The RISE *and* FALL *of* NEW TREATMENT OPTIONS *for* CHRONIC HEPATITIS C

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The rise and fall of new treatment options for chronic hepatitis C
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The rise and fall of new treatment options for chronic hepatitis C

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(met een samenvatting in het Nederlands)

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CONTENTS

CHAPTER 1

Introduction and outline of the thesis

Hepatitis C virus (HCV) is one of the most prevalent causes of chronic liver disease with an estimated 130-210 million people chronically infected worldwide (1-3). The severity of the disease varies from asymptomatic carriers to a progressive and life-shortening disease which may result in liver cirrhosis and hepatocellular carcinoma (HCC) (4;5). Successful eradication of the virus is associated with regression of histological abnormalities (6;7) and with reduction of complications of liver disease, such as development of cirrhosis, HCC and liver-related mortality (8-11). During the last 20 years treatment options have developed from monotherapy with interferon via combination therapy consisting of PEG-interferon and ribavirin to the addition of the direct acting antiviral agents in the near future. The sustained virological response (SVR) for naive chronic hepatitis C (CHC) patients has dramatically improved over these years: being 6% in the early nineties and reaching 70-90% in the era of the direct acting antiviral agents (12-17).

In the period the work described in this thesis was started, the standard of care consisted of a combination of PEG-interferon and the nucleoside analogue ribavirin. This regimen achieved a SVR in 55-64% of treatment-naive patients (12;13). However SVR proved to be dependent on HCV genotype and was significantly lower in genotype 1 patients reaching a SVR of only 45% after one year of intensive therapy (12;13). Moreover, retreatment of previous non-responders to interferon based therapies with the standard of care yielded a response rate of only 12-30% (18;19). Considering these poor treatment results, new experimental treatments for naive patients as well as for earlier interferon non-responders were developed and evaluated in this thesis aiming at improving previous mentioned SVR rates.

First, triple therapy combining high dose interferon therapy (induction therapy) in combination with ribavirin and the experimental addition of amantadine was evaluated in a double-blind, placebo-controlled, multicenter, randomized trial in treatment-naive HCV patients (CIRA study). In **chapter 2,** a literature overview on virological background and potential working mechanisms of induction therapy is given in which comparisons with the viral kinetics and treatment of human immunodeficiency virus (HIV) are being made.
The results of the CIRA study, a collaboration of 26 Dutch hospitals in which 297 CHC patients were randomized for amantadine or placebo, are described in **chapter 3.**

PEG-interferon and ribavirin combination therapy has considerable clinical side effects such as flu-like symptoms, severe fatigue, nausea, skin reactions, dyspnea, depression and other psychological side effects as irritability and sleeping disorders. Biochemical side effects include ALT flares (12;13). Furthermore, hematological side effects such as neutropenia, thrombopenia and anemia requiring dose-reduction or even discontinuation of therapy which result in diminished SVR, are described (12;13;20). The cause of anemia is probably

multifactorial; interferon may suppress bone marrow regenerative activity of erythroid progenitor cells and also inhibit erythropoietin production (21;22). In addition, ribavirin may induce dose-dependent hemolytic anemia (23-25). To elucidate the potential underlying mechanisms which cause hemolytic anemia during antiviral therapy potential changes in erythrocyte membrane phospholipid composition and susceptibility to osmotic or bile salt induced stress in anemic hepatitis C patients on anti-viral treatment were evaluated in vitro and are described in **chapter 4**. Anemia during anti-HCV treatment can be prevented or treated by the administration of recombinant erythropoietin in an effort to reduce the rate of dose reductions and ameliorate the quality of life of CHC patients during antiviral therapy (26-30). However, there are conflicting results about the effect of exogenous erythropoietin on SVR (28;31) and no prospective trials have been performed that demonstrate a definite positive impact on SVR. The question whether endogenous erythropoietin production is sufficient to compensate the anemia during anti-HCV treatment has not definitely been answered (32-34). Like in other chronic diseases, for example renal failure, HIV-infection and cancer, the endogenous erythropoietin production may be hampered (35-37). In **chapter 5**, the extent of anemia during anti-HCV treatment is related to the endogenous erythropoietic response during 24 weeks antiviral therapy in a large cohort of CHC patients included in the CIRA-study.

The eventual success of any therapy for CHC depends on virus related factors, including HCV genotype and pre-treatment viral load (14;38;39). Furthermore, host related factors such as age, histology, duration of HCV carriage and co-morbidity may have predictive value for eventual treatment success. Alpha-1 antitrypsin (A1AT) deficiency is an autosomal recessive disorder that is relatively rare and poorly recognized, which may lead to progressive liver disease by synthesis and accumulation of an abnormal A1AT protein in the liver. Retention of the inefficiently secreted mutant A1AT Z molecule in the endoplasmatic reticulum of liver cells triggers a series of events that are eventually hepatotoxic (40). The natural history of the disease is not completely known but it is generally thought that homozygous as well as heterozygous A1AT deficiency may influence the natural course of any other liver disease and is associated with an increased risk of liver cirrhosis and HCC (40-45).
The relation between CHC infection and A1AT deficiency is not clear. In one study, A1AT deficiency could be detected in only one of 1842 liver biopsies of chronic hepatitis B or C patients (46). However, higher prevalences of markers of hepatitis B and C infection were found in patients with A1AT deficiency associated chronic liver disease possibly due to an increased susceptibility to viral infection although the latter has never been proven (47). A1AT deficiency has been found to be more present in CHC patients compared to healthy controls (44;48). Moreover, A1AT deficiency seems to be associated with a worsening of the clinical course of CHC and an increased need for liver transplantation (49;50). In

chapter 6 the prevalence of A1AT deficiency in CHC patients was assessed as well as the possible association with viral clearance during anti-HCV treatment.

The second clinical trial in this thesis concerns an experimental treatment of extracorporeal whole body hyperthermia (EWBH) in CHC patients who were previous non-responders to interferon based regimens. Under propofol anesthesia, the body temperature was raised to 41.8°C using a veno-venous extracorporeal circuit that included a heater/cooler device. In **chapter 7** the efficacy, safety and feasibility of this treatment which had been proven in patients with HIV infection (51), is described for patients with CHC.

Finally the results described in this thesis are being discussed and summarized in **chapter 8.**

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CHAPTER 2

Treatment of chronic hepatitis C, lessons from human immunodeficiency virus dynamics

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ABSTRACT

Treatment of chronic hepatitis C (CHC) is a major problem. A sustained viral response (SVR) to interferon alpha monotherapy occurs in <20 % of patients.
Using a combination therapy of interferon alpha and ribavirin, the SVR in naive hepatitis C patients has increased to 31-47%.
The success of therapy for CHC depends on both virus- and host-related factors, such as age, histology, duration of hepatitis C virus (HCV) carriage and biochemical parameters.
During the last 5 years, insight into the dynamics of human immunodeficiency virus (HIV) has been obtained by analysing the changes in viral load after starting antiviral treatment. By using a mathematical model of the HIV kinetics as an example, an exponentially rapid decline in serum HCV RNA was seen after the first dose of interferon alpha, followed by a slower exponential decline: a so-called bi-phasic pattern.
The estimated virion half-life varies between 2.7 to 16.8 hours. The high virion turnover allows the generation of a heterogeneous quasi-species population of HCVs.
It is therefore supposed that initial aggressive treatment can be helpful to prevent the development of mutations that make the virus more defensible for the interferon alpha treatment. Various trials are now being conducted based on this principle of high induction antiviral therapy.

INTRODUCTION

Since its discovery in 1989, hepatitis C has been recognized as a major public health problem worldwide. It is a life-shortening disease associated with complex and expensive morbidity and a decrease in quality of life. It is estimated that nearly 170 million people in the world have been infected with the hepatitis C virus (HCV) (1). About 5 million people are estimated to be chronic carriers of HCV in western Europe (1). In industrialized countries, HCV is responsible for 20 % of cases of acute hepatitis, 70 % of cases of chronic hepatitis, 40% of cases of end-stage liver cirrhosis, 60 % of cases of hepatocellular carcinoma and 30 % of liver transplants (2).
HCV is an enveloped, positive-stranded RNA virus belonging to the family Flaviviridae. It can cause chronic hepatitis, demonstrated by persistence of HCV RNA in the blood, in 80% of infected patients. Chronic infection with HCV progresses in 20-30 % of patients to liver cirrhosis after 10-20 years of infection (3;4).
Decisions concerning which patients should be treated and which should not are difficult to make. According to the 1999 Paris Consensus (2), treatment is recommended for patients with moderate-to-severe necro-inflammation and/or fibrosis on liver biopsy, patients with persistently elevated aminotransferase levels in the blood, patients in whom virus RNA can be detected in the blood and also for patients positive for anti-HCV antibodies.

THERAPY

The single treatment that is widely accepted and has proven to be effective for CHC is interferon alpha. The exact effect of interferon alpha in CHC is unknown, but it induces several direct and indirect antiviral mechanisms, such as intracellular viral RNA degradation, inhibition of viral RNA translation and activation of key components of the cellular immune system important in viral recognition and prevention of susceptible cells from viral infection (5).
The effect of interferon alpha implies a decrease in aminotransferase activity and a decline in HCV RNA levels. The real goal of therapy is to stop the progression to cirrhosis, hepatocellular carcinoma and eventual death. These endpoints cannot easily be evaluated because the normal progression of CHC is slow. Therefore "surrogate" endpoints are currently being used, such as serum aminotransferase levels, HCV RNA levels and histological status. The results of interferon monotherapy, however, are not satisfactory: the initial response, defined as normalization of the serum alanine aminotransferase (ALT) level and loss of detectable HCV RNA, varies between 40-60% (6;7). However, >50% of patients show a biochemical relapse once treatment is discontinued. A sustained viral response (SVR), defined as no detectable HCV RNA at the end of treatment and at least 6 months after cessation of the treatment, has been found in <20% of patients treated with interferon

alpha monotherapy(8). Reichard *et al.* found that in the group of patients treated with a combination of interferon alpha and ribavirin, a higher number of patients achieved a SVR, compared to patients treated with interferon alpha alone (9). They observed that there were no differences in response in these two groups at the end of treatment. Thus they assumed that the better results in the combination group were due to a reduced relapse rate produced by ribavirin.

The exact working mechanism of the nucleoside analogue ribavirin is not certain. It seems that ribavirin does not have a direct antiviral effect but that it acts as an immuno modulator preserving Th1 and reducing Th2 cytokine production (10).

Poynard, Mc Hutchinson and co-workers have shown by means of multicenter randomized trials, that the combination of interferon alpha and ribavirin is more effective for naive CHC patients (11-13). They concluded that there is no subgroup of patients who will have benefit from interferon monotherapy compared to combination therapy with ribavirin (11).

According to the large randomized, controlled trials in naive hepatitis C patients, the SVR rate is increased to 31-47% by using the combination therapy of interferon alpha and ribavirin (14-16). The goal remains to investigate the best dose and duration of this combination therapy so that the sustained response can be further improved.

The eventual success of any therapy for CHC depends on both virus- and host-related factors, such as age, histology, duration of HCV carriage and biochemical parameters. There have been a lot of investigations to determine factors that can predict the success of the therapy, so that before treatment or during an early stage of treatment, predictions can be made and therapy can even be stopped if necessary.

The HCV genotype and the pre-treatment HCV-RNA serum value seem to have predictive importance (17;18). For the clinician, however, no single parameter or combination of parameters can definitely predict the response to antiviral therapy (18).

According to Zeuzem *et al.* the predictive value of the initial response to interferon alpha, as measured by quantification of the change in the serum HCV-RNA level in the first 4 weeks, is more significant than any combination of pre-treatment factors (19).

Poynard *et al.* concluded that five favourable factors are associated with a sustained virological response: genotype 2 and 3, low viral load, female gender, age <40 years and no liver fibrosis. They recommend 48 weeks of treatment for those patients who are polymerase chain reaction (PCR-) negative at 24 weeks and who do not have 4 or 5 favourable response factors. For patients who are PCR-positive at 24 weeks they reported a probability of obtaining sustained clearance of the virus of only 2% (11).

In order to further improve the results of treatment, it is necessary to understand the viral behaviour as a result of these many factors, and thus to study the viral kinetics and dynamics of the HCV before and after treatment with interferon alpha. Previously, a comparable process was performed for the human immunodeficiency virus (HIV), with remarkable therapeutic success.

VIRAL DYNAMICS OF HIV-1

During the last 5 years, considerable insight into the dynamics of HIV has been obtained by analysing the changes in viral load after starting antiviral treatment. Ho, Perelson and co-workers (20;21) described an initial lag, followed by a rapid exponential decline in the plasma concentration of HIV-1 in the first 2 weeks after starting therapy with a potent protease inhibitor as ritonavir.
The initial lag was a result of the pharmacokinetic delay. In addition to this kind of delay the lag appeared to be due to the working mechanism of protease inhibitors: to make the newly infected virions non-infectious, without preventing the production of virions by already infected cells or the infection of new cells by previously produced infectious virions. The viral decline after the initial lag was due to two separate effects: the clearance of virions from the plasma and the loss of virus-producing cells (20;22).
Despite the decline of the free virions in the plasma, genetic material of the virus persists in the body of the patient because of the capacity of the virus to integrate its genetic material into the genome of the host. To eliminate all these infected cells, the patient has to be treated until the last infected cell has died and disappeared from the body. Estimations of the life span of these infected cells vary from years to decades.
Using complex mathematical models, Ho and Perelson calculated the half-life of plasma-HIV to be as little as 6 hours. Productively infected blood cells were estimated to have a half-life of 1.6 days. By assuming that, without treatment, the virion production equals the virion clearance, the total daily virion production varied from 0.4×10^9 to 32.1×10^9 virions with a mean of 10×10^9 virions. These calculations make it clear that HIV-1 viraemia is a dynamic process with rapid virus replication and cell turnover.
As a consequence of this rapid virion turnover, an enormous genetic diversity is promoted, with the development of quasi-species (20;22). The mechanism of formation of such quasi-species may be a combination of inherent errors of transcription and selective genetic pressure created by an active immune response. The genetic diversity creates a significant challenge for the development of effective antiviral agents and vaccines.
Therefore, in summary, it was learned from HIV kinetics that effective therapy should induce rapid initial viral suppression in order to limit the formation of quasi-species.

VIRAL DYNAMICS OF HEPATITIS C VIRUS

Investigation of the hepatitis C viral half-life cycle, the structural and functional properties of the HCV proteins and their interactions with cellular proteins is limited by the lack of an efficient and reproducible cell culture. However, in connection with the investigations concerning HIV-1 dynamics, comparable studies have been made to determine the dynamics of

HCV. Kinetic information on virus load was obtained in groups of patients chronically infected with HCV and treated with interferon alpha monotherapy. All investigators initially observed first an exponentially rapid decline in serum HCV RNA levels after the first dose of interferon alpha, followed by a slower exponential decline: a so-called bi-phasic pattern (23-26) (Fig. 1). Before the viral decline, a 7-10 hours delay has occurred between the administration of interferon alpha and the actual loss of HCV RNA (23;24). This delay can be interpreted as a consequence of the pharmacokinetics of interferon alpha. The peak plasma concentrations will be reached 7-10 hours after the injection. This initial lag and bi-phasic pattern of decrease of viral load has also been seen in HIV, but with a big difference in rate. The rate of decline in HCV clearance is 10-fold faster compared with that of HIV.

The question 'By which mechanism does interferon alpha act?' can be answered by using the mathematical model created for investigating the kinetics of HIV (20-23) in combination with the bi-phasic clearance pattern. This model involves free virions, V, which can infect susceptible cells, T, at rate k. These infected cells, I, will again produce new free virions at rate p, and die at rate δ. Virions are being cleared from the serum at rate c. (Fig. 2).

The possible major effects of interferon alpha are either to block the production of virions by infected cells, to prevent de novo infections of susceptible cells or to increase the viral clearance rate.

Lam, Perelson and co-workers (23;25) showed a dose dependent decline in virions during the first day after interferon alpha administration in patients infected with HCV type 1. Twenty-four hours after the administration of interferon alpha, they observed mean percentage serum viral reductions of 41.4%, 63.7% and 85.5% after administration of 3, 5 and 10 MIU, respectively. They observed an increase in the HVC RNA levels and thus a decreased viral clearance at 48 h after administration of the drug. Considering virus clearance, administration of 15 MIU had no extra benefit.

Using the above-mentioned mathematical model, they concluded that a dose-dependent viral clearance during the first phase after administration of interferon alpha, can only occur by assuming that the major effect of interferon alpha is to block production or release of HCV virions. According to their model, Neumann *et al.* explained the bi-phasic pattern of the viral clearance by assuming that this blocking of new virions is not perfect. The initial rapid decline is a product of both viral clearance, c (Fig. 2), and the efficacy of interferon alpha. The second phase which shows a slower decline in the viral load, reflects on the one hand the death rate, δ (see Fig. 2), of the infected cell and on the other the efficacy of the therapy (24).

Furthermore Neumann *et al.* described a positive correlation of the baseline ALT level and the rate of decline in viral load during the second phase but no correlation with the initial rapid decline in viral load. This finding confirms their hypothesis concerning the working mechanism of interferon alpha: ALT elevation is a measure of the cell death of infected hepatocytes by direct virus-related or immunomediated processes (24).

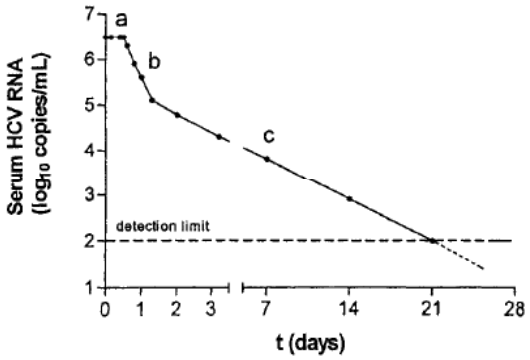

Figure 1: Model of the initial decline of serum HCV RNA. a = delay because of pharmacokinetics of interferon alpha; b = rapid, dose dependent decline (first phase); c = slow decline of serum HCV RNA level (second phase). Reprinted from Zeuzem [31] with permission from Munksgaard International Publishers Ltd., Copenhagen, Denmark.

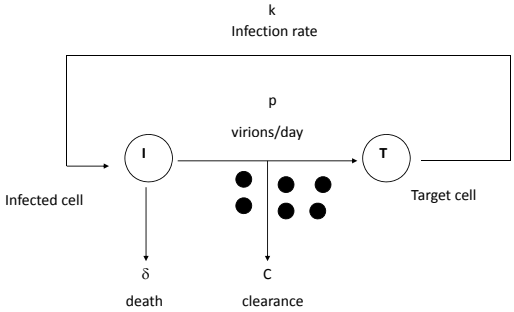

Figure 2: Schematic overview of HCV-kinetics. I = infected cell; T = target cel; k = infection rate of the target cell by the virion; p = production rate of new virions; δ = death rate of the infected cell; c = clearance rate of the virion from the serum. Reprinted from Perelson [25] with permission from Excerpta Medica Inc

The estimations of the half-life of infected cells also vary with the given dose of interferon alpha. These estimations showed a large interpatient variability, with the half-life varying from 3 to 69 days at 5 MIU interferon alpha, 4 to 17 days for 10 MIU and 2 to 6 days for 15 MIU (24).

The eradication of the virus depends on the death of all infected cells and thus high turn-over rates of the infected cells, possibly induced by enhancement of the immunoresponse by interferon alpha, may establish faster eradication.

Neumann *et al*. (24) showed an inverse correlation between infected cell death rate, δ (Fig. 2), and baseline viral load.

Zeuzem *et al*, in contrast, developed a mathematical model which implies an inhibition of de novo infection of susceptible cells as the major effect of interferon alpha (5).

According to their model the rate of HCV elimination from the serum after administration of the interferon alpha is determined by two processes: the clearance of HCV RNA per se and the elimination or suppression of virus-producing cells (5).

By comparing the viral kinetics after interferon alpha monotherapy and the combination of interferon alpha and ribavirin, they observed similar rates of production and release of free virions in the serum and of degradation of the free virions from the serum (26). This observation makes a synergistic antiviral effect of interferon alpha and ribavirin unlikely. In addition, it corresponds to the observations of Reichard *et al*. who demonstrated that at end of treatment there were no differences in response in these two therapy groups (9).

Ramratnam *et al*. investigated HCV clearance from serum by using large plasma apheresis (27). They observed a similar HCV clearance rate as that of Neumann et al (24). This makes an effect of interferon alpha on virion clearance less probable. Because the baseline viral load is relatively constant before treatment (28), the extracellular viral production rate has to be as high as the viral clearance rate in order to maintain the equilibrium. Again mathematical models, based on the analytical model of the dynamics of HIV-1, were used to estimate the virion half-life: values vary between 2.7 and 16.8 hours (5;23;24;27). Fukumoto *et al*. found a virion half-life of 4 hours after orthotopic livertransplantation (29). They suggested that the extrahepatic replication contributes little to the virus pool and the liver seems to be the principal site of virus replication (29). This HCV virus replication is like that of HIV-1; a continuous and highly productive process with an estimated virion production of 4×10^{12} per day (24).

The high virion turnover allows the generation of a heterogeneous quasi-species population of HCVs. The incidence of quasi-species is negatively associated with the responsiveness to interferon alpha therapy (30).

It is therefore supposed that initial aggressive treatment can be helpful to prevent the development of mutations that make the virus more defensible for the interferon alpha treatment. In this way, a higher success rate of the treatment should be obtained by the initial use of more aggressive (induction) therapy.

These insights in the complex viral kinetic system of HCV need to be further enlarged to develop better methods of treatment of CHC, which will improve the rate of SVR. It remains to be seen if the changes observed during the first phase of treatment can be maintained until, or can predict the response at, the end of treatment or better still, at the end of follow-up.

For now, the mathematical models and quantitative calculations, especially the early monitoring of the viral load of HCV and the changes after administration of interferon alpha and ribavirin, can be helpful in determining the efficacy of new forms of therapy for hepatitis C, such as higher induction therapy, higher and longer consolidation of antiviral effect and further extension of combination therapy, an approach that appeared successful for the treatment of HIV infection.

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CHAPTER 3

No beneficial effects of amantadine in treatment of chronic hepatitis C patients

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ABSTRACT

Background: Benefit of adding amantadine to antiviral therapy for hepatitis C is controversial. *Aims:* We aimed to examine whether such policy enhances sustained virological response (SVR) in treatment-naive patients. *Methods*: 297 naive hepatitis C patients were randomized for treatment with amantadine 200 mg or placebo, combined with weight-based ribavirin and 12-day high-dose interferon alpha-2b induction therapy, followed by PEG-interferon alpha-2b (1.5 µg/kg/week up to 26 weeks and thereafter, 1.0 µg/kg/week until week 52). Treatment was discontinued if hepatitis C virus (HCV) RNA was positive at week 24. *Results*: 49% of patients were (former) drug users. Genotype 1 occurred in 45%, high viral load in 70% and severe fibrosis/cirrhosis in 32%, without differences between amantadine or placebo groups. 90 patients prematurely discontinued treatment, mainly because of grade 3 or 4 toxicity. Intention-to-treat analysis revealed SVR in 47% and 51% of amantadine and placebo groups (p=0.49). Amantadine did not enhance SVR in patients with genotype 1 or high viral load nor did it improve primary non-response, breakthrough or relapse rates. Genotype non-1 and lower pre-treatment γGT levels were independent predictors for SVR. *Conclusion*: Adding amantadine to antiviral therapy of previously untreated chronic hepatitis C patients has no beneficial effects.

