

	YEAR	WORKLOAD
Hepatitis E infection among chronic hepatitis C-infected patients: risk factors and clinical outcome AASLD, Boston, USA	2012	12 hours
Chronic hepatitis C genotype 1 infection: the economic need for early intervention with antiviral therapy. AASLD, San Francisco, USA	2011	12 hours
A prognostic model to select patients for prophylactic SSRI therapy during antiviral treatment for chronic hepatitis C infection. AASLD, San Francisco, USA	2011	12 hours
Common bile duct dilatation, an erroneous and misleading sign in the diagnostic approach of methadone users. AASLD, San Francisco, USA	2011	12 hours
Access to Hepatitis C Testing and Treatment for Substance Users in Rotterdam, The Netherlands: results of a multidisciplinary approach. 1st International Symposium on Hepatitis Care in Substance Users, Zürich, Switzerland	2010	12 hours
Accelerated clinical trial design to assess safety, tolerability and antiviral activity of PHX1766, a novel HCV protease NS3/4A inhibitor, in healthy volunteers and chronic hepatitis C patients. AASLD, Boston, USA.	2009	12 hours

Courses and workshops

	YEAR	WORKLOAD
NIHES biostatistics for clinicians	2010	28 hours
NIHES regression analysis for clinicians	2010	38 hours
Survival analysis for clinicians	2010	38 hours
Good Clinical Practice (BROK) course	2008	20 hours
Ultrasonography course (Dutch Liver Week)	2008	8 hours

Teaching activities

	YEAR	WORKLOAD
Diagnosis and treatment of chronic hepatitis C. Third year Erasmus MC medical students participating in a 4-week Gastroenterology and Hepatology training program. Rotterdam, The Netherlands.	2011	6 hours
Treatment of chronic hepatitis C. Medical interns, the department of Gastroenterology and Hepatology. Rotterdam, the Netherlands.	2010	6 hours
Treatment of chronic hepatitis C. Medical interns, the department of Gastroenterology and Hepatology. Rotterdam, the Netherlands.	2009	6 hours

Treatment of chronic hepatitis C. Lecture for participating general practitioners, Rotterdam, the Netherlands.	2009	6 hours
Treatment of chronic hepatitis C. Lecture for addiction treatment nurses organized by Cluster Infectious Diseases, Public Health, Rotterdam, the Netherlands.	2010	6 hours

Dankwoord

Dit is het dan. Na een jaar of vijf werken aan onderzoek ligt nu warempel een proefschrift van ondergetekende op de plank. Tijd voor het dankwoord.

Rob, jij en ik, ik en jij. Samen begonnen we aan dit promotietraject waarvan voor mij totaal onduidelijk was wat aan studies te verwachten, bedenken en afronden viel. Je hebt hart voor de patiënt, je werk, hecht aan de mensen om je heen. Je bent veel doortastender en scherper in je observaties dan je aanvankelijk laat blijken. Belangrijker is dat jij weet wat zowel op het werk als daarbuiten het allerbelangrijkst is en dat is gewoon gelukkig zijn. Dit proefschrift bestaat zeker niet uit studies die reeds klaar lagen. Gedurende mijn promotietraject ben je gegroeid als onderzoeker en begeleider; zonder jouw support en commentaar was dit proefschrift er nooit geweest. Tevens was het niet zo'n ontzettend leuke tijd geweest. Dank voor je onvoorwaardelijke steun zowel in mijn promotietraject als bij mijn sollicitatie voor de opleiding; ik ben heel blij dat ik jou op deze wijze heb mogen leren kennen en wij houden altijd goed contact.

Harry, dank voor je vertrouwen in mij en je immer constructieve bijdrage aan mijn onderzoek. Je bent ongelooflijk slim, laat geen kans onbenut en gaat altijd uit van je eigen kracht. Jij creëert voorwaarden en verwacht inzet; aldus schep je een veilige, eerlijke en constructieve situatie voor je promovendi. Dat is prettig, want waar een promotor publicaties wil, geldt dat voor een promovendus minstens zo. Ik ben er trots op bij jou onderzoek te hebben mogen doen. Veel dank voor jouw steun bij mijn sollicitatie voor de opleiding en heel veel succes in Toronto!

Prof.dr. J.J. van Busschbach, Prof.dr. A. Verbon, Prof.dr. J.H. Richardus, Prof.dr. B. van Hoek en Prof.dr. C.A. Uyl-de Groot; dank voor jullie bereidheid zitting te nemen in mijn commissie.