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a major health problem (1). Current antiviral treatment with PEG-interferon and ribavirin achieves sustained virological response (SVR) rates of 55-64% in treatment-naive patients (2;3). HCV genotype affects SVR rates strongly (approximately 45% SVR in genotype 1 and 80-90% in genotypes 2 and 3) (2;3). Amantadine enhances immune response to various viruses as influenza A, dengue and herpes zoster (4-7) and is an effective prophylactic and therapeutic drug against influenza virus (4). Also, beneficial effects of amantadine on HCV have been reported. In one study, amantadine monotherapy induced on-treatment decreases in serum aminotransferases, without effects on viral load (8). Caronia *et al.* showed increased on-treatment virological responses after 3 months interferon alpha/amantadine combination, without differences in SVR rates (9). In contrast, a meta-analysis revealed significant increases in SVR with interferon-alpha/amantadine compared to interferon monotherapy (10). Also, in a small study in non-responders to interferon alpha monotherapy, Brillanti *et al*. found 0% SVR in patients treated with interferon alpha/ribavirin vs. 30% SVR for amantadine/interferon alpha/ribavirin triple therapy (11). Another study reports SVR of 18% in interferon alpha non-responders when amantadine was used as monotherapy (12). Berg *et al*. randomized 400 naive patients to amantadine sulphate (100 mg twice daily) or placebo, in combination with interferon alpha-2a and ribavirin (1000-1200 mg daily). On-treatment viral response rates at week 24 were significantly higher (70 vs. 59%, p=0.02) and SVR rates tended to be higher (53 vs. 43%, p=0.11) in the amantadine group (13). Nevertheless, there are also several negative studies on the effect of amantadine in HCV-infected patients (14-16). In the current double-blind, placebo-controlled, multicenter, randomized trial in naive HCV patients, we explored whether adding amantadine to PEG-interferon alpha and ribavirin could improve virologic outcome.

PATIENTS AND METHODS

Patient selection

Eligible subjects were previously untreated adult patients who tested positive for serum HCV-antibodies and HCV RNA, with alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) elevated at least once within 6 months before inclusion and liver biopsy (performed within 1 year before entry) consistent with chronic viral hepatitis. Minimal baseline hematological values were: hemoglobin (Hb) 6.5 mmol/L, white-blood-cells 2.5 x 10^9/L, neutrophils 1.5 x 10^9/L, platelets 70 x 10^9/L and serum creatinine <150 µmol/L. Exclusion criteria were Child-Pugh classification B or C, human immunodeficiency virus

(HIV) co-infection, active uncontrolled psychiatric disorders, significant dysfunction of the central nervous system, chemotherapy and/or systemic antiviral treatment in the preceding 6 months, other serious disease, pregnancy or intention to get pregnant or unwillingness to use contraception. (Former) drug users could be included if stable psycho-social situation, support and housing were available. The study was conducted according to recommendations of Good Clinical Practice and the Declaration of Helsinki. All patients provided written informed consent and the protocol was approved by the medical ethical committees of the UMC Utrecht and the other participating centers. This study was registered at ClinicalTrials.gov (identifier NCT00146016).

Study design

This double blind, placebo-controlled, randomized trial was conducted at 26 centers in the Netherlands from January 2001 to July 2007. Patients were randomly assigned in equal proportions to two treatment arms, stratified for HCV genotype (1 vs. non-1). Both treatment groups received the same interferon alpha induction therapy (from day 1 combined with ribavirin), consisting of interferon alpha-2b (Schering Plough B.V., Maarssen, The Netherlands) 10 MIU/day subcutaneously during the first 6 days, followed by 5 MIU/day for the next 6 days, followed by PEG-interferon alpha-2b (Schering Plough B.V.) 1.5 µg/kg/week subcutaneously up to 26 weeks and 1.0 µg/kg/week from week 26 to week 52. Oral ribavirin (Schering Plough B.V.) was given during the entire 52-week treatment period in two different doses: 1000 mg/day for body weight <75 kg and 1200 mg/day for body weight ≥75 kg. In the triple therapy group, oral amantadine hydrochloride (Pharmacy UMC Utrecht, Utrecht, The Netherlands) 100 mg twice daily was added. In the double therapy group, oral placebo of identical shape and taste was added. These groups will be referred to in the following as amantadine and placebo groups. Central randomization was implemented by the pharmacist of the coordinating academical center using a block size of 4. Investigators and patients were blinded to treatment assignment during the entire study and follow-up period. Follow-up occurred at 0, 1, 2 and 4 weeks and monthly thereafter during 1 year of active treatment and at 3-month intervals during 1 year post-treatment.
Serum HCV RNA testing was performed quantitatively before inclusion (Cobas amplicor HCV Monitor Test, version 2.0, detection limit 600 IU/ml, Roche Diagnostics) and qualitatively at weeks 24, 52 and 104 (Cobas Amplicor HCV test, version 2.0; detection limit 50 IU/ml, Roche Diagnostics) by the central study laboratory. Treatment was terminated if qualitative HCV RNA test was positive after 24 weeks of treatment. Genotyping was performed by sequence analysis of the 5′ untranslated region of the HCV genome by the Visible Genetics TrueGene Hepatitis C Assay.
Adverse events were graded mild, moderate, severe or potentially life threatening according to WHO recommendations (WHO-grade 1-4). Therapy was permanently discontinued for

life-threatening events or for WHO-grade 4 events (including Hb <4.0 mmol/L, leucocytes <1.0 x 10^9/L, granulocytes <0.5 x 10^9/L, platelets <25 x 10^9/L). In case of grade 3 toxicity (including Hb 4.0-4.9 mmol/L, leucocytes 1.0-1.9 x 10^9/L, granulocytes 0.5-0.9 x 10^9/L, and platelets 25-49 x 10^9/L) the dose of the responsible drug was discontinued until recovery to at least WHO-grade 2 toxicity level had occurred. Thereafter, the responsible medication dose was restored at 50% of the initial dose and further increased if laboratory values and clinical course allowed this. In case of recurrent WHO-grade 3 toxicity, further treatment within the trial was terminated.

Assessment of efficacy

Our primary study aim was to determine whether SVR rate as indicated by negative serum HCV RNA by qualitative PCR 1 year after cessation of the study medication, could be enhanced by adding amantadine to anti-HCV treatment. Two analyses were performed: 1) all patients who received at least one dose of the study medication i.e. intention-to-treat analysis (patients who were drop outs because of toxicity, unwillingness to have a proper follow-up or whose HCV RNA were missing were considered non-responders); 2) all patients who completed the study until a primary study endpoint had been reached (i.e. completion until end of follow-up (T=104) or HCV RNA positivity after 24 weeks or 52 weeks of treatment: per-protocol analysis). In addition to SVR rates, virologic response rates (negative HCV RNA at week 24), breakthrough rates (negative HCV RNA at week 24 and positive HCV RNA at week 52) and relapse rates (negative HCV RNA at week 24 and 52; positive HCV RNA at week 104) were calculated for each treatment group.

Statistical analysis

This study was designed to have 150 patients per group so as to achieve 80% power to detect at least a 20% difference in SVR rates (40% vs. 60%), at 5% level of significance between amantadine and placebo groups, when analyzed according to intention-to-treat principle. Values are expressed as means ± SD or, in case of non-parametric distribution, as medians and ranges. Differences in baseline characteristics between amantadine and placebo groups were compared by 2-tailed Fisher's exact test for dichotomous variables and unpaired t-test or Mann Whitney-U test for continuous variables. Various parameters of viral response in various subgroups were compared using 2-tailed Fisher's exact test. In order to identify predictive factors for SVR, univariate and multivariate logistic regression analyses were performed, with odds ratios and 95% confidence intervals depicted. First, possible predictive factors were examined in univariate analysis. Only determinants with p-values less than 0.2 were included in the subsequent backward multivariate logistic regression analysis. A two-sided p-value less than 0.05 was considered statistically significant.

RESULTS

Baseline patient characteristics

333 patients were considered for inclusion, and 321 patients were randomized for amantadine or placebo treatment (Fig.1). 24 of these patients did not use any medication for various reasons, leaving 297 patients actually treated with PEG-interferon alpha-2b/ribavirin in combination with amantadine hydrochloride (*N*=144) or placebo (*N*=153). 18% of patients exhibited positive serum antinuclear antibodies, 26% positive anti-smooth muscle antibodies and 2% positive anti-mitochondrial antibodies. There were no differences in baseline host and viral characteristics between both treatment arms (Table 1). Genotype 1 was most prevalent, whereas most patients exhibited high baseline viral load (>800.000 IU/mL). 49% were (former) drug users (intravenous heroin in 80%, including six patients

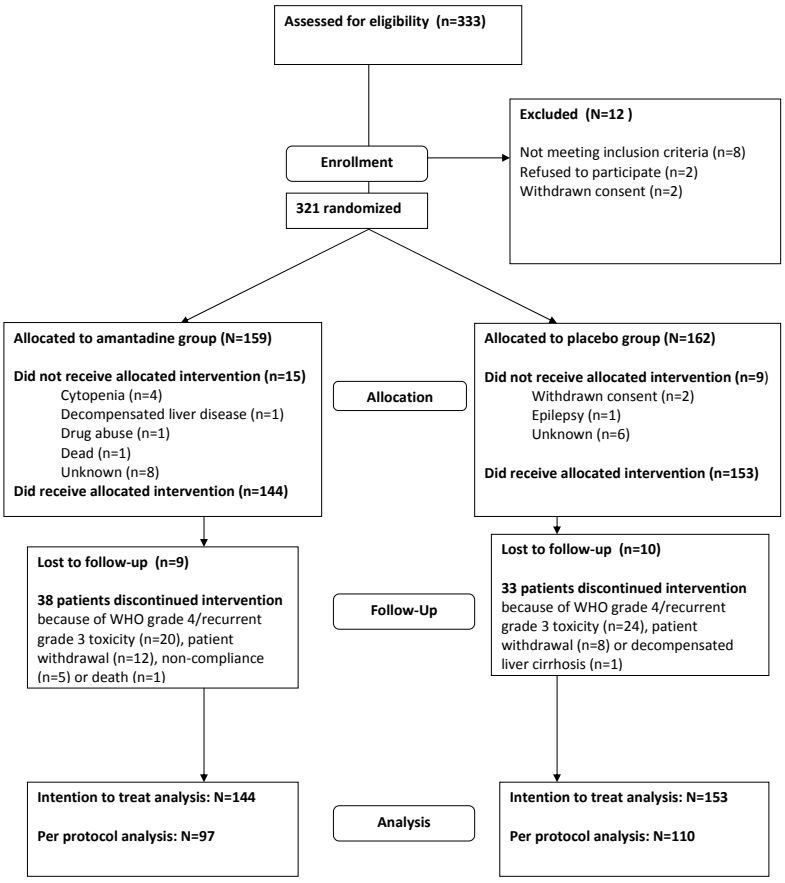

Figure 1: Flowchart with detailed information on patient numbers during inclusion and treatment periods

Table 1. Baseline characteristics of 297 naive chronic hepatitis C patients

Characteristic	ITT Amantadine (N=144)	ITT Placebo (N=153)	PP Amantadine (N=97)	PP Placebo (N=110)
Age, yrs (SD)	42.6 (9.1)	43.8 (9.2)	42 (8.8)	43.8 (9.4)
Male sex, *n* (%)	108 (75)	105 (69)	71 (73)	75 (69)
Weight, kg (SD)	78 (16)	76 (14)	77 (16)	76 (13)
HCV Genotype, *n* (%)				
1	65 (45)	70 (46)	45 (46)	52 (47)
2	13 (9)	17 (11)	10 (11)	11 (10)
3	53 (37)	51 (33)	34 (35)	34 (31)
4	12(8)	13 (9)	7 (7)	12 (11)
5&6&unk	1 (1)	2 (1)	1 (1)	1 (1)
Fibrosis score*, *n* (%)				
F0	25 (17)	33 (22)	18 (18)	24 (22)
F1	26 (18)	26 (17)	17 (18)	18 (16)
F2	33 (23)	33 (22)	25 (26)	27 (25)
F3	25 (18)	31 (20)	17 (18)	22 (20)
F4	15 (10)	13 (8)	8 (8)	10 (9)
Missing	20 (14)	17 (11)	12 (12)	9 (8)
HbsAg positive, *n* (%)	5 (4)	1 (1)	2 (2.1)	1 (1)
Anti Hbc positive, *n* (%)	60 (42)	55 (36)	41 (42)	39 (36)
ANA positive, *n* (%)	25 (17)	27 (18)	20 (21)	22 (20)
ASMA positive, *n* (%)	28 (19)	49 (32)	22 (23)	31 (28)
HCV viral load >800 000 IU/mL, *n* (%)	97 (67)	110 (72)	66(68)	82 (75)
HCV RNA, IU/mL (range)	2.2x10^{E}6 (970-2.4x10^{E}8)	2.6x10^{E}6 (16000-2x10^{E}8)	2.7x10^{E}6 (9500-2x10^{E}8)	3x10^{E}6 (16000-2x10^{E}8)
Source of infection, *n* (%)				
IV Drug use	65 (45)	82 (54)	43 (44)	55 (50)
Bloodtransfusion	36 (25)	39 (26)	22 (23)	27 (25)
AST, U/L (range)	59 (15-342)	63 (15-451)	59 (16-300)	62 (15-285)
ALT, U/L (range)	90 (18-595)	94 (31-686)	89 (18-595)	94 (31-507)
Albumin, g/L (SD)	42 (4)	43 (4)	42 (3)	42 (4)
GGT, U/L (range)	57 (9-700)	63 (5-473)	58 (9-700)	66 (5-473)
Bilirubin, umol/L (range)	10 (2-42)	11 (3-48)	10 (2-42)	12 (3-48)
Hemoglobin, mmol/L (range)	9.2 (7-11.7)	9.1 (4.4-10.9)	9.2 (7.7-11.7)	9.2 (6.3-10.8)
WBC, x10^{E}9 (range)	6.8 (3.3-20.6)	6.9 (2.4-16)	7.1 (3.8-20.6)	7.1 (3.4-15.1)
Platelets, x10^{E}9 (SD)	208 (68)	212 (64)	215 (70)	213 (61)

Values are expressed as means (SD). For values that are not sampled from a Gaussian distribution, medians (range) are depicted. ITT, intention to treat analysis; PP, per protocol analysis; HCV , hepatitis C virus; unk, unknown; HbsAg, hepatitis B surface antigen; Anti Hbc, anti-hepatitis B core antibodies; ANA, antinuclear antibodies; ASMA, anti-smooth muscle antibodies; IV, intravenous; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma glutamyltransferase; WBC, white blood cell count.

* F0 = no fibrosis; F1 = minimal fibrosis; F2 = periportal fibrosis; F3 = bridging fibrosis; F4 = cirrhosis or advanced fibrosis

with active IV heroin use during the study, cocaine in 52% and amphetamine in 18%). (Former) drug use was significantly associated with amount of previous alcohol consumption (p<0.001). 65% had stopped drug use at least 5 years, 15% 2-5 years, 15% 0-2 years before inclusion, and 5% exhibited active drug use during the study. Patients were provided with pharmacotherapy such as methadon, psychiatric support and antidepressants as needed. Overall mean weight was 77 kg (range 47-130): <65kg, 20%; 65-85 kg, 57%; >85-105kg, 18%; >105 kg, 5%, without differences between amantadine and placebo groups.

207 of 297 (70%) patients reached a primary study endpoint. Due to severe adverse events (grade 4 or persistent/recurrent grade 3 toxicity) 44 patients prematurely discontinued treatment according to the study protocol (20 amantadine and 24 placebo). Moreover, before reaching a primary endpoint**,** 19 patients (9 amantadine and 10 placebo) were lost to follow-up, 20 patients (12 amantadine and 8 placebo) decided to discontinue therapy and in 5 patients (5 amantadine and 0 placebo) therapy was withdrawn because of non-compliance, persistent IV drug use or subjective severe side effects. One patient exhibited decompensated cirrhosis 1 week after start of therapy and one patient was found dead due to drug overdose. There was no difference in time to drop out between amantadine and placebo groups: 56 patients (30 amantadine and 26 placebo) were drop outs before 24 weeks treatment, 27 patients (13 amantadine and 14 placebo) between week 24 and 52 after negative HCV RNA on week 24 and 7 patients (4 amantadine and 3 placebo) between week 52 and 104 after negative HCV RNA at week 52. Patients who never used drugs were less frequently dropouts than (former) drug users (27 vs. 34%). When those who stopped using drugs 2 years or longer before inclusion were considered non-drug users, frequency of dropout was 30% vs. 38% in those with active drug use within 2 years from inclusion. When those who stopped using drug 5 years or longer before inclusion were considered non-drug users, frequency of dropout was 29% vs. 38% in those with active drug use within 5 years before inclusion. Although significance was not reached, these data suggest higher dropout rates in (former) drug users.

Virologic responses

Intention to treat analysis

An overall SVR with undetectable HCV RNA levels at end of follow-up was achieved in 145 of 297 patients (49%). There were no differences in SVR between amantadine and placebo groups (47% vs. 51%, p=0.49). Accordingly, there were no differences in percentage of on-treatment virologic response, end-of-treatment HCV RNA negativity, primary non-response, breakthrough or relapse (Table 2). SVR in genotype 1 was significantly lower than in genotype non-1 (Table 3: all patients 40% vs. 56%, p=0.007: amantadine group 35% vs. 55%, p=0.02: placebo group 44% vs. 57%, p=0.15).

Table 2. Virologic response in 297 naive chronic hepatitis C patients to treatment with (PEG)-interferon and ribavirin in comparison with triple treatment consisting of (PEG)-interferon, ribavirin and amantadine hydrochloride

	Intention-to-treat			Per-protocol		
	Amantadine	Placebo	*p*	Amantadine	Placebo	*p*
	N=144	N=153		N=97	N=110	
Virologic response (HCV RNA negative)						
On Treatment (week 24)	99 (69)	105 (69)	1.0	80 (83)	88 (80)	0.7
End of Treatment (week 52)	79 (55)	86 (56)	0.9	75 (77)	83 (76)	0.9
End of follow-up (week 104)	67 (47)	78 (51)	0.5	67 (69)	78 (71)	0.9
Primary non-response (HCV RNA positive at week 24)	17 (12)	22 (14)	0.6	17 (18)	22 (20)	0.7
Viral breakthrough (HCV RNA negative at week 24 and positive at week 52)	5 (3)	5 (3)	1.0	5 (5)	5 (5)	1.0
Relapse (HCV RNA negative at weeks 24 and 52; positive at week 104)	8 (6)	5 (3)	0.4	8 (8)	5 (5)	0.4

Number of patients with virologic response with percentage between brackets. HCV, hepatitis C virus

SVR in patients with high viral load was not significantly different from patients with low viral load (all patients 47% vs. 52%, p=0.45; amantadine group 43% vs. 53%, p=0.29; placebo group 51% vs 51%, p=1.0). Also in difficult to treat subgroup of patients with genotype 1 and high viral load no benefit of adding amantadine could be detected (SVR 36% and 42% in amantadine and placebo groups respectively, Table 3). Similarly, we found no benefit of amantadine in the subgroup of patients with genotypes 1 and 4 combined (SVR 36% vs. 42% in amantadine and placebo arms, p=0.52) or in patients with these genotypes and high viral load (SVR 36% vs. 40% in amantadine and placebo arms, p=0.70). Although SVR increased progressively with decreasing baseline body weight (53% in group <65 kg, 51% in group 65-85 kg, 44% in group 85-105 kg and 31% in group >105 kg), these differences were not statistically significant. Also in the difficult to treat patients i.e. genotype 1 infected patients with high viral load, no statistically significant differences between the SVR rates of patients in the specified weight groups were found (data not shown).

Determinants associated with sustained virologic response at the 0.2 level in univariate regression analysis were: female sex, younger age, lower weight, absence of severe fibrosis/

Table 3. Sustained virologic response in 297 naive chronic hepatitis C patients to treatment with (PEG)-interferon and ribavirin in comparison with triple treatment consisting of (PEG)-interferon, ribavirin and amantadine hydrochloride

	Intention-to-treat			Per-protocol		
	Amantadine	Placebo	*p*	Amantadine	Placebo	*p*
	N=144	N=153		N=97	N=110	
*HCV Genotype**						
HCV 1	23/65 (35)	31/70 (44)	0.38	23/45 (51)	31/52(60)	0.42
HCV non-1	43/78 (55)	47/83 (57)	0.88	43/51 (84)	47/58 (81)	0.8
Baseline viremia						
Serum HCV RNA < 800.000 IU/mL	25/47 (53)	22/43 (51)	1.0	25/31 (81)	22/28 (79)	1.0
Serum HCV RNA > 800.000 IU/mL	42/97 (43)	56/110 (51)	0.33	42/66 (64)	56/82 (68)	0.6
*HCV Genotype and baseline viremia**						
HCV 1/HCV RNA < 800.000 IU/mL	7/20 (35)	11/22 (50)	0.37	7/12 (58)	11/14 (79)	0.4
HCV 1/HCV RNA > 800.000 IU/ml	16/45 (36)	20/48 (42)	0.67	16/33 (49)	20/38 (53)	0.81
HCV non 1/HCV RNA < 800.000 IU/mL	18/27 (67)	11/21 (52)	0.38	18/19 (95)	11/14 (79)	0.29
HCV non 1/HCV RNA > 800.000 IU/ml	25/51 (49)	36/62 (58)	0.35	25/32 (78)	36/44 (82)	0.77

Number of patients with virologic response with percentage in brackets. HCV, hepatitis C virus.
*In one patient genotype was missing.

cirrhosis (F3/F4), infection with HCV genotype non-1 and lower pre-treatment γGT (Table 4a). Multivariate analysis identified only infection with HCV genotype non-1 (p=0.03) and lower pre-treatment level of γGT (p=0.001) as independent predicting variables for sustained virologic response (Table 4b).

Per protocol analysis

207 of the 297 patients reached a primary endpoint. Baseline characteristics in the 97 patients in the amantadine group and the 110 patients in the placebo group did not differ (Table 1). In the whole group, SVR was achieved in 145 of 207 patients (70%) without differences between amantadine and placebo groups (69 vs. 71%, p=0.9; Table 2). Accordingly, there were no differences in percentage of on-treatment virological response, end-of-treatment HCV RNA negativity, primary non-response, breakthrough or relapse (Table 2). SVR in genotype 1 infected patients was significantly lower than in patients infected with genotype non-1 (all patients 56% vs. 83%, p<0.001; amantadine group 51% vs. 84%, p=0.001; placebo group 60% vs. 81%, p=0.02). SVR in patients with high viral load tended to be lower than in patients with low viral load, but significance was not

Table 4a. Univariate regression analysis of factors potentially associated with sustained virologic response in intention-to-treat analysis

Determinant	Coefficient	Odds Ratio	95% CI	*p*-value
Placebo	0.178	0.837	0.53-1.32	0.443
Female Sexe	0.476	1.595	0.958-2.654	0.073
Age	-0.02	0.980	0.955-1.005	0.117
Weight	-0.012	0.988	0.973-1.003	0.128
Ribavirin/kg bodyweight	0.062	1.064	0.953-1.188	0.266
ALT	0.000	1.0	0.998-1.002	0.985
GGT	-0.006	0.994	0.991-0.997	**<0.001**
Viral load < 800.000 IU/mL	0.195	0.823	0.501-1.350	0.440
F3/F4	-0.447	0.639	0.379-1.080	0.094
Genotype non-1	0.643	1.901	1.195-3.024	**0.007**
No alcohol use	0.192	0.825	0.518-1.315	0.419

ALT, alanine aminotransferase; GGT, gamma glutamyltransferase. F3 = bridging fibrosis; F4 = cirrhosis or advanced fibrosis. Bold style is used for those p-values that reach significance.

Table 4b. Independent predictive factors associated with sustained virologic response (intention-to-treat analysis)

Determinant	Coefficient	Odds Ratio	95% CI	*p*-value
GGT	-0.006	0.994	0.990-0.997	**0.001**
Genotype non-1	0.590	1.804	1.079-3.018	**0.03**

GGT, gamma glutamyltransferase. Bold style is used for those *p*-values that reach significance.

reached (all patients 66% vs. 80%, p=0.07; amantadine group 64% vs. 81%, p=0.11; placebo group 68% vs. 79%, p=0.34). In difficult to treat patients i.e. genotype 1 infected patients with high viral load, no benefit of adding amantadine could be found (Table 3). Similarly, we found no benefit of amantadine in the subgroup of patients with genotypes 1 and 4 combined or in patients with those genotypes and high viral load (not shown). Also, SVR rates in various weight classes were not different. Results of univariate and multivariate analysis to identify predictive factors for SVR were highly similar in the intention-to-treat and per-protocol analysis (not shown).

Side effects and reason for discontinuation of treatment

Side effects were reported in 98% of patients (Table 5). Except for fever and diarrhea, there were no differences between treatment groups (Table 5). According to the study protocol, 44 patients (20 amantadine and 24 placebo) had to discontinue treatment before reaching a primary endpoint due to severe toxic effects of the prescribed medication (Table 6). Of all 207 patients who reached a primary study endpoint, 44 patients (21%) had a decrease of PEG-interferon dose and 19 patients (9%) had their ribavirin dose lowered.

Table 5. Summary of adverse events during treatment in amantadine group (*N*=144) and in placebo group (*N*=153)

Symptoms	Amantadine	Placebo	*p*-value
	n (%)	*n* (%)	
Fatigue	100 (69)	118 (77)	0.17
Myalgia	83 (58)	102 (67)	0.14
Headache	81 (56)	86 (56)	1.0
Nausea	73 (51)	92 (60)	0.13
Fever	48 (33)	79 (52)	**0.002**
Pruritus	60 (42)	61 (40)	0.84
Depression	45(31)	54 (35)	0.54
Irritability	43 (30)	54 (35)	0.38
Cold Chills	39 (27)	48 (31)	0.49
Diarrhea	25 (17)	45 (29)	**0.02**
Dizziness	37 (26)	30 (20)	0.26
Sleep disorders	33 (23)	27 (18)	0.32
Poor appetite	26 (18)	33 (22)	0.54

Events that occurred in at least 20% of patients were listed according to frequency.
Bold style is used for those p-values that reach significance.

Table 6. Number of serious adverse events (SAE) during treatment in amantadine group (*N*=144) and in placebo group (*N*=153)

SAE	Amantadine	Placebo
Depression	5	4
Catatonic syndrome	1	0
Labyrynthitis	0	1
Pancreatitis	1	0
Rhabdomyolysis	0	1
Trombopenia	0	2
Leukopenia	3	0
Anemia	0	2
Granulopenia	4	12
Pancytopenia	2	0
Toxic hepatitis	2	0
Thyroid dysfunction	2	1
Gastroenteritis	0	1
Venous retinal occlusion*	1	0
Peri-anal abscess*	0	1
GGT elevation*	3	0
Exacerbations COPD*	1	1
Vomitus and Nausea*	2	1

*Serious adverse events for which medication was not discontinued. GGT, gamma glutamyltransferase

There were no differences in rate or extent of dose modification between amantadine and placebo groups. Also, average dosages of PEG-interferon and ribavirin used during the first 26 weeks and between week 26 and 52 did not differ between both groups, neither in intention-to-treat nor in per-protocol analysis. Baseline psychiatric symptoms occurred significantly more often in (former) drug users than in those who never used drugs; sleeping disorders (p=0.03), depression (p=0.02), fear (p=0.06), disturbance of concentration (p<0.001), irritability (p=0.04), emotional lability (p=0.06) and fatigue (p=0.19). During the study period, these differences were even more pronounced (overall psychiatric symptoms: p<0.001, including depression, p=0.001) and generally treated with antidepressants and/or psychiatric support.

DISCUSSION

The major finding of the current study is that, in treatment-naive HCV patients, adding amantadine to PEG-interferon alpha-2b and ribavirin does not enhance SVR rates. In line with our findings, amantadine affected neither RNA replication nor release or infectivity of HCV particles across a spectrum of HCV isolates and genotypes in recent *in vitro* studies (17). Also, in that study, p7 ion channel activity was not affected by amantadine, indicating that amantadine is not an HCV-selective antiviral medication. In our study, 90 patients discontinued treatment before reaching a primary endpoint, especially because of grade 4 or recurrent/persistent grade 3 toxicity. We hypothesize that high-dose interferon induction could have contributed to frequent grade 3 and 4 toxicity. Current criteria for treatment withdrawal in various guidelines are less strict, particularly for hematological toxicity (18). Furthermore, 49% of our patients were (former) drug users with tendency to higher dropout rates. Although in theory, our relatively high dropout rate could have led to type II error, we consider this improbable. First, there were no differences in dropout rates or time of dropout between amantadine and placebo groups. Second, in per-protocol analysis, no significant differences between amantadine and placebo treatment were found. Third, SVR rates turned out -in all probability by chance- to be somewhat better in placebo than in amantadine group (Table 3).

Favourable trends of triple therapy were previously described in literature especially for patients with HCV genotype 1 infections and/or high viral load (13;19). One may argue that in our study, beneficial effects of amantadine may have been missed due to type II error. Nevertheless, we found no trend toward better results in the amantadine-treated patients. In fact, results in these subgroups were (probably by chance) somewhat better in placebo-treated patients, again arguing against a beneficial effect of amantadine in these subgroups. Differences between our results and the recent study of Angelico *et al*. (who found a better response with amantadine added to PEG-interferon alpha-2a and ribavirin

in the subgroup of patients with genotype 1 and 4 and no early virologic response after 3 months induction therapy with PEG-interferon) could relate to differences in study design (20). In line with our results, von Wagner *et al*. found no beneficial effects of amantadine added to PEG-interferon alpha-2a and ribavirin in 705 naive HCV genotype 1 patients (21)**.** Formulation and/or dosage of amantadine could explain differences between various studies. However, in a meta-analysis, amantadine hydrochloride (the formulation used in our study) achieved better responses than amantadine sulphate, at least in combination with interferon (10). As far as amantadine dosage is concerned, the data by von Wagner *et al*. indicate that even the high dose of 400 mg amantadine per day in combination with PEG-interferon and ribavirin does not improve SVR rates, in line with our results (21). Our data do not exclude the possibility that adding amantadine to standard therapy could be beneficial in non-responder patients, as previously suggested (11;22;23). It should also be noted, that our study was designed in the period: 1999-2000, and therefore some aspects of the protocol differ from current practice. For example, high-dose induction therapy is now controversial, and currently not advised (24). Also, dose of PEG-interferon alpha-2b was decreased in the second half of the treatment period from 1.5 to 1.0 µg/kg/week. Although this is not current practice, ribavirin rather than PEG-interferon doses may be key to reaching SVR (25). Also, there is no evidence from controlled studies, that the lower PEG-interferon dose used in the second half of the treatment period is associated with lower SVR rates. In fact, in a randomized double-blind trial comparing various doses of PEG-interferon alpha-2b as monotherapy, there was no difference in SVR rates between the 1.0 and 1.5 µg/kg dose (26). Last, it is now generally accepted that 24 weeks of treatment is sufficient in patients with genotypes 2 or 3.

We identified in our multivariate analysis, genotype non-1 and lower pre-treatment levels of γGT as independent predictors for SVR. High γGT is known to be associated with more severe hepatic fibrosis in HCV patients (27). In our study 56% of patients had elevated γGT levels and γGT levels were associated with pre-treatment fibrosis score (r=0.22, p<0.001). Since in our multivariate analysis, fibrosis score did not turn out to be an independent predictor of SVR, one may speculate that γGT is a more sensitive marker for fibrosis than liver biopsy with its inherent risk of understaging (28). Alternatively, alcohol or metabolic syndrome (conditions associated with both high γGT and reduced success rates of antiviral therapy) could explain the apparent association of γGT with SVR rates in the present study.

In conclusion, we did not find any beneficial effects of adding amantadine to PEG-interferon alpha-2b and ribavirin in previously untreated chronic hepatitis C patients.

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CHAPTER 4

Clinical and basal aspects of anemia during antiviral therapy for hepatitis C

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ABSTRACT

Background: Anemia is a major side effect of combination therapy for chronic hepatitis C (CHC). In this study, severity, potential risk factors for and potential underlying mechanisms of anemia were evaluated. *Patients and Methods*: 44 CHC patients on interferon-ribavirin treatment were included. Anemia-related parameters were measured before and during treatment. Potential changes in membrane phospholipid composition of erythrocytes of patients on anti-viral treatment and potentially increased erythrocyte susceptibility to osmotic or bile salt induced stress were explored. *Results:* Anemia was almost universal during treatment, with evidence of hemolysis. Decrease of hemoglobin (Hb) after six months of therapy was 2.1 ± 0.1 mmol/L (range -0.6-4.1). Higher pre-treatment Hb, highest ribavirin dose (15-17.5mg/kg) and lower pre-treatment platelet level were independent risk factors for decrease of Hb. Serum erythropoietin levels increased during treatment with negative correlation to Hb levels at week 12 (r=-0.70, p=0.002) and 24 (r=-0.72, p=0.002). Erythrocyte membrane phospholipid composition did not differ between anemic patients and healthy controls. Also, resistance to osmotic or bile salt induced stress was normal in anemic patients. Phosphatidylserine exposure at the outer membrane leaflet did not change upon 24 hrs ex vivo incubation with pharmacological ribavirin concentration. *Conclusions:* Anemia is almost universal during anti-HCV treatment. The extent of anemia correlates with pre-treatment levels of thrombocytes and Hb and with high ribavirin dosing. Although we found hemolysis as contributing factor, our data do not indicate that altered membrane phospholipid composition is an important factor in pathogenesis of anemia.

INTRODUCTION

Chronic hepatitis C (CHC) is a life-shortening disease associated with significant morbidity and decreased quality of life. Current treatment (PEG-interferon alpha and ribavirin) achieves a sustained virological response (SVR) in 50-90% of cases, depending on hepatitis C virus (HCV) genotype (1;2). Treatment may cause anemia, requiring dose-reduction or even discontinuation of therapy in up to one third of patients (3). The cause of anemia is probably multifactorial: interferon might suppress bone marrow regenerative activity of erythroid progenitor cells and inhibit erythropoietin production (4;5). Also, ribavirin may induce dose-dependent hemolytic anemia (6-8). Ribavirin is converted into ribavirin-mono-, di- and triphosphate in all cell types but subsequent dephosphorylation back to ribavirin occurs exclusively in nucleated cells, not in erythrocytes. Accumulated phosphorylated ribavirin derivates within the erythrocyte might lead to relative intracellular adenosine triphosphate (ATP) depletion (9-12), impaired antioxidant defense and possibly, premature removal from the circulation (13).
The major structural phospholipids of the erythrocyte membrane outer leaflet are phosphatidylcholine and sphingomyelin. Phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol are mainly located in the inner leaflet (14-17). Such membrane asymmetry, which is dependent on flippase activity, is essential for membrane integrity and cellular function (18-20). Increased intracellular ribavirin could induce a change of phospholipid composition with enhanced signalling for red cell removal from the circulation. In this study, we explored potential changes in erythrocyte membrane phospholipid composition and susceptibility to osmotic or bile salt induced stress in anemic hepatitis C patients on antiviral treatment. We also determined serum erythropoietin (EPO) levels in a subgroup of patients and related these to various clinical parameters of anemia.

METHODS

Patients

44 treatment naive CHC patients participating in a multicenter, randomized placebo-controlled trial comparing standard therapy (PEG-interferon alpha/ribavirin combination therapy) with an experimental triple regimen (PEG-interferon alpha/ribavirin and amantadine), were included in this study. All patients provided written informed consent and the protocol was approved by the medical ethical committee of the UMC Utrecht. Baseline patient characteristics are given in Table 1. 36% of patients had severe fibrosis or cirrhosis corresponding with Metavir score F3-F4 (21). Treatment consisted of weight-based ribavirin (Rebetol®, Schering Plough B.V. Maarssen, The Netherlands: 1000 mg/day in case

Table 1. Baseline characteristics in 44 treatment naive patients with chronic hepatitis C.

Characteristic	
Age (yrs)	45 ± 1.4 (28-66)
Male/Female ratio	35:9
Amantadine/placebo	22:22
Weight (kg)	78.5 ± 2.3 (40-134)
Genotype 1/non-1 (% of patients)	21:23 (48:52)
High viral load* (% of patients)	57
AST (U/mL)	76 ± 6 (28-226)
ALT (U/mL)	111 ± 9 (22-260)
Albumin (U/L)	40 ± 1 (28-46)
Prothrombin time (sec)	12.4 ± 0.1 (10.5-14.5)
Bilirubin (mmol/L)	12 ± 0.7 (6-28)
Hemoglobin (mmol/L)	9.2 ± 0.1 (7-10.3)
Platelets (x10^9/L)	223 ± 10 (109-406)
Severe fibrosis/cirrhosis (% of patients)	36
Histological activity index	2.8 ± 0.3
Renal clearance** (ml/min)	113 ± 4 (45-182)

Data are presented as mean ± SEM with range in brackets.
* viral load >800.000 IU/ml. ** By Cockroft-Gault(50). AST, aspartate aminotransferase; ALT, alanine aminotransferase

of body weight <75 kg, 1200 mg/day in case of body weight >75 kg) and interferon (Intron A®, Schering Plough B.V. Maarssen, The Netherlands) 10 MIU/day during days 1-6, 5 MIU/day during days 7-12, thereafter 3 MIU/day until week 26 and 3 MIU TIW during weeks 27-52 in combination with amantadine hydrochloride 200 mg per day or placebo for 52 weeks. Amantadine was part of the treatment regimen in 50% of the cases. According to protocol, dose modification was indicated whenever hemoglobin (Hb) concentration was <4.9 mmol/L. Patients were grouped according to actually received ribavirin dose in three predefined subgroups: group A: ribavirin <13.5 mg/kg/day, group B: ribavirin 13.5-15 mg/kg/day or group C: ribavirin 15.1-17.5 mg/kg/day. Thirty-two patients (73%: 95% CI 60-86%) reached a SVR, defined as undetectable serum HCV RNA 12 months after discontinuation of antiviral treatment. One patient was lost to follow-up after 40 weeks of treatment. Of the remaining patients, five cases (11%: 95% CI 2-21%) had a persistently detectable HCV RNA after 24 weeks of treatment (non-response: 4 patients stopped antiviral therapy) and six patients (14%; 95% CI 4-24%) relapsed after week 52 after initially negative HCV RNA at 24 weeks.

Materials

Taurocholate was obtained from Sigma Chemical Co. (St. Louis MO, USA) and yielded a single spot upon thin-layer chromatography (butanol-acetic acid-water, 10:1:1 vol/vol/vol, application of 200 μg bile salt). 3α-Hydroxysteroid dehydrogenase for the enzymatic measurement of bile salt concentrations (22) and TRIS-HCl were purchased from Sigma. The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from BD Pharmingen (San Diego CA, USA). 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) was obtained from Sigma. All other chemicals and solvents were of ACS or reagent grade quality.

Clinical measurements

Hb levels were quantified by standard assay at baseline, at 1, 2, and 4 weeks after start of therapy and thereafter every four weeks during the entire study period. Anemia was defined as Hb <7.4 mmol/L for females and <8.6 mmol/L for males. Clinical anemia-related parameters were determined by standard assays before and after 12 weeks of anti-viral therapy. HCV RNA was tested by quantitative polymerase chain reaction (PCR) (Roche Amplicor HCV monitor Kit v2.0) and values >800.000 IU/mL were considered high viral load. HCV genotype was determined using Innolipa (Innolipa HCV II, Innogenetics, Ghent, Belgium).

Serum erythropoietin (sEPO) was quantified before, 12 and 24 weeks after start of anti-viral treatment in a subgroup of 16 patients. sEPO was measured using a chemiluminescent enzyme-labeled immunometric assay (Immunolite® EPO, Diagnostic Products Corporation (DPC, Los Angelos CA, USA)). The lower limit of detection for sEPO was 0.24 mU/mL and values between 3-20 mU/mL were considered normal (23). To determine whether the sEPO responses to the decreasing hematocrit were normal in our patients, their values were compared with the normal human response to anemia defined by the equation $\log_{10}EPO=4.609 – 8.7xHt$ (24;25).

Hemolysis induced by hypotonic solutions or taurocholate

Resistance of erythrocytes against osmotic and bile salt-induced stress of fresh human erythrocytes of anemic hepatitis C patients after twelve weeks of anti-viral treatment (Hb 6.2 ± 0.6 mmol/L) and from healthy controls (Hb 8.4 ± 0.3 mmol/L) was determined as described in detail before (26;27).

Phospholipid composition of the erythrocyte membrane

Fresh erythrocytes (aliquots of 10 mL blood) were sedimented three times by centrifugation during 15 min. at 3000 rpm. After discarding the plasma and the buffy coat, membrane phospholipids were extracted from the erythrocytes according to Reed (28). After separation by thin layer chromatography (chloroform:methanol:acetic acid:water – 50:25:8:3 vol/vol/vol/vol), phospholipid contents of separated spots were quantified according to Rouser (29).

Exposure of phosphatidylserine and hemolysis after ribavirin incubation

Phosphatidylserine normally localizes to the inner leaflet of erythrocyte membranes but becomes exposed to the cell surface in pathologic or aged cells, with subsequent removal from the circulation (30-33). Annexin V is a calcium-dependent phospholipid-binding protein that exhibits a high affinity for cell membranes exposing phosphatidylserine on the outer leaflets (34). Fresh erythrocytes of normal volunteers were incubated during 15, 30, 45, 60, 240 minutes and 24 hours at 37°C with solution containing 3.125 μg/mL ribavirin in order to mimic a therapeutic concentration of ribavirin in the tube (2.5μg/mL) (9). After addition of FITC labelled annexin V, phosphatidylserine exposure was measured by quantifying fluorescence in a Becton Dickinson Fluorescence Automate Cell sorter (35). During all incubations hemolysis was assayed by measuring absorbance of hemoglobin in the supernatant at 540 nm (36).

Statistics

Values are expressed as means ± SEM or in case of non-parametric distribution, as medians (range). Differences between groups were tested with unpaired *t*-tests or Mann Whitney-*U* tests as appropriate. Differences between pre-treatment and on-treatment data were tested with paired *t*-tests or with repeated measures anlaysis of variance (ANOVA). Correlation between parameters was tested for statistical significance by Pearson correlation tests or Spearman Rank test in case of non-Gaussian distribution. In order to identify risk factors for Hb decrease during the first 24 weeks (ΔHb), univariate and multivariate linear regression analyses were performed. Coefficients are expressed with 95% confidence intervals (C.I.). First, determinants were examined in univariate analysis. Only determinants with coefficients significant at the 0.2 level were included in subsequent multivariate analysis. In multivariate analysis, stepwise regression procedure was used. A two-sided *p*-value less than 0.05 was considered statistically significant.

RESULTS

Anemia during antiviral therapy

Mean pre-treatment level of hemoglobin was 9.2 ± 0.1 mmol/L. During antiviral treatment, mean hemoglobin decreased 2.6 ± 0.1 mmol/L (28%, range 11-44%) if pre-treatment Hb level was compared to lowest Hb level at any time point during treatment. 98% of patients developed anemia during antiviral therapy (see "Methods" for definitions), and 27 patients (61%) experienced a drop of hemoglobin of at least 2.5 mmol/L. Nevertheless, no dose-reduction was required for anemia in any patient. Since there was no difference between amantadine and placebo groups in extent of anemia or any other parameter of potential relevance, these groups are reported together in the following. Hb levels started to decline two weeks after the first medication was taken and minimum values were reached after a median of 24 (range 2-52) weeks (Fig. 1).

Mean ΔHb, defined as difference between pre-treatment Hb and Hb at 24 weeks, was 2.1 ± 0.1 mmol/L. ΔHb was not different between patients with or without severe fibrosis/cirrhosis, between genotype 1 and non-1 patients or between patients with 1000 and 1200 mg ribavirin/day. ΔHb did not correlate with age, transaminases or histological activity index. Although ΔHb was greatest in patients with highest weight-based dose of ribavirin, ΔHb did not differ significantly (p=0.44) between the various ribavirin doses (2.2 ± 0.2 mmol/L,

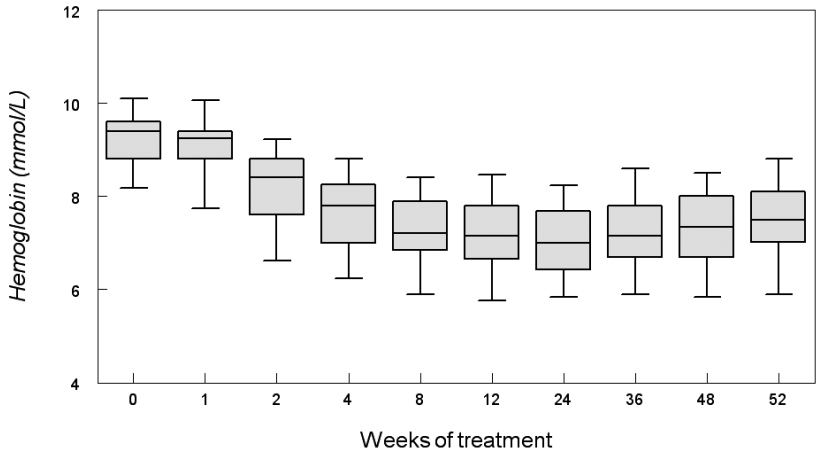

Figure 1: Box Whisker plots of hemoglobin levels during antiviral treatment for hepatitis C (N=40; patients who were treated during 52 weeks). Boxes represent the interquartile range (IQR): the boundaries of the box indicate the 25th and the 75th percentiles, the horizontal line in the middle of the box represents the median. The vertical lines from the ends of the box indicate the highest and lowest value. One way repeated measured ANOVA reveals a significant Hb decrease during therapy (p<0.0001).