Andre, toen ik zo'n anderhalf jaar bezig was kwamen we wat directer met elkaar in contact. Onze studie bij druggebruikers had een behoorlijk thinking-out-of-the-box gehalte en resulteerde in een mooi artikel. Ik kende je al een tijdje maar kreeg pas hoogte van je toen we samen gingen werken. Ik ken geen begeleider die zo toegewijd is. Inhoudelijk ben je enorm kundig, je hebt een groot analytisch vermogen en je weet je afdeling en mensen perfect te managen. Ik heb je zeer leren waarderen. Dank voor je begeleiding en je altijd positieve en onophoudelijke stimuleren, ook toen ik reeds in Deventer verkeerde. En dank voor het zitting nemen in mijn commissie!

Bart, aan ons de eer om een subanalyse van de POPS-studie uit te voeren. Je ziet altijd mogelijkheden en werkt ontzettend hard. Wat heerlijk om met iemand samen te werken die zo goed en toegewijd is! Je hebt mijn schrijven verbeterd. Van jou heb ik beter leren analyseren en je hebt me bijgebracht dat het glas halfvol zit. Basaal en belangrijk. Dank voor je begeleiding en ons mooie artikel.

Annemiek, laatste auteur op mijn laatste hoofdstuk. Ik kan ontzettend met je lachen en als ik je niet kan doen stoppen met praten kan ik wederom niet ophouden met lachen. Gedurende

mijn promotietraject is jouw functie uitgebreid. Je bent naar mijn mening een stuk zakelijker geworden en dat past je goed. Op charmante, soepele wijze managede je onze hepatitis E studie van relatief kleine analyse tot volwaardig en mooi artikel. Je bent zorgvuldig, opbouwend, professioneel en compleet. Veel dank voor je begeleiding en toewijding met betrekking tot onze studie; super dat je in de commissie zitting neemt.

Jeoffrey, dank voor je hulp bij onze common bile duct studie. Samen op locatie, mission undercover but not impossible; jij, ik en een echo-apparaat op de heroïnepoli. Het is een mooi artikel geworden! Dank voor je opbouwende kritiek en tomeloze energie.

Bettina, wat een geluk hebben wij dat wij een statisticus ter beschikking hebben en dan ook nog eens een die zo leuk is. Je bent niet alleen betrokken bij de statistiek, je brengt resultaten naar een hoger niveau. Je bent een topvrouw en dat kan na de mooie congressen en spin-offs in Napa Valley, Antwerpen en New York niet vaak genoeg gezegd worden. Dank voor al je hulp!

Thonie en Kim, de drugs study heeft wat grijze haren opgeleverd, maar ook een mooi artikel! Jullie labwerk is de basis. Dank hiervoor.

Suzan en Cédric, dank voor jullie hulp bij de hepatitis E studie.

Henk, Joep en Martine, dank voor de constructieve samenwerking. Het was soms een crime om patiënten te vinden, maar we kwamen er wel. Henk, met jou als laatste auteur heb ik twee mooie papers. Fase-1-trials zijn een vak op zich en je hebt daar veel ervaring mee. Dank voor je begeleiding. Joep, ik heb je kritische, constructieve blik op mijn eerste artikel enorm kunnen waarderen. Dank hiervoor.

Vrienden van de GGD, Jeanelle en Reinoud. Jeanelle, jou ken ik als de spil in Project Actief Testen!. Dankzij jouw rol in dit project en kritische en relativerende houding werd deze samenwerking van totaal verschillende partijen een succes. Ik denk nog wel eens terug aan ons drieën :) bovenop de berg in Zürich. Dank voor je betrokkenheid bij mijn onderzoek en hulp waar je die kon bieden. Reinoud, dank voor de constructieve samenwerking. Ingrid en Francis, verpleegkundigen van de hepatitis C poli van de verslavingszorg; dank voor jullie inzet en hulp. Zonder jullie was een groot gedeelte van de patiënten überhaupt niet gescreend geweest. Joanneke, dank voor je betrokkenheid bij onze studie naar verwijding van de galwegen bij methadongebruik.