2.1 ± 0.2 mmol/L and 2.5 ± 0.2 mmol/L in groups with ribavirin <13.5 mg/kg/day, 13.5-15 mg/kg/day and 15.1-17.5 mg/kg/day). Determinants associated with greater ΔHb at the 0.2 level in the univariate regression analysis were: weight, highest ribavirin dose (15-17.5 mg/kg), higher pre-treatment level of Hb, lower viral load and lower pre-treatment platelet level (Table 2a). Multivariate analysis identified only higher pre-treatment Hb, highest ribavirin dose (15-17.5 mg/kg) and lower pre-treatment platelet level as independent risk factors for decrease of Hb (Table 2b). As shown in Table 3, significant differences between pre- and 3 month treatment levels of mean corpuscular volume (MCV), ‰ of reticulocytes, immature reticulocyte fraction (IRF), plasma hemoglobin, bilirubin and lactate dehydrogenase (LDH) were found. Median pre-treatment level of serum erythropoietin measured in a subgroup of 17 patients, was 8 (5-48) mU/mL, increased to 51 (13-326) mU/mL after 12 weeks and to 67 (7-1590) mU/mL after 24 weeks of treatment (Fig. 2a: $p<0.001$). Baseline levels of serum erythropoietin were not associated with baseline levels of Hb or hematocrit (r=-0.23, p=0.37 and r=-0.32, p=0.21 respectively). In contrast serum erythropoietin levels at 12 and 24 weeks after start of treatment were negatively correlated with simultaneous Hb and Ht levels (r =-0.7, p =0.002 and r =-0.78, p=0.0004 for Hb and Ht levels at week 12: r =-0.72, p =0.002 and r =-0.79, p =0.0002 for Hb and Ht levels at week 24).

Table 2a. Univariate regression analysis of determinants associated with an increase in ΔHb

Determinant	Coefficient	95% C.I.	*p*-value
Age	0.017	-0.05 to 0.13	0.25
Viral load	-7.8E-8	-2.0E-7 to 4.0E-8	0.19
ASAT	0.002	-0.009 to 0.005	0.59
ALAT	0.002	-0.007 to 0.003	0.50
Weight	0.018	-0.035 to -0.001	**0.04**
Ribavirin <13.5 mg/kg	0.068	-0.713 to 0.578	0.83
Ribavirin 13.5-15 mg/kg	-0.117	-0.448 to 0.682	0.68
Ribavirin 15-17.5 mg/kg	0.579	-1.132 to -0.025	**0.04**
Platelets pre-treatment	-0.004	0 to 0.008	0.06
Hb pre-treatment	0.648	-1.037 to -0.259	**0.002**

Hb, hemoglobin. Bold style is used for those p-values that reach significance.

Table 2b. Independent factors associated with an increase in ΔHb in multiple regression analysis

Determinant	Coefficient	95% C.I.	*p*-value
Ribavirin 15-17.5 mg/kg	0.608	0.109 to 1.108	**0.018**
Platelets pre-treatment	-0.004	-0.008 to -0.001	**0.017**
Hb pre-treatment	0.677	0.211 to 1.144	**0.005**

Hb, hemoglobin. Bold style is used for those *p*-values that reach significance.

Table 3. Mean clinical anemia-related parameters before and during treatment

Determinant	Pre-treatment	On treatment	*p*-value
MCV (fL)	90 ± 2	101 ± 2	**<0.001**
Reticulocytes (‰)	11 ± 2	36 ± 6	**0.001**
IRF	0.19 ± 0.02	0.27 ± 0.03	**0.02**
Plasma hemoglobin (mg/L)	92 ± 10	38 ± 11	**0.005**
Bilirubin (mmol/L)	6 ± 0.4	9 ± 1	**0.04**
LDH (U/L)	475 ± 36	547 ± 24	**0.03**
Haptoglobin (g/L)	1.3 ± 0.2	0.9 ± 0.1	0.06
Folic acid (nmol/L)	21 ± 5	19 ± 5	0.68
Vitamin B12 (pmol/L)	307 ± 43	357 ± 66	0.17
Ferritin (ug/L)	130 ± 80	389 ± 167	0.07

MCV, mean corpuscular volume; LDH, lactate dehydrogenase; IRF,immature reticulocyte fraction. Bold style is used for those p-values that reach significance.

Also, ΔsEPO (sEPO at 24 weeks minus pre-treatment sEPO) correlated positively with ΔHb (r=0.5, p=0.047) and inversely with pre-treatment level of Hb (r=-0.8, p=0.001). ΔsEPO was not different between patients with or without histologically proven severe fibrosis/cirrhosis, between genotype 1 and non-1 patients or between males and females. Comparing the normal human response to anemia with the response in our population, no significant differences in the slope of hematocrit (x) versus $\log_{10}$EPO (y) (–8.7 versus –8.7) and y-intercept (4.719 vs. 4.609) (Fig. 2b) were found.

Effect of osmotic and bile salt induced stress on hemolysis

In Fig. 3, hemolysis of erythrocytes induced by hypotonic buffer is shown for hepatitis C patients with anemia due to anti-viral treatment and for healthy controls. In TRIS-buffer solutions with concentrations ranging from 150 to 110 mM, hemolysis proved to be negligible. Hemolysis increased progressively at lower concentrations. Nevertheless, no difference was found in osmotic resistance pattern between erythrocytes of anemic patients and healthy controls.

Although low concentrations of taurocholate exhibited no effects, incubation with ≥15 mM of this detergent bile salt induced progressive dose-dependent hemolysis. Again there were no differences between erythrocytes of anemic patients and healthy controls (results not shown).

Phospholipid composition of the erythrocyte membrane

No differences in the phospholipid composition of the red cells membranes or in the sphingomyelin (SM) / phosphatidylcholine (PC) ratio were found between healthy controls and

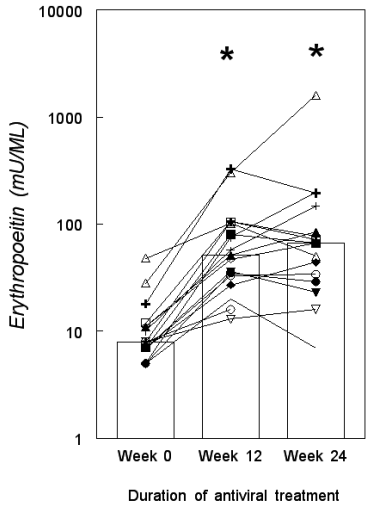

Figure 2a: Serum erythropoietin levels of individual patients (N=16) before and during antiviral treatment. Bars indicate median values at various time points.* $p<0.001$ compared to basal.

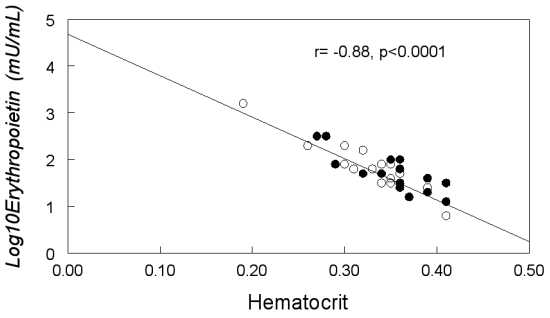

Figure 2b: Relationship between serum erythropoietin and hematocrit levels (r=-0.88, p<0.0001) in our population. For comparison, the regression line (–)of the normal compensatory erythropoietin response to anemia defined by the equation $\log_{10}$EPO=4.609 – 8.7xHt is also shown (24,25).
● observed value at week 12 (N=16), ○ observed value at week 24 (N=16).

anemic hepatitis C patients on antiviral treatment. Sphingomyelin comprised 27.3 ± 0.7% and 25.1 ± 1% of total cell membrane phospholipids in hepatitis C patients and controls (p=ns). For phosphatidylcholine, these values were 32.7 ± 0.7% and 34.9 ± 1.7%, for phosphatidylinositol/phosphatidylserine 14.1 ± 0.8% and 12.7 ± 0.3%, and for phosphatidylethanolamine 25.9 ± 1% and 27.2 ± 1.3% respectively (all not significantly different).

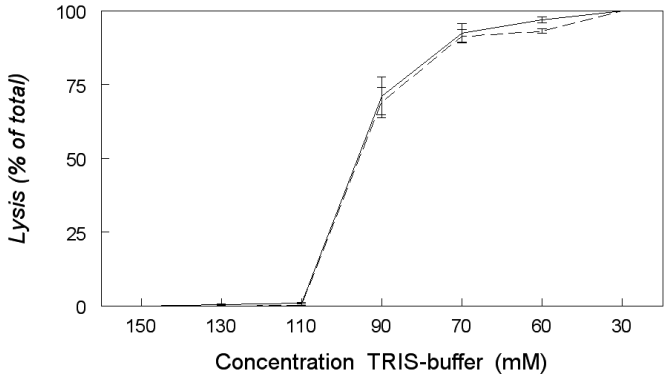

Figure 3: Resistance (mean ± SEM) against osmotic stress of erythrocytes obtained from anemic patients during antiviral therapy for hepatitis C does not differ from healthy controls.
Continuous line, patients (N=5); interrupted line, controls (N=4).

Annexin V assay

Phosphatidylserine exposure on the outer leaflet of the erythrocyte was not detected after incubation up to 24 hours with pharmacologically relevant ribavirin concentrations. Hemolysis after 24 h incubation with ribavirin and TRIS-buffer solution was respectively 5% and 6%.

DISCUSSION

Specific risk factors for developing anemia during antiviral therapy for hepatitis C are not well established. We found in our multivariate analysis higher pre-treatment Hb level, lower pre-treatment platelet count and highest dose of ribavirin (>15 mg/kg/day) to be independent factors associated with greater decreases of Hb. The correlation between pre-treatment Hb level and extent of Hb decrease during therapy has been described before (37;38) and could be the consequence of the fact that a certain -more or less fixed- fraction of all circulating erythrocytes might be removed from the circulation during a certain time period during therapy. In line with previous data (38), greater ΔHb was associated with lower pre-treatment platelet level, but not with histologically proven severe fibrosis/cirrhosis. One may speculate that low thrombocyte levels are a more sensitive marker for severe liver disease than liver biopsy. Indeed, thrombocytopenia is generally the first hematological abnormality to occur in patients with cirrhosis (39). Recent data suggest increased platelet breakdown in chronic liver disease and cirrhosis, and to a lesser extent decreased platelet production and platelet dysfunction (40). Total ribavirin dose per day

was not associated with magnitude of Hb decrease, and there were no significant differences in ΔHb between subgroups with low, intermediate or high weight-based ribavirin dose. This may be explained by the fact that there is a threshold ribavirin dosage of ≥1000 mg/day for development of anemia (41). In our study all patients received at least 11.2 mg ribavirin/kg bodyweight and no patient had a dose of less than 1000 mg/day. Nevertheless, in our multiple regression analysis, ribavirin dose >15 mg/kg/day proved to be an independent factor associated with greater ΔHb, in line with another recent study (42). Our study focused on baseline predictive factors for extent of anemia during antiviral treatment. It was recently reported, that early on-treatment extent of Hb-decline (after 2-4 weeks of therapy) can predict extent of anemia during the subsequent treatment period (43;44). We found in a post-hoc analysis in the current study, a highly significant correlation between Hb-decline after 2 (r =0.63, p<0.0001) and 4 weeks (r =0.71, p<0.0001) of therapy and maximal decrease of Hb during the entire study period, thus confirming the previous reports. In the current study we found clear evidence of hemolysis, with elevated levels of bilirubin and LDH and decreased haptoglobin levels. Serum ferritin levels also increased, in line with previous studies (45). So far there are conflicting results in the literature about serum erythropoietin response during interferon/ribavirin therapy (25;37;46). This issue has considerable clinical relevance, since erythropoietic growth factors are used to increase hemoglobin levels and to reduce the need of ribavirin dose reductions (47;48). Our results show no correlation between $\log_{10}$EPO levels and Ht in non-anemic patients before treatment but during the anemic period there was a significant inverse correlation between these parameters up to 24 weeks after initiation of therapy. Previous studies examined this correlation for shorter periods of maximal 12 weeks (25;37;46). Based on comparison with the normal human response to anemia, our data would suggest that serum erythropoietin response could be adequate in patients with anemia during antiviral therapy (Fig. 2b). Since all available studies are hampered by relatively small patient numbers with severe anemia, and considering the appreciable inter-individual variation in normal serum erythropoietin response to anemia (49), further research is warranted on this issue.

We did not find changes of erythrocyte membrane phospholipid composition or decreased resistance to osmotic or bile salt-induced stress in anemic hepatitis C patients. Furthermore, after *ex vivo* incubation with ribavirin during 24 hours, there was no enhanced exposure of phosphatidylserine on the outer leaflet of the membrane. Although we cannot exclude that longer incubation times could lead to different results, metabolites of ribavirin are already formed within a few hours of incubation (10). Also, normal phosphatidylserine exposure in patients with hemolytic anemia from other causes has been described, in line with our findings (30).

It should be noted that our study was already designed in the year 1999 and executed in 2000. Therefore some aspects differ from current practice. For example, PEG-interferon alpha rather than interferon is now generally used for treatment of hepatitis C. Also, high

dose interferon induction therapy as applied in our study is now controversial, and SVR is now defined as negative HCV RNA 24 rather than 48 weeks after the end of therapy. Nevertheless, it is generally thought that ribavirin rather than (PEG-) interferon alpha is the most important factor in development of anemia, the topic of the current study. Since we used the standard dose of ribavirin and there was a similar rate of anemia in the PEG-interferon alpha and standard interferon groups in the two main registration trials we assume that our results would have been similar if PEG-interferon alpha had been used (1;2).

In conclusion, anemia occurs in most patients during anti-HCV treatment. Extent of anemia correlates with pre-treatment levels of thrombocytes and Hb and becomes aggravated by high ribavirin dosing. Although we found clear hemolysis as contributing factor, our data do not indicate altered membrane phospholipid composition as an important factor. Further research is needed to explore whether serum erythropoietin response is adequate during antiviral therapy.

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CHAPTER 5

Suboptimal endogenous erythropoietin response in chronic hepatitis C patients during ribavirin and PEG-interferon treatment

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ABSTRACT

Background: During the treatment of chronic hepatitis C (CHC), anemia may necessitate PEG-interferon alpha and ribavirin dose reductions with reduced sustained viral response rates. Although erythropoietic growth factors are frequently used to improve anemia, it is controversial whether endogenous erythropoietic response is insufficient under these circumstances. We aimed to identify risk factors for more pronounced anemia and to evaluate endogenous erythropoietic response during antiviral therapy. *Methods:* One hundred forty-five naive CHC patients on PEG-interferon alpha/ribavirin treatment were evaluated for hemoglobin, hematocrit, serum ribavirin and erythropoietin levels. *Results:* About 99% of patients developed anemia, with maximal decrease in hemoglobin of 2.5 ± 1.0 mmol/L (range 0.3-5.5). Older age, lower baseline creatinine clearance, higher baseline hemoglobin, more pronounced hemoglobin decrease after 2 weeks and higher week 24 serum ribavirin concentrations were independent risk factors for more pronounced anemia. Serum erythropoietin levels increased from median 12 IU/L (range 4-63) at baseline to 41 IU/L (range 12-683) after 12 weeks of therapy and to 43 IU/L (range 7-3238) at week 24 (p<0.001). Erythropoietin levels at baseline, week 12 and week 24 negatively correlated with hematocrit. The erythropoietic response to anemia in our study population was significantly different from the normal human response to anemia. *Conclusion:* Older age, lower baseline creatinine clearance, higher baseline hemoglobin, more pronounced hemoglobin decrease after 2 weeks and higher week-24 serum ribavirin concentrations were independent risk factors for more pronounced anemia during antiviral therapy. Endogenous erythropoietin production is suboptimal during antiviral therapy, supporting use of erythropoietic growth factors.

INTRODUCTION

Chronic hepatitis C (CHC) leads to significant morbidity and mortality, and decreased quality of life. Current treatment (PEG-interferon alpha and ribavirin) achieves sustained virological response (SVR) in 50-80% of cases, depending on hepatitis C virus (HCV) genotype (1;2). Nevertheless, antiviral therapy is associated with significant side effects. Indeed, the majority of CHC patients becomes anemic, and in 9-22% of the patients dose reduction is needed for this reason (1;2). The cause of anemia is probably multifactorial. Ribavirin may induce dose-dependent hemolytic anemia (3-5). In addition, interferon may suppress bone marrow regenerative activity of erythroid progenitor cells and inhibits erythropoietin (EPO) production (6;7). SVR rates are lower in patients who require dose modifications (8;9), and the use of hematologic growth factors such as EPO-α can reduce anemia and need for dose modification (10;11). However, little is known about the endogenous erythropoietic response in CHC patients on antiviral therapy. Some studies suggest a normal response (12), while others suggest a blunted response (13;14) similar to the impaired response seen in patients with renal failure, HIV-infection and cancer (15-17). These studies were often relatively small, explored changes in erythropoietin response during a follow-up of 8 weeks or shorter and used suboptimal statistical analyses. In this study, we relate extent of anemia to endogenous erythropoietic response during 24 weeks antiviral therapy in a large cohort of naive CHC patients with the aid of sophisticated statistical methodology. We also explored potential risk factors for more pronounced anemia during treatment.

PATIENTS AND METHODS

Patients

In this study, we analyzed treatment naive CHC patients participating in a multicenter, double-blind randomized placebo-controlled trial on potential benefit of adding amantadine to PEG-interferon alpha-2b and ribavirin combination therapy (18). All patients received interferon alpha induction therapy (from day 1 combined with ribavirin), consisting of interferon alpha-2b (Schering Plough B.V., Maarssen, The Netherlands) 10 MIU/day subcutaneously during the first 6 days, followed by 5 MIU/day for the next 6 days, followed by PEG-interferon alpha-2b (Schering Plough B.V.) 1.5 µg/kg/week subcutaneously up to 26 weeks and 1.0 µg/kg/week from week 26 to week 52. Treatment was terminated if qualitative HCV RNA test was positive after 24 weeks of treatment. Oral ribavirin (Schering Plough B.V.) was given from day 1 during the entire 52-week treatment period in two different doses: 1000 mg/day for body weight less than 75 kg and 1200 mg/day for body weight of at least 75 kg. In the triple therapy group, 100 mg of oral amantadine hydrochloride (Dept

of Pharmacy UMC Utrecht, Utrecht, The Netherlands) was added twice daily. In the double therapy group, oral placebo of identical shape and taste as amantadine hydrochloride was added. There were no differences between the amantadine and placebo groups in baseline hemoglobin (Hb), decrease of Hb during treatment, increase of EPO levels during treatment or any other parameters of potential relevance. Therefore, data of amantadine and placebo groups were combined. Follow-up occurred at 0, 1, 2 and 4 weeks and thereafter monthly during 1 year of active treatment and at 3-month intervals during 1 year post-treatment. At each visit, actual body weights and doses of ribavirin and PEG-interferon alpha-2b were recorded and standard laboratory tests, including Hb and hematocrit (Ht) were performed. Anemia was defined as Hb less than 8.6 mmol/L in men and less than 7.4 mmol/L in women. ΔHb was defined as the difference between Hb at baseline and after 24 weeks of treatment. Hb nadir was defined as the lowest Hb level observed during the first 24 weeks of treatment. Baseline creatinine clearance was calculated according to the Cockcroft-Gault equation (19). Adverse events were graded mild, moderate, severe or potentially life threatening according to the WHO recommendations (WHO grade 1, 2, 3 and 4 respectively). Therapy was permanently discontinued for life-threatening events or for WHO-grade 4 events (including Hb <4.0 mmol/L, leucocytes <1.0 x 10^9/L, granulocytes <0.5 x 10^9/L, and platelets <25 x 10^9/L), which were considered related to antiviral medication. In case of grade 3 toxicity (including Hb 4.0-4.9 mmol/L, leucocytes 1.0-1.9 x 10^9/L, granulocytes 0.5-0.9 x 10^9/L, and platelets 25-49 x 10^9/L) the administration of the causative agent was discontinued until recovery to at least WHO-grade 2 toxicity level took place. Thereafter, the responsible medication dose was restored at 50% of the initial dose and further increased if laboratory values and clinical course allowed this. In case of recurrent WHO-grade 3 toxicity, further treatment within the trial was terminated. None of the patients received blood transfusions during the study period. During the treatment period, no adjuvant therapy with exogenous erythropoietin was available for any of the patients.
To be eligible for the current study, patients had to meet the following criteria: 1) antiviral therapy during at least 24 weeks completed: 2) presence of recorded Hb and Ht levels at each follow-up visit: 3) availability of baseline serum samples: 4) availability of serum samples at 12 and/or 24 weeks of treatment: 5) absence of cancer, HIV-infection or renal insufficiency. A total of 178 patients received antiviral therapy during at least 24 weeks according to the study protocol and Hb/Ht levels were known in all. Out of the 178 patients, 145 were eligible for analysis, because stored serum (-80°C) was available for measurement of serum EPO at baseline and week 12 and/or 24. In available serum samples at week 24 (98% of all patients), serum ribavirin concentrations were also determined. One hundred and thirty-eight patients were treated for a period of 1 year.

METHODS

Serum HCV RNA testing was performed quantitatively before inclusion (Cobas Amplicor HCV Monitor Test, version 2.0, detection limit 600 IU/mL, Roche Diagnostics, Almere, The Netherlands). Values greater than 800.000 IU/mL were considered high viral loads. Baseline liver biopsies were performed in 125 patients (86%) and assessed according to the METAVIR scoring system (20). Qualitative serum HCV RNA testing was performed at weeks 24, 52 and 104 (Cobas Amplicor HCV test, version 2.0; detection limit 50 IU/mL, Roche Diagnostics) by the central study laboratory. Genotyping was performed by sequence analysis of the 5' untranslated region of the HCV genome by the TrueGene Hepatitis C Assay (Visible Genetics, Suwanee GA, USA). Serum EPO levels were determined by chemiluminescent immunoassay (Immulite 2000, Siemens, Los Angeles CA, USA) in serum samples stored at -80°C at baseline and at week 12 and/or week 24. This assay was calibrated to the WHO EPO reference preparation (second IRP 67/343) and is linear for values less than 200 IU/L. The assay has an excellent inter-run precision with coefficients of variance around 3% at 14 IU/L and at 53 IU/L. The lower detection limit of the assay is 1 IU/L, and serum EPO levels between 3 IU/L and 20 IU/L are considered normal. Samples were diluted in case of EPO value greater than 200 IU/L. A total of 402 serum samples were analyzed. Serum concentrations of ribavirin were analyzed by a validated high-performance liquid chromatography assay with UV detection. In brief, to 125 µL of serum 1.5 mL acetonitrile was added. The sample was mixed on a vortex mixer for 1 min, followed by centrifugation at 11000 rpm for 5 min. Afterwards, the organic phase was evaporated at 37^{0}C under a gentle stream of nitrogen gas, and reconstituted in 125 µL of mobile phase (20 mmol/L phosphate buffer pH 3.23). The resulting solution (25uL) was run on a 4.6*150 mm Atlantis T3 5 µm reversed phase C18 column (autosampler column oven 35^{0}C, flow rate 1.0 mL/min) and ribavirin was detected by use of a UV detector (λ 235 nm) (UV2000 ThermoFisher Scientific, Breda, The Netherlands). Accuracy values were 104.6, 105.2, 101.5, 100.9 and 100.8% at 0.300, 1.00, 4.00, 10.0 and 12.0 mg/L, respectively. At the same concentrations, the precision values (within and between days, coefficient of variation) were below 6.0%. The calibration curve was linear over a concentration range of 0.3-12.0 mg/L. One hundred and six possible co-medications were tested on this assay; none interfered with ribavirin. The CIRA study conforms to the ethical guidelines of the 1975 declaration of Helsinki and was approved by the medical ethical committees of all participating hospitals. All patients provided written informed consent. The CIRA study was registered at ClinicalTrials.gov (identifier NCT00146016).

Statistics

Values are expressed as means ± SD for data with parametric distribution. In case of non-parametric distribution, medians and range are given. Changes in Hb, Ht and EPO during treatment were compared by repeated measures analysis of variance (ANOVA). Univariate and multivariate linear regression analyses were performed to identify risk factors for more extensive Hb decline after 24 weeks of treatment. Univariate and multivariate logistic regression were performed to identify risk factors for significant anemia (Hb <6.0 mmol/L). Only variables with *p*-value less than 0.2 in univariate analyses were included in the multivariate analyses with backward selection. A two-sided *p*-value less than 0.05 was considered statistically significant. Levels of $\log_{10}$EPO were plotted against the associated Ht levels from the same patients at the same time points. Linear regression was used to infer the relation between $\log_{10}$EPO and Ht in our study population. As in earlier studies (12-14), this relation was compared with the normal human response to anemia as defined by a historic population of iron deficiency patients. We compared our data with the data of Erslev (21;22). In this study the relation between EPO and Ht was obtained from 175 normal blood donors and patients with iron deficiency anemias, those with renal disease, rheumatoid arthritis and solid tumours having been excluded. In our study, Tobit regression was applied for statistical inference to allow for the truncation of EPO levels below 4 IU/L ($4 = 10^{0.60}$) (23) The regression lines obtained for either study population were compared by their 95% confidence intervals. These confidence intervals were obtained by bootstrapping (10 000 samples). In addition, a likelihood ratio test was used to test for the significance of the perceived differences (24). SPSS for Windows, version 15.0.1 (SPSS Inc., Chicago, Illinois, USA) and R, version 2.7.2 (R Foundation, Vienna, Austria) were used for statistical analysis.

RESULTS

Patient characteristics

Baseline characteristics of the 145 included patients are given in Table 1. Median actual ribavirin dose during the first 24 weeks of treatment was 15.1 mg/kg/day (range 7.0-21.3 mg/kg/day) with the following distribution: 18% less than 13.5 mg/kg/day, 29% 13.5-15 mg/kg/day and 53% greater than 15 mg/kg/day. Variability in ribavirin dose was mainly caused by different baseline body weight: ribavirin dose was 18.2 ± 1.5 mg/kg/day in case of weight less than 65 kg, 14.8 ± 1.7 mg/kg/day for weights between 65 kg and 90 kg: and 12.4 ± 1.3 mg/kg/day for body weight greater than 90 kg. Median week 24 serum ribavirin concentration was 2.8 mg/L (range 0.2-5.7 mg/L). Ribavirin concentrations were

less than 2 mg/L in 13% of patients, 2-3 mg/L in 46%, 3-4 mg in 29% and greater than 4 mg/L in 12% of patients. Median PEG-interferon alpha-2b dose after induction therapy was 1.5 µg/kg/week (range 0.75-1.55 µg/kg/week). During the first 24 weeks, dose reductions of ribavirin were applied in 14 patients (10%) because of anemia. Dose reductions for PEG-interferon alpha were applied in 30 patients (21%) because of leucopenia, granulocytopenia and/or thrombocytopenia. One hundred and nineteen patients (82%) reached a SVR, defined as undetectable serum HCV RNA 1 year after discontinuation of treatment. Nineteen patients (13%) relapsed, two patients (1%) were non-responders at week 24 and five patients (4%) were dropouts after week 24 for various reasons. In the group of patients who reached SVR, significantly less patients were infected with HCV genotype 1 or 4 (47% vs. 76%, p=0.01) compared with the non-SVR group. Between the groups with and without SVR, no significant differences in baseline Hb, Hb decrease and EPO increase during treatment, actual ribavirin dose, 24-week serum ribavirin concentrations or actual PEG-interferon dose were identified.

Table 1. Baseline characteristics in 145 treatment naive chronic hepatitis C patients

Characteristic	
Age, yrs	43.0 (18–77)
Male gender, n	101 (71%)
Genotype 1 or 4, n	74 (51%)
HCV viral load >800.000 IU/mL, n	103 (72%)
Fibrosis score F3 or F4, n[a]	36 (29%)
Body weight, kg	76.0 (47–129)
ALT, U/L	89 (18–468)
GGT, U/L	51 (7–331)
Bilirubin, µmol/L	11 ± 6 (2–42)
Albumin, g/L	42.1 ± 3.5
Prothrombin time, sec[b]	12.5 (8.5–29.4)
Hemoglobin, mmol/L	9.1 ± 0.8
Hematocrit	0.43 ± 0.03
Leucocytes, $x10^9$/L	7.0 (3.4–15.1)
Platelets, $x10^9$/L	213 ± 66
Creatinine, µmol/L	79 ± 15
Creatinine clearance, mL/min	113 ± 31

Data are represented as means ± SD or medians (range).

ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HCV, hepatitis C virus.

[a]: liver biopsy was performed in 125 patients.

[b]: Two patients used anticoagulants for atrial fibrillation and thrombosis of the brachial vein, respectively.

Levels of hemoglobin and hematocrit and incidence of anemia during treatment

Ninety-nine percent of the patients developed anemia during treatment. In 44 patients (30%) Hb levels less than 6.0 mmol/L were observed, and in 12 patients (8%) levels less than 5.0 mmol/L. Figure 1 shows the mean Hb levels during antiviral therapy. The Hb fell from an average of 9.1 ± 0.8 mmol/L at baseline to 7.1 ± 0.9 mmol/L at week 12 and 7.0 ± 0.9 mmol/L at week 24 (p<0.001). The Ht fell from 0.43 ± 0.03 to 0.35 ± 0.04 at both weeks 12 and 24 (p<0.001). Mean ΔHb was 2.1 ± 1.0 mmol/L, mean Hb nadir was 6.7 ± 1.0 mmol/L. Mean maximal decrease of Hb during antiviral treatment was 2.5 ± 1.0 mmol/L (range 0.3-5.5 mmol/L), which was 27% of the baseline level (range 4-56%). Hb nadir was reached after a median time of 16 weeks (range 4-24 weeks). During the second half of the treatment period there was no further decrease of Hb.

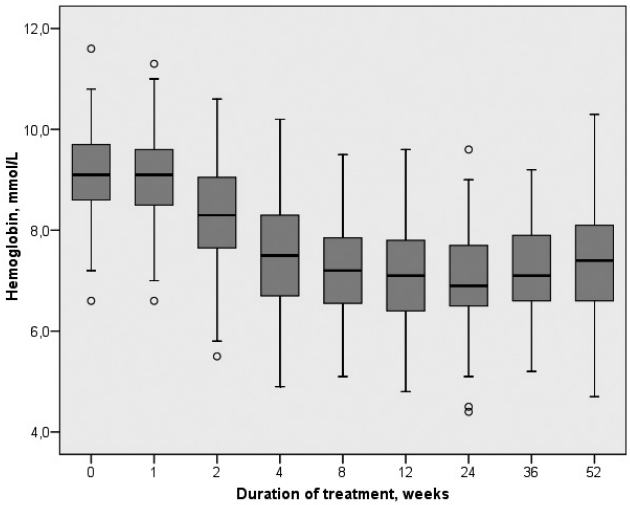

Figure 1: Hemoglobin levels in 145 CHC patients during antiviral treatment
Boxes represent the interquartile range (IQR): the boundaries of the box indicate the 25th and the 75th percentiles, the horizontal line in the middle of the box represents the median. The vertical lines from the ends of the box indicate the highest and lowest value, respectively observed within 1.5 IQR, circles represent outliers. Data for weeks 36 and 52 apply to the 138 patients who continued treatment during 52 weeks.

Factors associated with more pronounced decline of hemoglobin

In Table 2 the week 24 Hb decreases are given according to various baseline and on-treatment determinants. In univariate analysis (Table 3), there was a significant difference in ΔHb between male and female patients and between genotype 1 or 4 vs. genotype 2

Table 2. Week 24 hemoglobin decreases according to various baseline and on-treatment determinants

Determinants	Week-24 Hb decrease (mmol/L)	*p*-value
Age		
< 43 years	1.9 ± 0.8	0.06
≥ 43 years	2.3 ± 1.1	
Gender		
male	2.3 ± 1.0	0.09
female	1.7 ± 0.8	
Genotype		
1 or 4	2.3 ± 1.0	0.22
2 or 3	1.9 ± 0.9	
Fibrosis		
F0/1/2	2.0 ± 0.9	0.48
F3/4	2.1 ± 0.9	
Body weight		
< 76 kg	1.8 ± 0.9	0.58
≥ 76 kg	2.3 ± 1.0	
Creatinine clearance		
<108 mL/min	2.4 ± 1.1	**0.01**
≥ 108 mL/min	1.9 ± 0.8	
Baseline hemoglobin		
< 9.1 mmol/L	1.7 ± 0.9	**< 0.01**
≥ 9.1 mmol/L	2.4 ± 1.0	
Week-2 hemoglobin decrease		
< 0.8 mmol/L	1.6 ± 0.9	**< 0.01**
≥ 0.8 mmol/L	2.6 ± 0.8	
Week-24 ribavirin concentration		
< 2.8 mg/L	1.8 ± 0.8	**< 0.01**
≥ 2.8 mg/L	2.4 ± 1.0	

Median values were used as cutoff in case of continuous variables.
Data indicate mean ± SD.
Bold style is used for those *p*-values that reach significance.

or 3 patients. Age, Hb levels and creatinine clearance at baseline, decrease of Hb after 2 weeks and 24-week serum ribavirin concentrations were also significantly associated with ΔHb. In multivariate linear regression analysis, only older age, lower creatinine clearance, higher baseline Hb, more pronounced Hb decrease after 2 weeks and higher 24-week serum ribavirin concentrations could be identified as independent risk factors for increase in ΔHb at week 24 (Table 4). Multivariate logistic regression analysis with significant anemia (defined as Hb level <6 mmol/L during treatment) as outcome revealed essentially the same results with older age, lower baseline Hb, more pronounced week 2 Hb decrease and higher ribavirin levels as independent predictors of significant anemia (data not shown).

Serum ribavirin levels after 24 weeks exhibited no significant correlation with baseline Hb, body weight or creatinine clearance. However, a significant correlation between serum ribavirin levels and actual ribavirin dose was found (r=0.19, p=0.02). Actual ribavirin dose was not identified as an independent predictor of ΔHb.

Table 3. Univariate regression analysis of determinants associated with a more pronounced increase in Δ Hb

Determinant	Coefficient[a]	95% CI	*p*-value
Age, years	0.03	0.02–0.05	**< 0.01**
Gender, male vs. female	0.54	0.19–0.89	**< 0.01**
Genotype 1 or 4, yes vs. no	0.33	0.003–0.65	**0.048**
Fibrosis F3/F4, yes vs. no	0.11	-0.26–0.48	0.56
Body weight, baseline, kg	0.01	0.00–0.02	**0.05**
Creatinine clearance, baseline, mL/min	-0.006	-0.011–-0.001	**0.02**
Platelets, baseline, x10^9/L	-0.001	-0.003–0.001	0.43
Hemoglobin, baseline, mmol/L	0.56	0.38–0.74	**< 0.01**
Hemoglobin decrease, week 2, mmol/L	0.76	0.60–0.93	**< 0.01**
Starting ribavirin dose, mg/kg/day	-0.04	-0.11–0.03	0.28
Actual ribavirin dose, mg/kg/day during 24 weeks	-0.05	-0.12–0.02	0.15
Week-24 serum ribavirin concentration, mg/L	0.43	0.27–0.59	**< 0.01**
Actual PEG-interferon dose, µg/kg/day during 24 weeks	0.04	-1.10–1.18	0.95

Hb, hemoglobin

[a]Regression coefficients indicate the increase in ΔHb in mmol/L in case of an increase with one unit in one of the determinants.

Bold style is used for those p-values that reach significance.

Table 4. Independent predictors of a more pronounced Δ Hb in multivariate regression analysis

Determinant	Coefficient[a]	95% CI	*p*-value
Age, years	0.02	0.004–0.03	**0.01**
Creatinine clearance, baseline, mL/min	-0.004	-0.08–-0.001	**0.02**
Hemoglobin, baseline, mmol/L	0.46	0.33–0.59	**< 0.001**
Week-2 hemoglobin decrease, mmol/L	0.51	0.38–0.65	**< 0.001**
Week-24 serum ribavirin concentration, mg/L	0.34	0.22–0.45	**< 0.001**

Hb, hemoglobin

[a]Regression coefficients indicate the increase in ΔHb in mmol/L in case of an increase with one unit in one of the determinants.

Bold style is used for those *p*-values that reach significance.

Erythropoietin levels

In 112 patients (77%) samples at baseline, weeks 12 and 24 were available for measurement of EPO. In 28 patients (20%) only baseline and week 24 samples were available, and in 5 patients (3%) only baseline and week 12 samples were available.
Levels of EPO increased from a median of 12 IU/L (range 4-63 IU/L) at baseline to 41 IU/L (range 12-683 IU/L) after 12 weeks of antiviral therapy and further to 43 IU/L (range 7-3238 IU/L) at week 24 (p<0.001). In Fig. 2, the EPO levels at the three different time points are shown. At baseline, 89% of the EPO levels were considered normal, compared with 14% and 12% after 12 and 24 weeks of treatment. Serum EPO levels at baseline, weeks 12 and 24 negatively correlated with Hb (r =-0.186, p=0.03, r=-0.538, p<0.001 and r=-0.296, p<0.001) and Ht (r=-0.195, p=0.02, r=-0.531, p<0.001 and r=-0.312, p<0.001, Fig. 3). ΔsEPO (defined as the difference between serum EPO at baseline and after 24 weeks of treatment) did not differ significantly between males and females, between patients with genotype 1 or 4 and 2 or 3 infection, between patients with high and low viral load or between patients with or without severe fibrosis or cirrhosis. Furthermore, no significant correlations between ΔsEPO and baseline Hb, actual ribavirin or PEG-interferon alpha dose, serum ribavirin levels or weight were found.

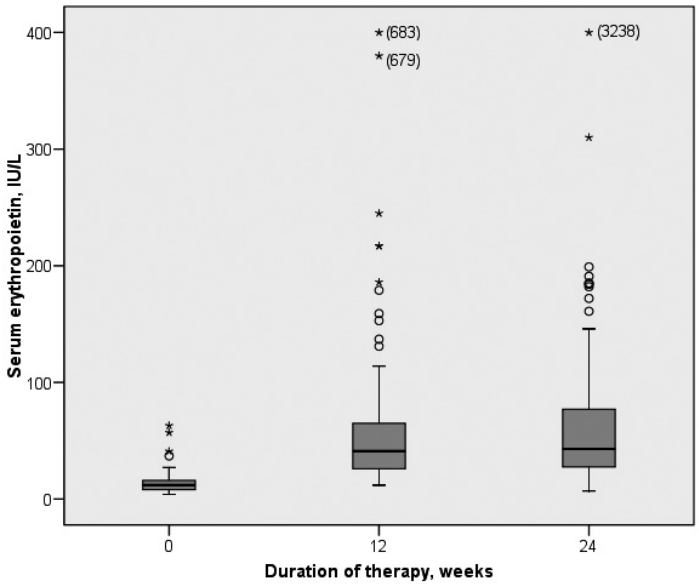

Figure 2: Erythropoietin levels at baseline and after 12 and 24 weeks of antiviral therapy. Circles represent outliers (>1.5 interquartile rang (IQR)), asterisks represent extreme values (>3 IQR). Values greater than 400 IU/L are given between brackets.

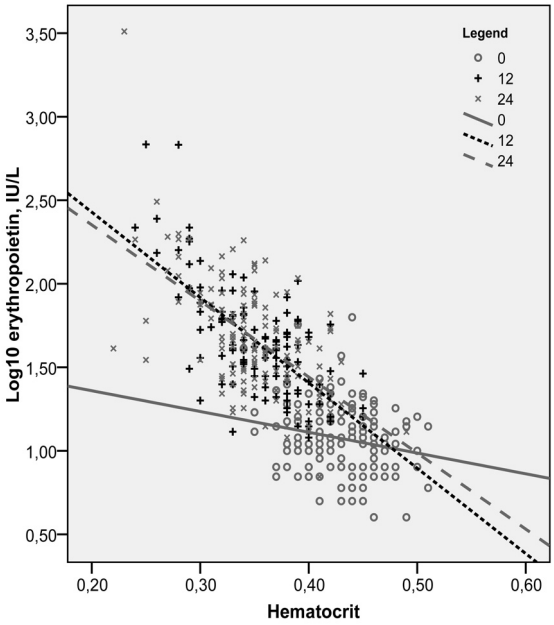

Figure 3: Correlation between hematocrit and erythropoietin levels at baseline, weeks 12 and 24.
Hematocrit at baseline, weeks 12 and 24 plotted against associated $\log_{10}$ erythropoietin levels, including regression lines.

Figure 4 shows the $\log_{10}$EPO levels plotted against the corresponding Ht levels. The regression equation for the erythropoietic response to anemia in CHC patients during antiviral treatment is given by $\log_{10}EPO = 3.55 - 5.52 * Ht$. The figure also shows the regression lines for our dataset and those for the dataset of Erslev, which represents the normal human response to anemia (21;22). There is significant overlap of the individual data; however, the regression estimates for either dataset clearly exclude each other. A likelihood ratio test confirmed this observation with a *p*-value of 1×10^{-11}.

DISCUSSION

Our data clearly indicate that although there is an increase in EPO levels in CHC patients during antiviral treatment, this increase is significantly less than the normal human response to anemia. Our finding that endogenous EPO production is suboptimal during antiviral treatment provides a theoretical basis for the use of EPO under these circumstances. In the past, three relatively small studies on this subject have been published, with opposite results. As in this study, all these earlier reports compared their data with the data of a historic control population of iron deficiency anemia patients. Durante Mangoni *et al.* (12)

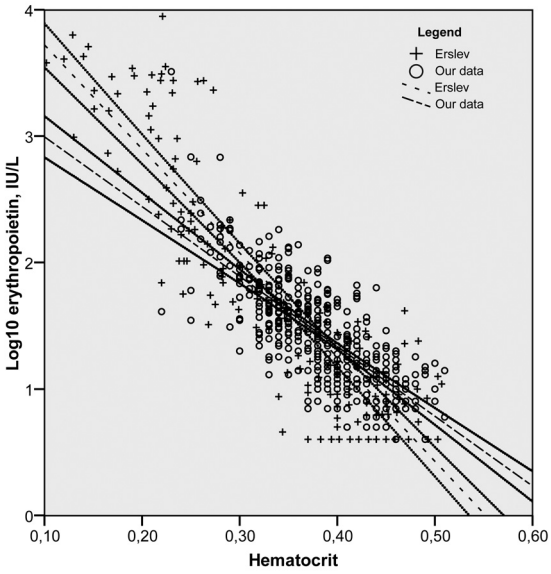

Figure 4: Erythropoietic response in 145 CHC patients treated with ribavirin and PEG-interferon alpha-2b compared with the normal human response to anemia. Each circle represents one serum sample. Interrupted lines indicate the regression equations, given by $\log_{10}$EPO=3.55–5.52Ht for our study population and $\log_{10}$EPO=4.609–8.7Ht for the normal human response to anemia, as described by Erslev. Solid black lines mark the 95% confidence intervals for these equations.

found a normal response, based on data of 18 HCV-patients prospectively studied during 12 weeks therapy. Trivedi *et al.* (14) found an impaired response in 43 patients during 4-week therapy. Similarly, Balan *et al.* (13) found an impaired response in 97 patients during 8-week treatment. These studies were relatively small with limited follow-up and used suboptimal statistical analysis. This study with its 145 subjects and 24 weeks follow-up is the largest study carried out on this topic so far. None of our patients were treated with EPO during the study, as is current practice in case of anemia. In addition, they did not receive blood transfusions which could interfere with the EPO determinations. We compared our data with an earlier study on EPO response in blood donors and patients with iron deficiency anemia (21;22), with the aid of sophisticated statistical analyses. The difference in the regression equations for the Ht and EPO levels found between normal and hepatitis C patients was confirmed by a highly significant likelihood ratio test outcome. The underlying mechanisms for suboptimal endogenous erythropoietin production are supposed to be the inhibitory effects of interferon, and other cytokines on EPO producing cells (6;7). Experimental data have shown subnormal increases in erythropoietin production, under hypoxic conditions, after interferon injection in a murine model (14;25).

The second aim of our study was to identify patient-related and treatment-related risk factors for a greater decrease in Hb during antiviral therapy. By multivariate analysis, we found

that older age, lower baseline creatinine clearance, higher baseline Hb, more pronounced Hb decrease after 2 weeks and higher serum ribavirin concentrations at 24 weeks were independent risk factors for more pronounced anemia. Higher baseline Hb is a well-known risk factor for larger Hb decline (26;27). One can imagine that this is because of the fact that a certain percentage of circulating erythrocytes is removed from the circulation as a result of ribavirin-induced hemolytic anemia. Older age and lower creatinine clearance are also well-known risk factors for anemia during antiviral therapy (26;27). Creatinine clearance is probably an independent predictor for anemia because of its influence on serum ribavirin levels. However, only 27% of variability in serum ribavirin levels could be explained by differences in body weight, sex, age and serum creatinine (the variables used in the Cockcroft-Gault equation) (28). Age itself was an independent predictor of anemia as well. Older patients are probably more prone to exhibit a larger Hb decline as a result of their impaired compensation mechanisms. More pronounced Hb decrease after 2 weeks of treatment has earlier been suggested as a predictive factor for more severe anemia (26;29). This finding may have some clinical relevance, as Hb levels after 2 weeks may predict a need for dose reductions and/or treatment with erythropoietic growth factors during the subsequent course of therapy. We also found high serum ribavirin concentrations after 24 weeks to be an independent risk factor for extent of anemia, which was not the case for actual ribavirin dose (even if corrected for body weight). This is in line with recent literature (30;31). Interestingly, ribavirin doses could explain little of the variability in serum ribavirin concentrations (r^2=0.04). We speculate that other determinants, such as factors influencing absorption, transport and intracellular metabolism of ribavirin could explain the weak correlation between serum ribavirin concentrations and actual ribavirin dose and contribute to the high interpatient variability in serum ribavirin concentrations.

A possible limitation of the current and all earlier studies is the comparison of EPO data in hepatitis C patients during antiviral therapy with those from an earlier study in iron deficiency patients. Although the ideal disease-control group would have been a group of contemporaneous hepatitis C patients without antiviral therapy with similar extent of iron deficiency anemia, this is not a feasible option. The approach in this and earlier studies (12-14) is therefore a reasonable alternative. In addition, we measured serum ribavirin concentrations after 24 weeks because this time point reflects a steady state level with high reliability. However, according to earlier literature, steady state concentrations may in most cases already have been reached after 8 or even 4 weeks (32).