Hepatitis C onderzoek doe je in het Erasmus MC niet alleen. In wisselende samenstelling werkte ik samen met Geert, Jilling, Mark, Robert, Ludi, Ad en Michelle. Geert, Jilling en Mark, dank voor het mij introduceren in de wereld van HCV. Geert, dank voor je bijdrage aan de POPS. Robert, ik moest even aan je wennen, maar na een paar congressen en een enkel skiweekend was het illustere beat-the-virus team toch geboren. Gelukkig weet jij altijd dat je werkt om te leven en niet andersom, dat een goed cholesterolgehalte (en laag gamma-gt!) mooi is om te zien en Duits een hilarische taal blijft; dank voor de gezellige samenwerking! Ad, je werkt superhard en streeft niet alleen naar het beste maar verbetert. Mooie aan jou is dat je je ingevingen niet voor jezelf houdt. Dank voor je hulp met betrekking tot mijn onderzoek waar mogelijk en de goede samenwerking. Ludi, ik kan ontzettend met en om je lachen. Ook al zijn we heel

anders, we hebben het altijd super gehad tijdens congressen en op het dak met als hoogtepunt ons verblijf op de Kurfursterdam. Je brutaliteit leidt soms tot botsingen, maar brengt je ook ver, zelfs tot de Mayo Clinic. Ik hoop je in de opleiding te zien! Michelle, mijn aimabele HCV-collega op het lab gewapend met fnab. Het leek een onmogelijke taak om mensen terug te roepen voor een fnab, maar de narlaprevir follow-up studie werd toch een mooie paper. Dank voor je hulp hierbij en de gezellige samenwerking!

Collega arts-onderzoekers (Claudia, Edmee, Jorie, Renate, Aafke, Attija, Jildou, Margot, Judith, Esmee, Desiree, Nicoline van Heel, Nicoline Schepers, Lisette, Vera, Veerle, Angela, Lisanne Plompen, Lisanne Holster, Milan, Erik, Jurrien, Vincent, kleine Vincent, Jerome, Dr. Viv, Leonie, Aaf, Femme, Susanne, Nicoline, Michelle, Jehan, WP, Pauline, Lieke, Edith Kuiper, Edith Koehler, Ludi, Robert, Ad, Wim, Roeland, Florine, Celine, Aria) van het Erasmus MC; als je dan 4 jaar met elkaar op een werkplek zit die duiventil genoemd wordt, dan kan je maar beter zorgen dat het leuk is. Gelukkig waren er genoeg congressen, borrels, skireizen, fietsweekenden, practical jokes en serieuze tips 'n tricks; ik heb een heerlijke tijd gehad met jullie, dank hiervoor.

Mijn roomies van weleer, Erik, Milan, Paul en Lisanne, in wisselende samenstelling. Het was veel chocola, koffie en gezelligheid en dat is belangrijk. Erik, ik ken niemand die meer zichzelf is dan jij. In al je zorgzaamheid en organisatie hielp je me waar mogelijk; veel dank daarvoor toen ik net begon. En voor je superdikke superhandige statistiekboek welke ik je nooit terug heb gegeven :). Paul, drie jaar (!) lang 8 uur per dag op elkaars lip. Ondanks onze veelbesproken stroeve start hebben we het ontzettend gezellig gehad. Als je zoveel samen bent deel je veel. Met ups en downs is ook jouw proefschrift afgekomen; dank voor je gezelligheid en support tijdens het promotieonderzoek. Milan, je werklust en perfectionisme zijn bewonderenswaardig. Je laat niet snel het achterste van je tong zien, maar volgens mij konden wij het heel goed vinden. Je nonchalance is slechts een pose; je 1-persoons flesje wijn met boek voor mij toen ik naar Deventer mocht emigreren zeiden genoeg. Dank voor je hulp bij mijn onderzoek en je statistische input! Lisanne, eindelijk een proefschrift dat Echt over M-D-L gaat ;)! Dat je een goede dokter bent wisten we al. Binnenkort kan je je energie en toewijding kwijt in de opleiding. Ik benijd degene die jou later als maat krijgt en wens je alle succes in de opleiding. Lisanne (Plompen), Jorie, Aafke, Leonie en Jildou, jullie deden geen HCV-onderzoek en waren niet mijn kamergenoot, maar ik heb jullie enorm gewaardeerd. Glamper girls, Pauline, Edith en Michelle, onze tour in een 7.5 meter lange camper drie weken door Californie, Utah en Nevada na de AASLD was fantastisch. Het was heerlijk zo samen! Dit was denk ik wel de kers op de promotietaart en ik denk er nog vaak genoeg aan terug; de verhalen zijn eindeloos. Excuses nog voor de botsing waardoor we onze borg kwijt raakten…

Marion en Margriet, dankzij jullie loopt alles in het Ha-gebouw en op de rest van de afdeling van de Hepatologie op rolletjes. Dank voor jullie hulp, zeker aan het einde van het promotietraject, met alles dat geregeld moet worden.