In conclusion, our study identifies older age, lower baseline creatinine clearance, higher baseline Hb, more pronounced Hb decrease after 2 weeks and higher serum ribavirin concentrations at 24 weeks as independent risk factors for more pronounced anemia during antiviral therapy for hepatitis C. Our data also indicate that endogenous EPO production is suboptimal in CHC patients during ribavirin and PEG-interferon alpha therapy. These

findings provide a sound theoretical basis for the use of hematologic growth factors such as EPO-α during antiviral therapy.

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CHAPTER 6

Influence of alpha-1 antitrypsin heterozygosity on treatment efficacy of HCV combination therapy

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ABSTRACT

Background: The role of heterozygosity for alpha-1 antitrypsin (A1AT) alleles in patients with chronic hepatitis C (CHC) is unclear. There is limited evidence to suggest that there is an increased prevalence of heterozygous A1AT carriers in CHC, but it is unclear how this affects treatment success. *Aim:* To investigate the (1) prevalence of A1AT heterozygosity among two CHC cohorts and (2) its effect on treatment outcome. *Methods:* We performed a retrospective cohort study using two different cohorts. Cohort 1 consisted of 678 German CHC patients, 507 of them were treated for CHC with standard therapy. Cohort 2 consisted of 370 Dutch CHC patients of which 252 CHC patients were part of a clinical trial (treatment with amantadine or placebo, in combination with PEG-interferon alpha-2b and ribavirin) whereas 37 CHC patients received standard therapy. We analyzed A1AT status using direct sequencing of the A1AT gene (cohort 1) or isoelectric focusing of serum (cohort 2). In addition, we measured A1AT serum levels (cohort 2). *Results:* In total we included 1048 CHC patients; 986 (94%) were wildtype (protease inhibitor (Pi) MM), whereas 61 (6%) were heterozygous for a mutant A1AT allele (41 Pi MS, 20 Pi MZ). Mean A1AT serum levels (370 patients) were lower in A1AT heterozygous patients (1.68 vs. 1.36 g/L), (p<0.05) compared with wildtypes. Sustained viral response (SVR) after treatment was equal between the wildtypes and heterozygotes (54% vs. 56%). *Conclusion:* We found a heterozygosity rate of 0.06, in line with healthy controls in other studies. Serum A1AT levels from A1AT heterozygous CHC patients are significantly lower compared with wildtype patients, although they do not discriminate on an individual level. Finally, SVR in A1AT wildtypes was not different from SVR in A1AT heterozygotes.

INTRODUCTION

Alpha-1 antitrypsin (A1AT) deficiency is a hereditary disease that is characterized by the hepatic synthesis of an abnormal A1AT protein that cannot be released into the plasma completely. Accumulation of mutant A1AT protein in hepatocytes causes tissue damage and eventually ensues in liver and pulmonary disease. The A1AT protein is encoded by the protease inhibitor (Pi) locus located on the long arm of chromosome 14q31-32.3. This locus is highly polymorphic, and so far more than 100 different A1AT isotypes have been identified. They can be detected by iso-electrophoresis of the protein or by mutational analysis of the allele (1). The wild type allele (Pi MM) results in a functionally normal protein with normal serum A1AT levels. In Western Europe there are two frequent variants p.G342K (denoted as Z allele) and the p.G264V (commonly referred to as the S allele) (2). For example, in the general Dutch population, the allele frequency for these A1AT variants is estimated to be 0.02 (Z allele) and 0.04 (S allele) (3-5). The Z allele variant results in polymers that are retained in hepatocytes, whereas the S variant of the A1AT molecule has less deleterious effect on the protein (6). Homozygosity for A1AT deficiency is associated with liver and pulmonary disease, but the picture is less clear for A1AT heterozygosity (7-11).

Hepatitis C virus (HCV) is mainly transmitted through contact with blood and blood products. More than 80% of all HCV-infected patients will develop chronic hepatitis, and in 20% this will lead to liver cirrhosis (12). Treatment of chronic hepatitis C (CHC) aims to eliminate the virus and current standard treatment regimen consist of PEG-interferon alpha and ribavirin (13). Treatment success greatly depends on viral characteristics such as HCV genotype and viral load, but also host-related factors play a role. For example, the presence of the ACC IL-10 promoter diplotype increases the likelihood of reaching a sustained viral response (SVR) by more than three-fold (14).
If we focus on the role of A1AT in CHC, studies have merely investigated the prevalence of A1AT heterozygosity in HCV-induced liver disease. Studies in pre-transplantation CHC patients showed presence of Pi MZ in 10-13% of the patients, compared with 2.8% in controls, suggesting that A1AT heterozygosity influenced the development and/or course of the liver disease in CHC (10;11). A case-control study in Egyptian CHC patients found a relatively high frequency of Pi MS (15). In contrast, other studies failed to conform this (16-18).
In-vitro studies showed that A1AT plays a central role in inflammation, both as a regulator of proteinase activity and as a signalling molecule for the expression of pro-inflammatory molecules (19). Furthermore, A1AT has a role in viral clearance, as it inhibits human immunodeficiency virus (HIV) type 1 replication (20). We hypothesize that A1AT heterozygosity puts the host at a higher risk for infection with HCV and decreases viral elimination after treatment because of the decreased anti-inflammatory response. As a consequence, A1AT genotype status might be associated with the infection rate and treatment outcome.

Therefore, the aim of our study was (1) to investigate the prevalence of A1AT heterozygosity in a large cohort of Dutch and German CHC patients and to assess (2) its consequence on treatment outcome.

METHODS AND MATERIALS

Patients

We selected two different retrospective cohorts of patients. Cohort 1 consisted of 678 German CHC patients; 507 of them were treated for CHC and completed therapy. Cohort 2 consisted of 370 Dutch CHC patients; 289 patients of cohort 2 were treated and completed therapy. Patients were included regardless of their degree of liver fibrosis. We did exclude patients with a hepatitis B or HIV co-infection. Our study was approved by the medical ethical committee.

Chronic hepatitis C Treatment

Patients from cohort 1 were treated with interferon monotherapy, a combination of interferon and ribavirin or with PEG-interferon in combination with ribavirin. A total of 252 patients of cohort 2 were part of a nation-wide, double blind, placebo-controlled, randomized multicenter study (CIRA-study) (21). Patients were treated with amantadine or placebo, in all cases combined with weight-based ribavirin and high-dose interferon induction therapy followed by PEG-interferon alpha-2b. The remaining 37 patients (cohort 2) were treated with standard combination therapy. Treatment was given for 24-52 weeks depending on genotype. SVR was defined as a negative qualitative HCV RNA test at 1 year after the end of treatment.

Assays

A1AT status was analyzed in all patients. We performed mutational analysis (cohort 1) and isoelectric focusing and nephelometric measurement of A1AT serum levels (cohort 2).

Mutational analysis (Cohort 1)
DNA was extracted from peripheral blood leukocytes. Primer sequences used for polymerase chain reaction (PCR) were as follows: exon 5 (PiS), 5′-GATGAGGGGAAAC-TACAGCACCTCG-3′ and 5′-GGGCCTCAGTCCCAACATGG-3′ and for exon 7 (PiZ), 5′-GCATAAGGCTGTGCTGACCATCGTC-3′ and 5′-AGGGTTTGTTGAACTCGACCTC-3′. An automated thermal cycler (Biometra, Göttingen, Germany) was used. A1AT heterozygos-

ity was determined using restriction fragment length polymorphism (RFLP). Amplification of the desired products was confirmed by direct DNA sequencing. DNA sequences were analyzed by sequencing both strands. The reaction products were loaded into an ABI 373A fluorescence sequencer (Applied Biosystems, Weiterstadt, Germany) (22).

Isoelectric focusing (Cohort 2)

Isoelectric focusing was performed in serum by iso-electric focusing (Phast-system, GE Healthcare, Munchen, Germany) and subsequent protein silver staining. Patterns were compared with MM, MZ, MS, SZ and ZZ controls. MS and MZ designated patterns were subsequently validated by an isoelectric focusing with an immunofixation method in an independent laboratory (23;24).

Nephelometric plasma measurement (Cohort 2)

Determination of the serum A1AT level was performed by nephelometry. The A1AT reagent was used from the Immage Immunochemistry system (Immage Beckmann-Coulter, Woerden, The Netherlands), and a calibrator with an assigned A1AT value and a human quality control sample (Biorad, Liquichek, Hercules, CA, USA) were used (25).

Hepatitis C virus testing

HCV RNA testing was performed qualitatively (Cobas amplicor HCV Monitor Test, version 2.0, detection limit 600 IU/ml, Roche Diagnostics, Almere, The Netherlands) and quantitatively at weeks 24, 52 and 104 (Cobas Amplicor HCV test, version 2.0; detection limit 50 IU/ml, Roche Diagnostics). Genotyping was performed by sequence analysis of the 5' untranslated region of the HCV genome by the Visible Genetics TrueGene Hepatitis C Assay.

Statistical Analyses

The HCV characteristics (genotype) of the wildtype patients and heterozygous patients were analyzed using Pearson χ^2 test and Fisher's exact test where appropriate. The difference in age at the start of treatment was analyzed using Student's *t*-test. Differences between serum levels of the wild type patients (Pi MM) and heterozygous patients (Pi MS, Pi MZ and Pi SZ) were analyzed using Student's *t*-test.

We used Pearson $\chi 2$ test for analysis of the differences in SVR between wildtypes and heterozygous patients and for analysis of the degree of fibrosis in wildtypes and heterozygotes. All statistical analyses were performed using GraphPad Prism 4 (Version 4.02, GraphPad Software Inc., San Diego, California, USA). A two-sided *p*-value less than 0.05 was considered statistically significant.

RESULTS

Genotyping

Genotyping was performed in all 1048 patients. On account of technical difficulties, isoelectric focussing failed in a single patient, resulting in 1047 completely analyzed patients. We found 986 Pi MM (94%), 41 Pi MS (4%) and 20 Pi MZ (2%) patients (Fig. 1). Genotype results were within the Hardy-Weinberg equilibrium.

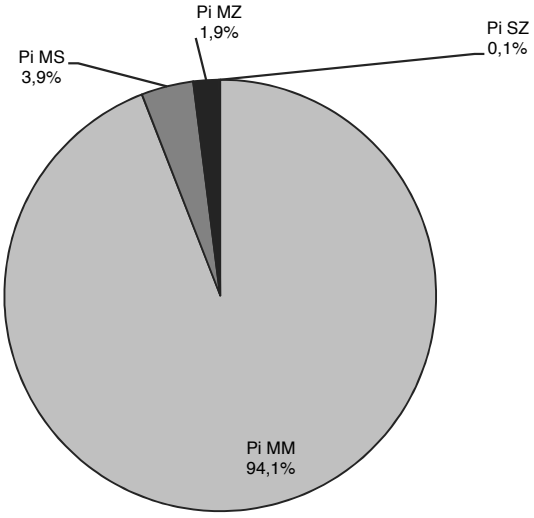

Figure 1: Prevalence of different alpha-1 antitrypsin carrier rates in 1047 patients with hepatitis C virus. Pi, protease inhibitor.

Serum levels

A1AT levels were measured in serum from 370 CHC patients from cohort 2 drawn before treatment. A1AT serum levels were higher in Pi MM carriers (340 patients, mean 1.69 g/L, 95% confidence interval (CI): 1.66-1.71 g/L) compared with Pi MS carriers (22 patients, mean 1.43 g/L, 95% CI 1.34-1.53 g/L) or Pi MZ carriers (seven patients, mean 1.14 g/L, 95% CI 0.80-1.49 g/L) respectively. The differences between the wildtypes and heterozygous patients were statistically significant ($p<0.01$); this also holds for the differences between the heterozygous Pi MS and Pi MZ patients ($p<0.01$) (Fig 2). We detected A1AT serum levels above 1.0 g/L in all heterozygous Pi MS patients and in five of seven (71%) of the heterozygous Pi MZ patients.

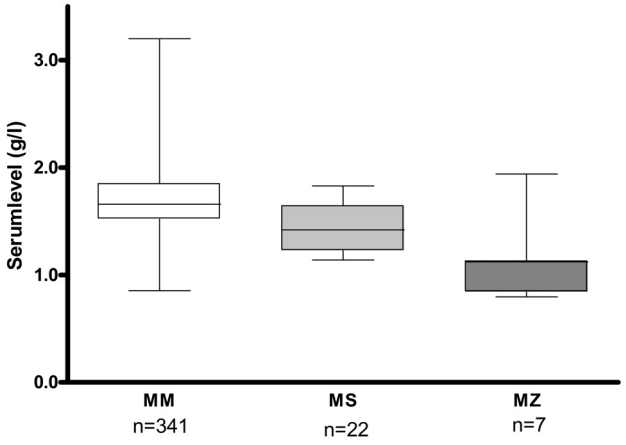

Figure 2: Alpha-1 antitrypsin (A1AT) serum levels in 370 patients with chronic hepatitis C arranged to the different A1AT phenotypes ($p<0.05$ for all comparisons). Pi, protease inhibitor.

Degree of fibrosis

We assessed the stage of fibrosis using the Metavir score in liver biopsies obtained from 409 patients. There was no statistically significant difference in the degree of fibrosis between wildtypes and heterozygous patients (Table 1).

Table 1. Degree of fibrosis in the different A1AT groups (*N* = 409)

Metavir score *	PI MM	PI MZ and Pi MS
	N=383 (%)	N=26 (%)
F0 - F2	269 (70)	19 (73)
F3 - F4	114 (30)	7 (27)

A1AT, alpha-1 antitrypsin; Pi, protease inhibitor.
*F0 = no fibrosis; F1 = minimal fibrosis; F2 = periportal fibrosis; F3 = bridging fibrosis; F4 = cirrhosis or advanced fibrosis

Hepatitis C virus characteristics and treatment response

A total of 796 patients completed anti-HCV treatment. A1AT carrier status and qualitative HCV RNA 1 year after the end of treatment were not available for 10 patients (one cohort 1; nine cohort 2). These patients were excluded from further analysis; and data from 786 CHC patients remained available for analysis. The distribution of the HCV genotypes at baseline did not differ among the groups with different A1AT genotypes (Table 2).

Table 2. Distribution of the HCV genotypes and age in the different A1AT states (*N*=795)

	Pi MM	Pi MZ	Pi MS	Pi SZ	*p* value *
	N=745 (%)	N=17 (%)	N=32 (%)	N=1 (%)	
HCV genotype					
1	450 (60,4)	10 (58,8)	20 (62,5)	0 (0)	
2	56 (7,5)	1 (5,9)	4 (12,5)	0 (0)	
3	170 (22,8)	5 (29,4)	4 (12,5)	0 (0)	0.89
4	34 (4,6)	1 (5,9)	2 (6,3)	0 (0)	
6	1 (0,1)	0 (0)	0 (0)	0 (0)	
unknown	34 (0,46)	0 (0)	2 (6,3)	1 (100)	
Mean age (yrs)	46,6	47,9	45,5	59	0.93

A1AT, alpha-1 antitrypsin; HCV, hepatitis C virus; Pi, protease inhibitor.
* Pi MM vs Pi MZ, Pi MS and Pi SZ

SVR was reached in 395 of 736 (54%) wildtype patients, in eight of 17 (47%) A1AT Pi MZ patients and in 20 of 32 (62%) of A1AT Pi MS patients. One Pi SZ patients did not achieve SVR. These differences were not statistically significant (Fig 3). The addition of amantadine to the combination of interferon and ribavirin in patients from cohort 2 did not affect treatment response (26).

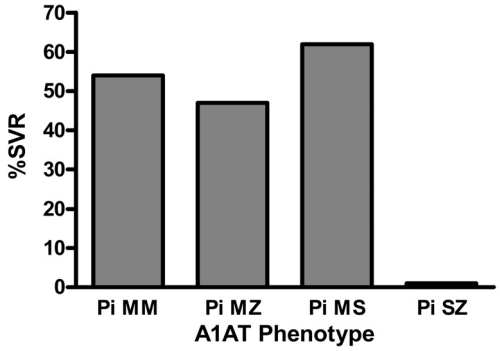

Figure 3: Hepatitis C virus treatment response in 736 alpha-1 antitrypsin (A1AT) wildtype (Protease inhibitor (Pi) MM) patients, 17 A1AT heterozygous Pi MZ patients, 32 A1AT heterozygous Pi MS patients and one Pi SZ patient. SVR, sustained virologic response.

DISCUSSION

This study shows that the efficacy of anti-HCV combination treatment with (PEG-)interferon and ribavirin is independent of carrier state of A1AT, as heterozygous (Pi MZ / Pi MS / Pi SZ) HCV patients had a similar treatment response compared with wildtype patients (Pi MM). In addition, we did not detect an association between A1AT heterozygosity and the

degree of liver fibrosis. We found a high rate of SVR in our study population, which can be explained by the fact that we included only patients who finished treatment; we did not include patients who stopped treatment for any reason or who were lost to follow-up. There are no previous studies documenting HCV treatment response in A1AT heterozygosity. Most of the studies in this field have focused on apparent differences in A1AT allele frequency between those with and without a certain liver disease (10;11). The common denominator is that a higher prevalence of heterozygotes is found in different causes of liver disease; this is particularly true for HCV and alcoholic liver disease in pre-transplantation patients (10;11). We could not confirm the finding of higher A1AT heterozygosity rates in HCV patients, and our data indicate that the A1AT carrier distribution in HCV is in line with the prevalence of control populations in The Netherlands and Germany (3-5). Our results are at odds with an Egyptian study that found higher prevalence of Pi MS in CHC cirrhotic patients compared with normal controls (15). This particular A1AT allele is rare in Germany and The Netherlands (27).

It is tempting to speculate why other case control studies in this field documented differences in A1AT carrier rate between patient and controls. We surmise that there are three possible explanations. First, the variation might depend on real population differences. In contrast, it cannot be excluded that bias in selecting controls and patients might have affected the results. In addition, the low prevalence of patients with heterozygote genotypes might have introduced a type II error giving rise to spurious results.

Serum A1AT levels were statistically significantly lower in heterozygous carriers compared with A1AT wildtype patients. There was no cutoff point that discriminated between heterozygotes and wildtypes. In some laboratories in The Netherlands, genotyping is only performed when the serum level is below 1.0 g/L. This strategy will miss obvious cases; in our data set, all Pi MS heterozygotes and 71% of the Pi MZ heterozygotes. Previous studies also indicated the variability of A1AT serum levels in heterozygous carriers (28;29). Some case reports document A1AT heterozygotes with concomitant HCV (30) or alcohol induced liver disease (31) who had consistent normal A1AT serum levels but A1AT globules in their liver biopsy specimen. It is possible that the liver inflammation is a driver for A1AT production, hence giving rise to "falsely" elevated A1AT serum levels.

Our study was adequately powered to detect differences in treatment outcome and to calculate the prevalence of A1AT heterozygosity in CHC patients in The Netherlands and Germany. Nevertheless, our study comes with several limitations. The lack of a control population does not allow a firm estimate of the allele frequency in relation to the background population.

Although A1AT does not a play a role in the anti-HCV treatment response, it is possible that there are other important genetic factors. For example, hereditary hemochromatosis (HFE) is a common genetic liver disease that appears to be overrepresented in CHC patients

(15). Currently, it is not known whether possession of the mutant HFE allele affects treatment response.
In conclusion, we found no evidence for an increased prevalence of A1AT heterozygosity in CHC patients. Serum A1AT levels from A1AT heterozygous CHC patients are significantly lower compared with wildtype patients, however they do not discriminate on an individual level. Finally, we could not confirm our hypothesis that CHC patients with A1AT heterozygosity deficiency had worse outcomes on anti-HCV combination therapy.

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CHAPTER 7

Extracorporeal Whole Body Hyperthermia in patients with chronic hepatitis C virus infection; Results of a pilot study

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ABSTRACT

Background: Systemic hyperthermia can reduce human immunodeficiency virus (HIV) RNA concentrations in humans. In addition, a sustained virologic response (SVR) was observed in one hepatitis C co-infected case. We studied the efficacy, safety and feasibility of whole body hyperthermia as experimental therapy for chronic hepatitis C (CHC) patients.
Methods: Thirteen patients with CHC infection, genotype 1, underwent extracorporeal whole body hyperthermia at 41.8^{0}C induced by a veno-venous extracorporeal heater/cooler device. The endpoints with respect to efficacy were a 90% viral load reduction and an undetectable HCV-RNA at the end of study. The primary and secondary safety endpoints were the absence of WHO grade 4 and 3 adverse effects. *Results:* Eight patients reached the primary endpoint of viral reduction of at least 90% at the end of treatment. None of these decreases were maintained and none of the patients achieved a sustained virologic response at 24 weeks. Four hundred and one adverse events occurred, 42 of them serious. Four patients developed irreversible neuropathy and one patient developed acute liver failure that resolved spontaneously. *Conclusions:* Considering the non-sustained decline in HCV RNA, the high incidence of (serious) adverse events and the potential for liver damage, hyperthermia has no therapeutic potential in the treatment of chronic hepatitis C infection.

INTRODUCTION

The use of systemic hyperthermia has been studied extensively in the field of oncology. Its therapeutic potential is based on the observation that cancer cells are more susceptible to heat than normal cells (1-3). Hyperthermia has been used for a variety of malignancies, including sarcomas, melanomas, gastrointestinal tumors, lung tumors and breast tumors. Although variably effective, it is considered a relatively safe method (4-7). Experience with hyperthermia has also been obtained in treatment of viral diseases. In a study in patients with Acquired Immunodeficiency Syndrome (AIDS), whole body hyperthermia was able to induce an increase in Human Immunodeficiency Virus (HIV) RNA concentration initially, which was followed by a decrease to undetectable levels in 2 of 6 patients (8).
In 1999, a phase II study was performed testing hyperthermia in HIV/AIDS patients who had failed prior drug therapy. One of the patients in this study was co-infected with the hepatitis C virus (HCV) and had not responded to previous interferon alpha therapy. Treatment with whole body hyperthermia resulted in a steady decrease in hepatitis C viral load which became in fact undetectable by week 12 (<0.2 Meq/L (31.746 IU/ml)) (9). This observation led to the hypothesis that hyperthermia may have therapeutic potential in hepatitis C infected patients.
Chronic hepatitis C (CHC) infection has become the predominant cause of chronic liver disease in the world and accounts for significant morbidity and mortality (10). Current treatment, consisting of a combination of pegylated (PEG-) interferon and ribavirin, results in a sustained virologic response (SVR) in 50-60% of naive patients (11;12). Retreatment of previous non-responders to interferon with a combination of PEG-interferon alpha and ribavirin yields a response rate of only 12-30% (13;14). These results are clearly unsatisfactory. Therefore new therapeutic strategies for non-responders are being developed (15).
In search of a new therapy, we assessed the efficacy, safety and feasibility of whole body hyperthermia as treatment for patients with CHC infection, genotype 1, who were non-responders to previous antiviral treatment.

PATIENTS AND METHODS

Patients

This study was approved by the medical ethical committee of the UMC Utrecht and it was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all patients. Adult patients with CHC infection (genotype 1) and a previous non-response to antiviral treatment with standard interferon alpha with or without ribavirin were eligible for this study. All patients were

tested positive for serum HCV RNA by quantitative polymerase chain reaction (PCR) (Roche Amplicor HCV monitor Kit v2.0, detection level of 600 IU/ml) and tested positive for HCV genotype 1 (Innolipa HCV II, Innogenetics, Ghent, Belgium). All patients had abnormal serum alanine (ALT) and/or aspartate aminotransferase (AST) levels during 6 months prior to inclusion and a Karnofsky performance (16) score of at least 70%. Exclusion criteria for hyperthermia included the presence of cardiac, pulmonary or neurological disease. Patients suffering from decompensated liver cirrhosis, anemia, leucocytopenia, granulocytopenia, thrombocytopenia, renal disease, HIV co-infection, malignancies, other major liver disease, psychiatric disorders, hemophilia, current use of non-steroidal anti-inflammatory drugs and any type of uncontrolled medication abuse, drug or alcohol abuse in the previous 6 months were excluded from the study.

Study protocol

Patients were subjected to a single session of extracorporeal whole body hyperthermia (EWBH) under general anesthesia using a veno-venous extracorporeal circuit that included a heater/cooler device (TEMET®, First Circle Medical, Inc. Minneapolis, MN, USA). Temperature monitoring sites included the rectum, esophagus, nasopharynx, pulmonary artery and jugular venous bulb. Target temperature was defined by either an esophageal or rectal temperature of $\geq 41.6^{0}$C. After reaching the target temperature, the core body temperature was maintained at 41.8 ± 0.2^{0}C for 120 min. Patients were then actively cooled to 39^{0}C after which the extracorporeal bypass was discontinued.
Hemodynamic monitoring during the procedure included the use of a pulmonary artery catheter. In addition, continuous 2-channel electroencephalography and transcranial Doppler ultrasonography of both middle cerebral arteries were used to measure seizure activity and arterial cerebral blood flow velocity.
Blood was drawn for laboratory and virologic measurements immediately before start of extracorporeal circulation, at multiple intervals throughout the procedure and during a 24-week follow-up.
After the procedure, patients were admitted to the ICU overnight. If the clinical condition allowed, patients were discharged from the hospital the next day.
Adverse events were scored for severity according to the WHO toxicity grading scale.
An adverse event was defined as serious (SAE) if it was fatal, life threatening, significant or permanent disabling and/or required prolonged hospitalization.

Outcomes

The antiviral effect and safety of EWBH treatment was assessed over a period of 24 weeks. The primary and secondary endpoints with respect to efficacy were a viral load reduction

of 90% compared to baseline values and an undetectable HCV RNA at the end of study, respectively. The primary and secondary endpoints with respect to safety were the absence of WHO grade 4 and 3 side effects.

Statistical analysis

Values are expressed as means ± SD or, in case of non-parametric distribution, as medians and interquartile range (IQR). Physiological and laboratory parameters were recorded at fixed intervals during and after the EWBH procedure. Differences between various time-points were tested for statistical significance by repeated measures analysis of variance (ANOVA). When a significant difference was detected, results were further compared using Tukey-Kramer Multiple Comparisons test as post-test. Comparisons between pre-treatment and post-treatment values were made by paired samples *t*-tests. Correlation between temperature and mean arterial pressure was tested using the Pearson correlation test (17). A two-sided *p*-value less than 0.05 was considered statistically significant.

RESULTS

Fifteen patients were screened for this study. One patient was excluded because of psychiatric disease. A second patient withdrew consent after she had finished the screening protocol. As a result 13 patients were treated with EWBH. The baseline characteristics of these patients are shown in Table 1.

Antiviral Efficacy

Median level of HCV RNA decreased during the plateau phase of EWBH treatment but did not reach the 90% reduction, which was determined as our primary endpoint. Within one day the median level of HCV RNA returned to pre-treatment levels. Eight of 13 patients reached the primary endpoint of viral reduction of at least 90% by the end of plateau phase or thereafter, as shown in figure 1. None of these decreases in viral load were sustained during follow-up. Therefore, the secondary endpoint regarding efficacy was not fulfilled in any patient.

Adverse effects during and following EWBH

As shown in Table 2, 359 adverse and 42 serious adverse events (SAE) occurred in 13 patients. The most frequently reported adverse events were mild to moderate, transient side effects, such as fatigue, headache, nausea, diarrhea, skin lesions such as blisters and

Table 1. Baseline characteristics of 13 chronic hepatitis C patients

Characteristic	
Age (yrs)	41 ± 8
Male (%)	77
Weight (kg)	79 ± 11
Log_{10} HCV RNA	6.45 ± 0.7
HCV Genotype (n)	
1a	4
1b	8
1	1
Source of infection (n)	
Transfusion	4
Intravenous drug use	7
Unk	2
Previous therapy (n)	
Interferon monotherapy	9
Interferon/Ribavirin 6 mo.	2
Interferon/Ribavirin 12 mo.	2
AST (U/mL)	67 ± 34
ALT (U/mL)	96 ± 61

Values are expressed as means ± SD. HCV , hepatitis C virus; unk, unknown; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

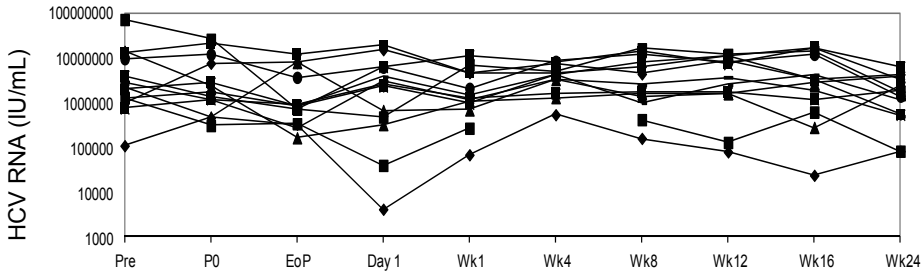

Figure 1: Individual viral kinetics of 13 patients. Pre=pre-treatment, P0=start plateau phase, EoP=end of plateau phase, Day 1=1 day after EWBH, Wk= weeks after EWBH

Table 2. Adverse events after EWBH treatment in all 13 patients

System	Grade 1	Grade 2	Grade 3	Grade 4
Cardiac system	1	1		
Dermatologic system	34	22	4	1
Hepato-Gastrointestinal system	21	8	9	23
Hematologic system	26	9	4	
Neurologic system	6	5	2	2
Musculo-Skeletal system	9	5	4	10
Psychological system	6	2	2	
Respiratory system	13	1		
General	22	34	6	4
Cannulation site	12		1	
Electrolytes	40	12		
Coagulation/Bleeding disorders	27	7	3	2
Infection		1		
Total	**217**	**107**	**35**	**42**

Cannulation site: hematomas at the v. femoralis, v. jugularis and v. subclavia cannulation sites. **Cardiac**: ischemia and murmur. **Dermatologic system:** Skin pressure points, abscess, pustel, pruritus and eczema. **Electrolytes:** hypokalemia, hypomagnesemia, hypophosphatemia, hypocalcemia, elevated bicarbonate, hyperglycemia, hypochloremia, hyperphosphatemia, hypercalcemia and hyperchloremia. **Hepato-gastrointestinal system:** diarrhea, abdominal pain, rectal blood loss, nausea, vomiting, hyperbilirubinemia, elevated alkaline phosphatase, AST, ALT and LDH and liver failure. **General:** fatigue, edema, malaise, hypoalbuminemia, low total protein, sweating and dizziness. **Hematologic system**: anemia, thrombocytopenia, leukocytosis, thrombocytosis. **Coagulation/ bleeding disorders:** prolonged prothrombin time (PT), prolonged Activated Partial Thromboplastin Time (aPTT), thrombin time (TT), elevated and decreased fibrinogen, hematoma conjunctivum. **Infection:** reactivation herpes simplex. **Neurologic system:** headache, paresthesia, sensibility loss, ataxia and amytriptilin intoxication. **Musculo-skeletal system:** myalgia, stiffness and paralysis, elevated CK. **Psychological system:** agitation, depression and confusion. **Respiratory system:** cough, cold, otitis, wheezing and sinusitis.

abrasions around pressure points at the back of the head, the elbows and the nose, facial edema and edema of the extremities, myalgia especially in the upper arms and hematomas at the cannulation sites.

During the procedure, jugular venous temperature was on average 0.3 ± 0.04°C and 0.6 ± 0.18°C higher compared with the esophageal or rectal temperatures respectively, but never exceeded 42.1°C. We did not find evidence of cerebral ischemia by jugular venous oxygen saturation and lactate measurements. In addition, we did not observe any signs of seizure activity in the 8 patients who were monitored with 2-channel electroencephalography. During heating, transcranial Doppler ultrasound measurements indicated a 1.8-fold increase in middle cerebral arterial blood flow velocity, loss of normal cerebrovascular autoregulation responses, and a flow pattern consistent with raised intracranial pressure due to cerebral edema. Clinically, five patients transiently showed signs of central nervous system toxicity, as evidenced by agitation and disorientation after the procedure, which

was severe in one case. This patient was later found to suffer from alcoholism, which he had denied during his screening interview.
Three patients suffered from loss of sensibility and tingling in hands and/or feet. In one patient a neuropathy of the median nerve was confirmed by electromyography. The neuropathic complaints diminished but persisted during 24 weeks of follow-up. One patient suffered from severe neuropathy of the femoral nerve and developed weakness and persistent pain in his right leg. The neuropathic pain was treated unsuccessfully with amitriptylin. As a result of high dosages of amitriptylin, he developed impaired gastrointestinal motility, nausea, vomiting and stupor. These side effects disappeared after amitriptylin was discontinued. After several months the pain and weakness in the right upper leg diminished but did not resolve completely.
The most frequently reported SAEs (*N*=32) were related to cell damage resulting in elevated ALT, AST, creatine kinase (CK) and lactate dehydrogenase (LDH) levels. These levels normalized spontaneously in all patients within two to three weeks.
One patient showed signs of acute liver failure with AST and ALT levels of respectively 8221 and 8752 U/L, a prothrombin time of 35 seconds and a bilirubin of 179 μmol/L. This patient developed ataxia on the fourth day after the EWBH procedure, presumably caused by a carbamazepin intoxication that was used for manic periods that were experienced in the past. Due to impaired clearance function, serum level of carbamazepin was 22 mg/L. CT scan of the brains did not show any other possible cause for the ataxia nor signs of central nervous hemorrhage.
This patients' transaminase levels returned to pre-treatment levels in 3 weeks while his bilirubin level was normalized after 8 weeks. The ataxia resolved within 3 days.
One patient underwent a hair transplantation because of persisting alopecia due to secondary infection of a pressure point blister.
Mean hospitalization time was 3 ± 0.5 days including 1 night in the intensive care unit.

Feasibility

At admission the patients' mean rectal temperature was 37.2 ± 0.5^{0}C. The intracavital temperatures interrelated closely with each other, the esophageal temperature changing the most rapidly and the rectal temperature the most slowly in the warm-up phase as well as in the cooling down phase. The intravascular temperature increased more rapidly than the body temperature measured in the rectum and esophagus.
After a mean warm up phase of 89 ± 27 minutes, the intracavital and intravascular temperatures were maintained between 41.3-42^{0}C for 120 minutes (plateau phase), as shown in (Fig. 2a-d).
Active cooling by the TEMET® heater-cooler took place until a core body temperature of 39^{0}C was reached after a mean time of 33 ± 12 minutes.

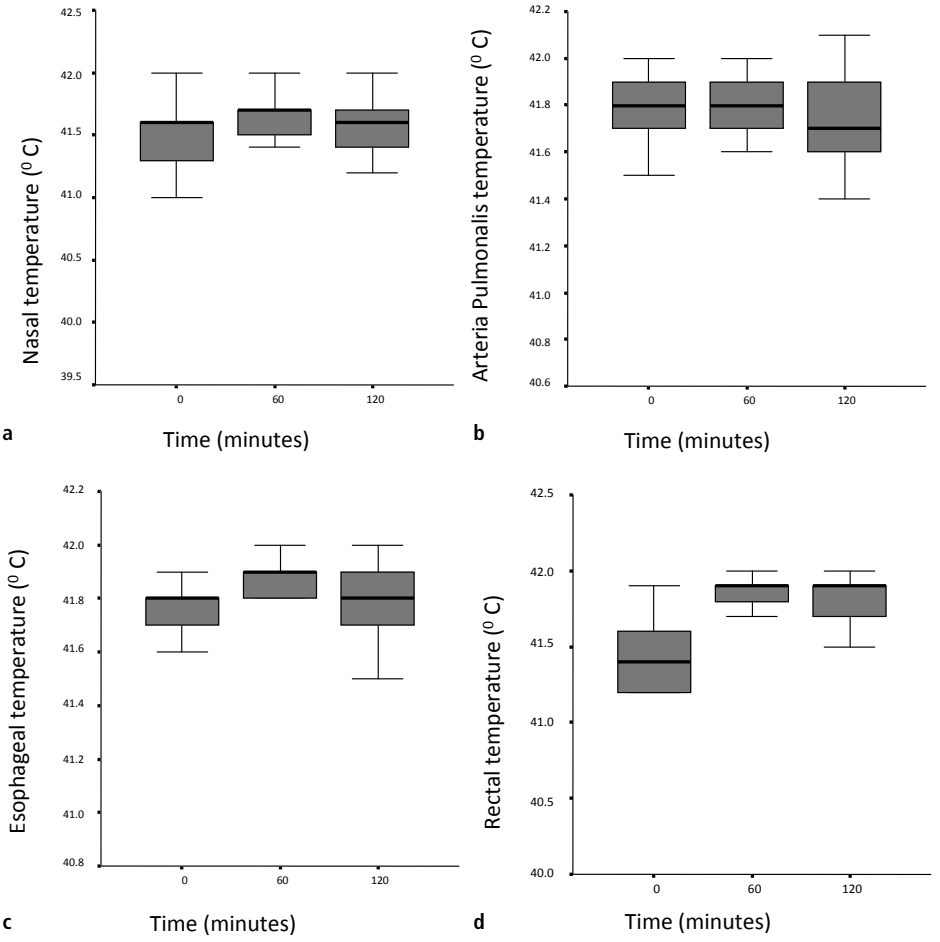

Figure 2a-d: Body Temperatures during plateau phase. Boxes represent the interquartile range (IQR): the boundaries of the box indicate the 25th en 75th percentiles, the horizontal line in the middle of the box represents the median. The vertical lines indicate the highest and lowest value. Temperatures are given at the beginning of the plateau phase (0), half way during the plateau phase (60) and at the end of plateau phase (120).

At the moment of ICU admission the bodytemperature had returned to pre-treatment value.

Figure 3 shows hemodynamic parameters during the EWBH procedure. The heart rate increased steadily while the patients were heated and reached a maximum at the end of the plateau phase (mean 121 ± 11 beats per minute). Mean arterial blood pressure (MAP) was negatively correlated (r=-0.96, p<0.001) with the core body temperature and reached a minimum at the end of the plateau phase.

Phenylephrine was administered in all but two patients to maintain mean arterial pressure stability. Norepinephrin was used in two patients because of a tachyphylaxis for phenyl-

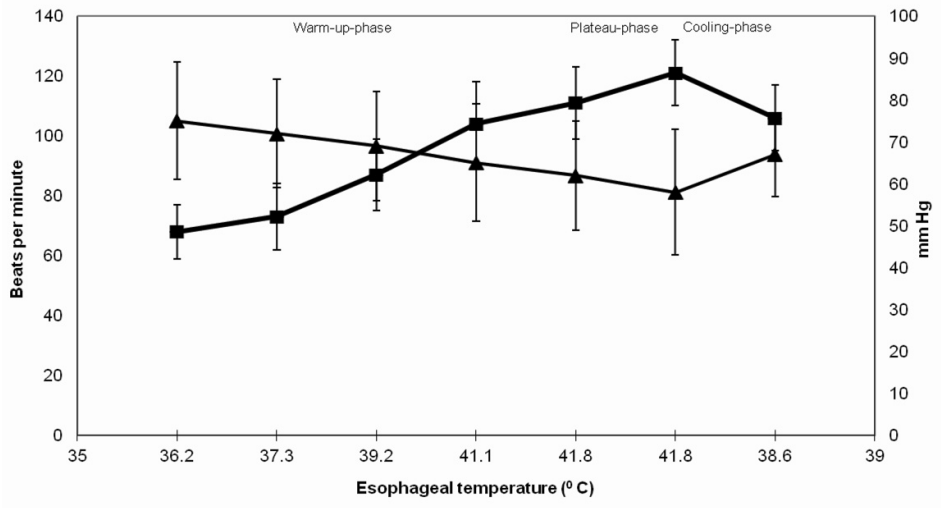

Figure 3: Hemodynamic data during EWBH treatment.
Values are means ± SD. ▲: MAP=mean arterial pressure in mmHg, ■:heart rate in beats per minute.

ephrine. Fluid administration was guided according to the central venous pressure. A mean amount of 10.6 ± 4.5 liters of fluids (cristalloid and/or colloid) was used to compensate for fluid losses.

EWBH induced biochemical changes

Table 3 shows the aminotransferase levels during the EWBH procedure. A 37 to 76 -fold increase in AST level was observed with the maximum levels in the second part of the first day after EWBH. Median ALT level increased up to 2145 (269-3549) U/L on the second day after EWBH. All patients had a pronounced increase of their CK levels. These started to rise just after the treatment, reached their maximum at day 1 and were completely normalized in the second week after treatment. Also, the levels of LDH markedly increased. The maximum of 2306 (1662-10495) U/L was reached on the first day after treatment. Within two weeks LDH levels returned to normal in 12 out of 13 patients. Serum creatinine levels increased with 54 ± 13% during the procedure, reaching a maximum at the end of the treatment and returned to normal levels within one day. Lactate levels did not differ significantly throughout the procedure.

Table 3. Biochemical parameters before, during and after EWBH

	Screening	Pre-ECC	End of treatment	4-6 h after EWBH	1 day after EWBH	7 days after EWBH	14 days after EWBH	End of follow-up
AST (U/L)	50 (42-93)	45 (34-54)	63 (42-106)	311 (178-381)	632 (499-2222)*	60 (49-88)	48 (41-69)	49 (42-57)
ALT (U/L)	80 (51-134)	64 (53-77)	80 (50-110)	201 (114-324)	506 (303-3071)*	226 (95-623)	91 (63-161)	79 (47-96)
LDH (U/L)	463 (433-600)	342 (320-387)	444 (428-521)	1238 (794-1979)	2306 (1662-10495)*	640 (601-840)	504 (459-583)	485 (422-561)
CK (U/L)	75 (57-94)	50 (38-83)	52 (37-84)	Not done	3914 (2197-7668)*	96 (62-147	46 (36-78)	77 (52-127)

Values are medians (25^{th}-75^{th} percentiles).

AST: aspartate aminotransferase, ALT: alanine aminotransferase, LDH: lactate dehydrogenase, CK: creatin kinase, Pre-ECC: before extracorporeal circulation

* $p<0.001$ when value is compared to pre-treatment level

DISCUSSION

EWBH did not result in a persistent antiviral effect or an early viral response defined as a 2-log decrease in viral load as seen in interferon-based treatment regimens. The initial decrease in hepatitis C viral load during and immediately after EWBH treatment may reflect a heat induced destruction of the virus or may be an effect of dilution. HCV RNA levels returned to pre-treatment levels within one day of hyperthermia without any decrease during follow-up suggesting that there is no anti-HCV activity in the 24 weeks after EWBH. This finding is in contrast with the findings reported by Blick et al. who documented a slow but persistent anti-HCV effect in a HIV/HCV co-infected patient after EWBH (9). One possible explanation for this discrepancy is the sensitivity of the Chiron bDNA-2 assay that was used by Blick *et al*, since this assay measures RNA concentrations in the upper quantitative range ($2x10^5$ to $5x10^7$ copies of RNA/mL). The assumed viral response may therefore represent a false negative result (18;19). With the exception of the case-report by Blick, the anti-HCV effect of hyperthermia has not been investigated in vivo.

In vitro several heat experiments have been performed on HCV and bovine viral diarrhea virus (BVDV), which may be used as a model for HCV on the basis of physicochemical similarities (20). These studies showed that BVDV was not inactivated to below detectable limits when exposed to 60^0C for 16 minutes (21). By pasteurization (heat treatment until 60^0C for 10 hours in a stabilized aqueous solution) BVDV and HCV could both be inactivated (22;23). HIV proved to be heat-sensitive at lower temperatures than HCV or BVDV (24-26). Although 56-60^0 C is required for a quick total inactivation of HIV, the viral inactivation can also be obtained by prolonged incubation at lower temperatures. McDougal found a 10-fold reduction in viral load when HIV was incubated for 116 hours at 37^0C and for 3.3 hours at 45^0C (24).

Pennypacker *et al*. analyzed mathematically the effects of heat on the in vivo inactivation of HIV (27). They investigated whether the hyperthermia induced decline in viral load was sufficient to decrease the negative effects of infection or to completely eliminate the infection. The authors concluded that WBH monotherapy given under general anesthesia, considered to be a prerequisite to reach a core body temperature of $\geq 42^0$C, couldn't be effective because treatment would need to be given daily and life-long since the virus will not be removed completely and can be reactivated as soon as the therapy would be stopped. Based on our data and supported by the in vivo and in vitro data of BVDV, HCV and HIV we conclude that extracorporeal whole body hyperthermia with heating until 41.8^0C is not able to eradicate HCV in humans. Possibly, the addition of antiviral medication to one session of hyperthermia might result in a SVR. However, concerning the multiple side effects of hyperthermia such a combination seems not harmless.

Although EWBH proved feasible in patients with CHC, it did not appear to be safe as it induced 42 serious and 359 other adverse events in 13 patients.

All patients experienced mild to moderate adverse events, which resolved spontaneously. The origin of the observed post-treatment agitation may be multifactorial and due to a combination of anesthetic influences, pre-treatment medication use (methadone), heat sensitivity of the central nervous system and possibly cerebral edema.
Hyperthermia induced damaging effects on the nerve vasculature leading to edema and ischemia are required for induction of moderate to severe neurologic abnormalities (28). Whether demyelization has occurred in these patients remains unclear since the electromyography did not show any typical pattern. All patients with neurological side effects experienced persistent sequelae of sensibility and tingling disorders.
Extracorporeal hyperthermia induced significant cell damage in our patients resulting in high, but reversible, elevated levels of AST, ALT, CK and LDH. These elevations were regarded as serious adverse events (WHO grade 4) and were observed in all 13 patients.
The marked increase in aminotransferase levels was not found in patients treated with WBH for cancer and/or HIV-infection (8;29;30). The reason why our patients developed this significant elevation of transaminase levels remains speculative. Elevations can be caused by an ischemic reaction due to impaired perfusion of the liver parenchyma. Secondly, a general inflammatory reaction may have been provoked by hyperthermia, most pronounced in the area where high concentrations of antigens are present i.e. HCV antigens in the liver. Thirdly, hyperthermia itself can be an important cause of liver cell toxicity through the mechanism of oxidative stress (31). Another possible cause for the marked increase in aminotransferase levels may be the fact that liver cells, infected by HCV, are more sensitive to heat than normal healthy liver cells. A comparable effect has been found in HIV infected cells, which was selectively enhanced by TNF-alpha (32).

In conclusion, this first EWBH study in CHC patients showed only transient changes in viral load and failed to achieve a SVR. Considering the serious adverse events, including liver failure, trials with hyperthermia with or without antiviral medication as treatment for chronic HCV infection need to be postponed until the antiviral efficacy of hyperthermia has been demonstrated in pre-clinical studies.