Poli-assistentes, met name Wilma, dank voor jullie hulp bij ons HCV-spreekuur.

Research verpleegkundigen Heleen, Lucille, Melek, Cokkie en Henny, jullie waren top. Dankzij mij zagen jullie de meest bijzondere patiënten :) en jullie veroordeelden ze nooit. We hebben veel gelachen en jullie dachten altijd mee wat betreft de logistiek van het doen van onderzoek waarvoor veel dank. Irene, Edith, Judith, Elke en Wanda van het Clinical Research Bureau, dank voor jullie hulp, gezelligheid en de constructieve samenwerking.

Enige overlap van onderzoek en kliniek vond plaats toen ik juni 2012 mocht starten met de opleiding in Deventer Ziekenhuis. Theo Diekman, nu al begrijpen wij elkaar; dank voor de briljante stelling.

Paranimfen, Meike en Pauline. Meike, je bent er voor me wanneer dan ook en hoe dan ook. Mijn onderzoeksdingen interpreteer je perfect, je sarcasme is onverbeterlijk. Ik ben er trots op dat jij vandaag naast mij staat en we gaan er een feestje van maken! Dank voor hoe je er altijd voor me bent geweest, ook vandaag. Pauline, we hebben elkaar in de afgelopen jaren heel goed leren kennen. Je hebt me in veel verschillende situaties op het werk gezien en weet hoe ik over een p-waarde denk :). Dank voor het feit dat je plaats wil nemen achter de katheder met mij en er voor me bent geweest de afgelopen tijd.

Edith, ongeveer een jaar nadat ik begonnen was met onderzoek kwam jij. Wij hadden het meteen prima; congressen, skivakanties, kamers delen, alles was goed. Jij en ik zijn allebei wazig en warrig, maar ambitieus. De maand waarin je bij mij logeerde was de leukste en meest alcoholische tot op heden en we gaan een mooie tijd tegemoet in Deventer. Eerst deze promoties; ik ben blij dat ik dit met jou mag delen!

Lieve familie en vrienden, dank voor het feit dat jullie er voor me zijn en altijd bereid zijn naar mijn onderzoeks- en ziekenhuisgeneuzel te luisteren!

Ouders, wat fijn dat jullie er ALTIJD zijn! Ik prijs me zeer gelukkig met het feit dat ik jullie heb. Jullie zijn altijd bereid om me te helpen of hebben dat al gedaan als ik het even niet door had. Dank voor jullie hulp tijdens de overlap van mijn baan in Deventer en het afronden van mijn onderzoek.

Lieve Sjors, je bent degene die het minst en het meest snapt van wat ik de afgelopen 5 jaar met betrekking tot onderzoek mee heb gemaakt. We zijn zo verschillend en toch ook weer niet. Zoals altijd ben je er ook de afgelopen 5 jaar voor me geweest en hebt me geholpen waar je dat kon. Dank voor alles!

Tenslotte, patiënten die deel hebben genomen aan onderzoek betreffende dit proefschrift; veel dank voor jullie bijdrage.

CURRICULUM VITAE

Daphne Marije Hotho werd op 10 mei 1983 geboren te Warnsveld. In 2001 rondde zij het VWO af aan 't Rhedens te Rozendaal. Ze studeerde geneeskunde aan de Erasmus Universiteit Rotterdam. In 2006 behaalde ze haar doctoraal nadat ze haar afstudeeronderzoek deed op de afdeling Nefrologie van het Erasmus MC. Haar keuze co-schap Heelkunde deed ze in Blantyre in Malawi en haar oudste co-schap vond plaats op de afdeling Maag-, Darm- en Leverziekten van het Albert Schweitzer ziekenhuis te Dordrecht. Na haar artsexamen in april 2008 begon ze haar promotieonderzoek bij de afdeling Maag-, Darm- en Leverziekten van het Erasmus MC onder begeleiding van dr. Robert J. de Knegt en prof.dr. Harry L.A. Janssen. Juni 2012 is ze gestart met de opleiding tot Maag-, Darm- en Leverarts (opleider Dr. Rob A. de Man). Op dit moment is zij in het eerste jaar van haar vooropleiding interne geneeskunde (opleider interne geneeskunde: dr. Cees J. Vermeij, opleider Maag-, Darm- en Leverziekten: dr. Frank ter Borg) te Deventer Ziekenhuis.