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CHAPTER 8

Summary and conclusion

Since its discovery in 1989, hepatitis C virus (HCV) has been recognized as a leading cause of chronic liver disease. It is a life-shortening disease associated with a complex morbidity pattern varying from minimal changes to liver cirrhosis and hepatocellular carcinoma (HCC). This leads to high costs for society. Progression to cirrhosis is dependent on co-factors such as age at acquiring HCV infection, gender and alcohol intake (1). It is a slow process that takes decades and occurs in 10-40% of patients (1-5). Decompensation of cirrhosis and HCC occurs at an incidence of 3-4% and 1-5% per year, respectively, leading to a liver-related mortality rate of 3% per year (6-8). The main goal of treatment for chronic hepatitis C (CHC) is to prevent liver-related morbidity and mortality. A sustained virologic response (SVR), defined as an undetectable HCV RNA level 24 weeks after treatment withdrawal, is the short-term surrogate for long-term efficacy of anti-HCV treatment and generally regarded as recovery from chronic infection.
In this thesis, new treatment options and their unsuccessful outcome for CHC ("rise and fall") are described.

In **chapter 2**, the hepatitis C viral kinetics with and without interferon and ribavirin therapy was studied. This knowledge is mandatory to further improve the results of treatment. HCV replication, like Human-Immunodeficiency Virus (HIV) replication, has been shown to be a continuous process with a high turnover rate of hepatitis C virions. The estimated virion half-life is between 2.7 and 16.8 hours and the daily estimated virion production 4 x 10^{12}. The high virion turnover allows the generation of a heterogeneous quasi-species population of HCVs which are negatively associated with responsiveness to interferon alpha therapy. Interferon alpha induces a bi-phasic decline in HCV load, after an initial lag due to pharmacokinetic properties of interferon. The decline in viral load in the first phase has been proven to be interferon alpha dose-dependent (9;10), suggesting that high dosing can induce a more pronounced decline. This observation became the theoretical basis for high dose interferon alpha induction therapy.

Amantadine enhances immune response to various viruses as influenza A, dengue and herpes zoster (11-14) and is an effective prophylactic and therapeutic drug against influenza virus (11). Also, beneficial effects of amantadine on HCV have been reported especially in previous non-responders to interferon alpha therapy (15;16). In **chapter 3**, we prospectively investigated in a double-blind, placebo-controlled, multicenter, randomized trial, whether the addition of amantadine to (PEG-)interferon alpha and ribavirin could improve sustained virologic outcome in treatment-naive CHC patients. A total of 297 patients from 26 Dutch hospitals were randomized between treatment with amantadine 200 mg (*N*= 144) or placebo (*N*=153) combined with weight-based ribavirin and a 12-day scheme of interferon alpha-2b induction therapy, followed by weekly PEG-interferon alpha-2b for a total of 1 year. Amantadine did not enhance SVR in treated patients (SVR 47% in the

amantadine group vs. 51% in the placebo group) nor in special subgroups such as patients with HCV genotype 1 and/or a high viral load. Moreover, amantadine had no effect on primary non-response, breakthrough or relapse rates. HCV genotype non-1 and lower pre-treatment γGT levels were independent predictors for a SVR. Although all patients in both treatment arms received a 12-day course of interferon alpha induction therapy, SVRs in this study were not better than those in the landmark studies using PEG-interferon alpha and ribavirin (17;18) which suggests that there is no benefit of high induction therapy. In our study, 90 patients prematurely discontinued treatment, mainly because of grade 3 or 4 toxicity in hematological parameters such as leucopenia, neutropenia and anemia. This high drop-out rate was not different between the amantadine and placebo groups and might have been due to the induction with high dose interferon leading to a more pronounced bone marrow depressive effect.

In **chapter 4**, severity, potential risk factors for and underlying mechanisms of anemia induced by PEG-interferon alpha and ribavirin therapy were evaluated in 44 CHC patients. Hemoglobin (Hb) levels decreased to anemia levels in 98% of patients with a mean decrease of 2.6 ± 0.1 mmol/L. In 61% of patients a drop of Hb level of 2.5 mmol/L or more was noted. A decrease of Hb was associated with higher pre-treatment Hb, ribavirin dose >15mg/kg bodyweight/day and lower pre-treatment platelet levels. Elevated levels of bilirubin and LDH and decreased levels of haptoglobin were found during antiviral therapy suggesting hemolysis as probable cause of anemia. It is generally thought that the accumulation of ribavirin derivates in erythrocytes leads to a relative adenosine triphosphate (ATP) deficiency which may damage the antioxidant defense system and can induce red blood cell membrane changes (19). In our study, in vitro experiments using thin layer chromotography showed no differences in membrane phospholipid composition between erythrocytes of anemic patients on anti-viral treatment and those of healthy controls. Therefore, changes in membrane phospholipid composition seem not to have been an important role in the development of ribavirin induced hemolytic anemia. Furthermore, the red blood cells of CHC patients on antiviral treatment were not more susceptible to osmotic or bile-salt induced stress than the erythrocytes of healthy persons without antiviral treatment, making an increased vulnerability of erythrocytes as underlying cause of the observed anemia unlikely. Further studies are however needed to reveal the true underlying pathophysiologic mechanisms of anemia development in these patients. These studies should probably focus on the role of inosine triphosphatase (ITPase) since a genome-wide-association study in a large CHC cohort recently identified a strong relationship between Hb reduction and 2 functional variants in the ITPA gene causing ITPase deficiency (20-22).

In **chapter 5**, we identified possible risk factors for more pronounced anemia during anti-HCV treatment and evaluated endogenous erythropoietin response in 145 naive CHC patients on PEG-interferon/ribavirin treatment. Anemia occurred in 99% patients during

anti-HCV treatment. More pronounced anemia was associated with older age, lower baseline creatinine clearance, higher baseline hemoglobin levels, a deeper decrease in hemoglobin levels after 2 weeks and higher serum ribavirin concentrations at week 24 of antiviral therapy. Hb decrease was not associated with the final treatment result and was not different between patients with a SVR and those without.
Serum erythropoietin levels, measured by a chemiluminescent immunoassay, increased from 12 IU/L at baseline to 43 IU/L at week 24 and inversely correlated with hematocrit (Ht). Comparison of rise in erythropoietin (EPO) levels in our study population with the normal human response to anemia as defined by a historical population of iron deficiency patients (23;24), showed a suboptimal endogenous EPO response in CHC patients during PEG-interferon/ribavirin treatment.
Although the administration of exogenous EPO had not been shown to improve SVRs (25), the observation of a suboptimal endogenous EPO production during antiviral treatment provides a theoretical basis for the use of EPO to prevent anemia during anti-HCV treatment.

The relationship between CHC infection and alpha-1 antitrypsin (A1AT) deficiency is not clear. A1AT deficiency has been reported to be more common in CHC patients with advanced liver disease than in healthy controls (26;27). Moreover, A1AT deficiency seems to be associated with a worsening of the clinical course of CHC and an increased need for liver transplantation (28;29). As A1AT plays a central role in inflammation as acute phase protein that inhibits proteolytic enzymes like elastases and other proteinases that are secreted by neutrophils, a deficiency of A1AT may be associated with an impaired HCV clearance before and during antiviral treatment. The prevalence of A1AT alleles heterozygosity and its effect on anti-HCV treatment outcome were retrospectively studied among two different CHC cohorts of 1048 patients and described in **chapter 6**. A1AT heterozygosity was found in 6% of patients and did not affect SVR rates. Although mean A1AT serum levels were lower in A1AT heterozygous patients, no effect on the degree of fibrosis was found.

The efficacy, safety and feasibility of extracorporeal whole body hyperthermia (EWBH) was studied in 13 CHC patients with a previous non-response to antiviral treatment. The rationale for this treatment was based on studies in patients with HIV/Acquired Immunodeficiency Syndrome (AIDS). In one study, EWBH was able to induce an initial increase in HIV RNA, which was followed by a decrease to undetectable levels in 2 of 6 treated patients (30). A second study with EWBH showed transient reductions in HIV load and a positive effect on the frequency of AIDS defining events, Karnofsky score, and weight maintenance (31). In 1999, a phase II study was performed testing hyperthermia in HIV/AIDS patients who had failed prior drug therapy. One of the patients in this study was co-infected with HCV and had not responded to previous interferon alpha therapy. Treatment with whole

body hyperthermia resulted in a steady decrease in hepatitis C viral load which became in fact undetectable by week 12 (<0.2 Meq/L (31.746 IU/ml)) (32). This observation led to the hypothesis that hyperthermia may have therapeutic potential in hepatitis C infected patients. The results of our experimental study on EWBH are described in **chapter 7**. Primary and secondary endpoints with respect to efficacy were a HCV RNA reduction of 90% compared to pre-treatment levels and an undetectable HCV-RNA at the end of study, respectively. Eight of 13 patients reached the primary endpoint of viral reduction of at least 90% at the end of plateau phase or thereafter. However, within one day, levels of HCV RNA returned to pre-treatment levels and the decrease in viral load was not sustained in any of the patients during follow-up.

The primary and secondary endpoints of this study with respect to safety were the absence of WHO grade 4 and 3 side effects, respectively. EWBH induced 359 adverse and 42 serious adverse events (SAE) in 13 patients. The most frequently reported adverse events were mild to moderate, transient side effects such as fatigue, headache, nausea, diarrhea, skin lesions particularly blisters and abrasions around pressure points at the back of the head, the elbows and the nose, facial edema and edema of the extremities, myalgia especially in the upper arms and hematomas at the cannulation sites. In most patients, these adverse events resolved without persistent sequelae. Following EWBH, 4 patients developed neuropathy, possibly due to edema and ischemia of the nerve vasculature. Symptoms such as sensitivity disorders and tingling persisted in all 4 patients and did not resolve within the 24 weeks of follow-up. Most of the SAEs were related to muscle and liver cell damage resulting in elevated levels of serum alanine (ALT) and/or aspartate aminotransferase (AST), creatine kinase (CK) and lactate dehydrogenase (LDH). These levels normalized in all patients within two to three weeks. One patient developed acute liver failure with AST and ALT levels of 8221 and 8752 U/L, respectively, a prothrombin time of 35 seconds and a bilirubin of 179 μmol/L. Transaminases returned to pre-treatment levels within 3 weeks and the bilirubin level was normalized after 8 weeks.

EWBH under propofol anesthesia was proven to be feasible; after a warm up phase of 89 ± 27 minutes, core body temperatures were kept between 41.3-42^{0}C for 120 minutes.

Nonetheless, it was also concluded that EWBH is associated with many severe adverse events and that it had no beneficial effect on the treatment of CHC.

CONCLUSION: THE "RISE AND FALL" OF NEW TREATMENT OPTIONS FOR CHC

The studies presented in this thesis represent a period of 10 years of searching for improvement of the treatment for CHC.

- Being extensively studied in the nineties of the past century, the results of viral kinetics and dynamics have shown that HCV replication is a dynamic process with a high virion

turnover and the formation of quasi-species. This finding resulted in the promising idea of high dose induction interferon alpha therapy. Although effective in inducing a dose-dependent decrease in HCV load, induction therapy has not been found to be able to increase SVR rates. The field of viral kinetics and dynamics will be of new importance in the upcoming era of new protease- and polymerase inhibitors for the treatment of CHC that is lying ahead of us.

- Although amantadine appeared promising in the beginning of the year 2000, we have clearly shown in this thesis that there is no role for amantadine in the treatment for CHC in treatment-naive patients. The discussion whether it may be of benefit in previous non-responders, will probably no longer be actual in the near future with the introduction of the new direct acting antivirals, which have already shown promising results.
- The exact pathophysiologic mechanism of the observed (hemolytic) anemia during anti-HCV treatment remains controversial and probably multifactorial. Our finding of suboptimal endogenous erythropoietin production in CHC patients during ribavirin and PEG-interferon therapy will be even more interesting in the near future, as the new direct acting antivirals may seriously aggravate anemia in these patients. Moreover, our finding that older age, lower baseline creatinine clearance, higher baseline hemoglobin levels, a deeper hemoglobin decrease after 2 weeks and higher serum ribavirin concentrations at 24 weeks are independent risk factors for more pronounced anemia during treatment, may help to carefully select the ideal candidate for the "new" antiviral therapy with protease inhibitors in combination with the "older" treatment standards.
- The co-presence of two diseases, i.e. alpha-1 antitrypsin alleles heterozygosity and HCV infection was not found to aggravate the severity of the liver disease and not to affect treatment outcomes. This means that testing of CHC patients for this genetic disorder is not indicated.
- EWBH for CHC, was found to only transiently reduce the viral load, but failed to achieve a SVR. Moreover, taking into account the multiple (serious) adverse events induced by EWBH, it became clear that trials with hyperthermia should no longer be undertaken for this infectious disease. This trial showed that these types of experimental trials can only be performed when previous laboratory research has shown convincing evidence for a possible positive effect of this treatment. Even then, it is of utmost importance that maximum care and safety measures should be taken in these types of phase-1 trials in order not to expose patients to unwanted (serious) adverse events.

It can be concluded from this thesis that this last decade gave us some directions for improvement of care of patients with HCV infection, but also many disappointments in that suggested improvements were not effective (amantadine) and/or were associated with severe, life-threatening side effects (EWBH).

Hopefully, the introduction of the direct acting antivirals will bring us what is really needed; an effective treatment with an acceptable safety profile. It should however always be kept in mind that the hope of the future effective treatments is built on the lessons that we learned in the past.

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Samenvatting en conclusie

Het hepatitis C virus (HCV) werd ontdekt in 1989 en wordt beschouwd als een van de belangrijkste oorzaken van chronische leverziekte. Chronische hepatitis C (CHC) verlaagt de algemene levensverwachting en kan ernstige morbiditeit en daardoor hoge kosten voor de samenleving geven. De ernst van de ziekte varieert van minimale leverafwijkingen tot lever cirrose en hepatocellulair carcinoom (HCC). Progressie van hepatitis naar cirrose is een langzaam proces dat enkele tientallen jaren duurt en bij 10-40% van de chronisch geïnfecteerde patiënten optreedt. Dit proces is afhankelijk van een aantal co-factoren zoals leeftijd ten tijde van het oplopen van de infectie, geslacht en alcoholgebruik (1-5). Gedecompenseerde cirrose en HCC hebben een incidentie van respectievelijk 3-4% en 1-5% per jaar en resulteren in een aan de lever gerelateerde mortaliteit van 3% per jaar (6-8). Het doel van de behandeling van CHC is het voorkómen van aan de leverziekte gerelateerde morbiditeit en mortaliteit. Het behalen van een aanhoudende (sustained) virologische response (SVR), gedefinieerd als het ondetecteerbaar zijn van HCV RNA 24 weken na het stoppen van de therapie, wordt beschouwd als genezing van CHC infectie en is een surrogaat marker voor effectiviteit van de antivirale therapie op lange termijn.
In dit proefschrift beschrijven wij de resultaten van onderzoek naar nieuwe behandelingsmogelijkheden voor CHC die veelbelovend leken maar uiteindelijk niet succesvol bleken te zijn ("rise and fall").

Om een beter inzicht in het daadwerkelijke gedrag van het virus te krijgen en daardoor eventueel de respons op antivirale therapie te kunnen verbeteren, werden de resultaten van kinetiek- en dynamiekstudies van het HCV bestudeerd en beschreven vanuit de literatuur in **hoofdstuk 2**. De ontwikkeling hierin is vergelijkbaar met de kennisontwikkeling van het humaan immunodeficiëntie virus (HIV). Bij HIV heeft dit geleid tot therapeutisch succes in de vorm van de zogeheten "highly active anti-retroviral treatment" (HAART-therapie). De replicatie van het HCV, is net als dat van het HIV, een continu proces met een hoge turnover van hepatitis C virusdeeltjes. De geschatte halfwaardetijd van de virusdeeltjes varieert tussen 2.7 and 16.8 uur en de dagelijkse productie van virusdeeltjes bedraagt 4×10^{12}. Door de hoge turnover van virusdeeltjes ontstaat er een heterogene groep quasi-species. De vorming hiervan is negatief geassocieerd met de response op interferon alfa therapie. Interferon alfa induceert, na een initiële fase zonder daling in HCV RNA die het gevolg is van de farmacokinetische eigenschappen van interferon alfa, een bi-fasische daling in het aantal HCV deeltjes. In de eerste fase is de daling in het aantal virusdeeltjes afhankelijk van de gegeven dosis interferon alfa (9;10). Naar aanleiding van deze observatie ontstond de hypothese dat een hoge inductie dosering interferon een sterkere daling in het aantal virusdeeltjes zou kunnen bewerkstelligen en daarmee de kans op SVR zou kunnen verhogen.

Amantadine verhoogt de immuunrespons tegen verscheidene virussen zoals het influenza A virus, dengue virus en herpes zoster virus (11-14). Daarnaast is amantadine effectief

als profylaxe tegen en behandeling van influenza (11). Voor de behandeling van CHC bleek amantadine effectief met name bij patiënten met eerdere non-respons op interferon alfa therapie (15;16). In **hoofdstuk 3** onderzochten we, prospectief, in een dubbelblinde, placebo-gecontroleerde, gerandomiseerde multicenter studie of de SVR kon worden verbeterd door de toevoeging van amantadine aan (PEG-)interferon alfa en ribavirine. In totaal werden 297 niet eerder behandelde CHC patiënten afkomstig uit 26 Nederlandse ziekenhuizen, gerandomiseerd tussen behandeling met amantadine (*N*=144) of placebo (*N*=153), in combinatie met, op lichaamsgewicht gedoseerde, ribavirine en een 12-dagen durend schema met interferon alfa-2b inductie therapie, gevolgd door wekelijks PEG-interferon alfa-2b gedurende een totale behandelingsduur van 1 jaar. De toevoeging van amantadine leidde echter niet tot een hogere SVR in de patiëntengroep in zijn geheel (SVR 47% in amantadine groep en 51% in placebo groep) en ook niet in specifieke subgroepen zoals patiënten geïnfecteerd met genotype 1 en/of patiënten met een hoog aantal virusdeeltjes. Bovendien had amantadine geen invloed op de primaire non-respons, virale "breakthrough" of mate van relapse. Infectie met genotype non-1 en een lagere uitgangswaarde van γGT bleken onafhankelijke voorspellers van een SVR. Ondanks het feit dat alle patiënten in beide onderzoeksgroepen gedurende 12 dagen werden behandeld met een hoge dosis interferon alfa inductie therapie, was de SVR niet hoger in vergelijking met de respons die eerder was gevonden in PEG-interferon alfa registratie studies (17;18). Deze bevinding suggereert dat een hoge dosis interferon alfa inductie therapie geen toegevoegde waarde heeft voor het bereiken van een SVR. In onze studie staakten 90 patiënten voortijdig de behandeling, voornamelijk door graad 3 of 4 hematologische bijwerkingen zoals leukopenie, neutropenie en/of anemie. Dit hoge uitvalspercentage is mogelijk het gevolg van de hoge dosis inductie therapie met interferon alfa en daardoor een meer uitgesproken beenmerg onderdrukkend effect en was niet verschillend tussen de amantadine en placebo groep.

In **hoofdstuk 4** werden de ernst, potentiële risicofactoren en mogelijke onderliggende oorzaken van de, door PEG-interferon alfa en ribavirine geïnduceerde, anemie geëvalueerd bij 44 CHC patiënten. Bij 98% van de patiënten daalde het hemoglobine (Hb) gehalte tot op het niveau waarbij wordt gesproken van anemie. De gemiddelde Hb-daling bedroeg 2.6 ± 0.1 mmol/L en 61% van de patiënten had een Hb-daling van tenminste 2.5 mmol/L. De daling in het Hb gehalte bleek geassocieerd met een hogere uitgangswaarde van het Hb, met een ribavirine dosis van >15mg/kg lichaamsgewicht per dag en met een lagere uitgangswaarde van het aantal trombocyten. Tijdens antivirale therapie werden verhoogde waarden van het bilirubine en LDH gehalte gevonden, wat kan wijzen op hemolyse als mogelijk onderliggende oorzaak van de anemie. Algemeen wordt aangenomen dat de accumulatie van ribavirine derivaten in de erytrocyt aanleiding geeft tot een relatieve adenosinetrifosfaat (ATP) deficiëntie. Hierdoor ontstaat er schade aan het anti-oxidante

verdedigingsmechanisme van de cel en kunnen veranderingen in de celmembraan van de erytrocyt optreden (19).
In onze studie toonden in-vitro experimenten met behulp van dunnelaagchromotografie geen verschillen in de fosfolipiden samenstelling van de erythrocyten membraan van anemische patiënten tijdens antivirale behandeling in vergelijking met die van gezonde controles. Veranderingen in fosfolipiden samenstelling van de erytrocyten membraan lijken derhalve geen belangrijke rol te spelen bij het ontstaan van de door ribavirine geïnduceerde hemolytische anemie. Bovendien waren de erytrocyten van patiënten die met antivirale therapie werden behandeld niet verhoogd gevoelig voor osmotische of galzoutgeïnduceerde stress in vergelijking met de erytrocyten van gezonde personen. Hiermee lijkt een verhoogde kwetsbaarheid van de erytrocyten als onderliggende oorzaak voor het ontstaan van de gevonden anemie niet aannemelijk. In de toekomst zullen daarom meer studies naar het onderliggende ontstaansmechanisme van anemie bij behandeling met ribavirine noodzakelijk zijn. De focus van dergelijke studies zal waarschijnlijk liggen op de rol van inosine trifosfatase (ITPase) gezien het feit dat genoomwijde associatiestudies (GWAS) een sterke relatie hebben gevonden tussen de daling in het Hb gehalte bij CHC patiënten met antivirale therapie en 2 functionele varianten van het ITPA gen dat ITPase deficiëntie veroorzaakt (20-22).

In **hoofdstuk 5** werden risicofactoren voor ernstige anemie gedurende antivirale therapie geïdentificeerd. Tevens werd de endogene erythropoëtine (EPO) respons bij 145 eerder onbehandelde CHC patiënten geëvalueerd gedurende behandeling met PEG-interferon alfa en ribavirine.
Tijdens antivirale therapie ontstond bij 99% van de patiënten anemie. Het ontstaan van anemie was geassocieerd met een hogere leeftijd, een lagere uitgangswaarde van de creatinine klaring, een hogere uitgangswaarde van het Hb gehalte, een meer uitgesproken daling van het Hb 2 weken na het starten van de therapie en een hogere serum ribavirine concentratie op week 24 van de behandeling. Daling van het Hb gehalte was niet geassocieerd met het behandelingsresultaat en verschilde niet tussen patiënten die wel en geen SVR behaalden.
Het serum EPO gehalte dat werd gemeten d.m.v. een chemiluminescentie immunoassay nam toe van 12 IU/L aan het begin van de behandeling tot 43 IU/L op week 24 en was omgekeerd evenredig met de waarde van de hematocriet (Ht). Bij vergelijking van de stijging in het EPO gehalte in onze studie populatie met de normale EPO respons op het ontstaan van anemie, gedefinieerd in een historische populatie van patiënten met ijzergebreksanemie (23;24), was er sprake van een suboptimale endogene EPO respons in CHC patiënten gedurende therapie met PEG-interferon alfa en ribavirine.
Hoewel niet bewezen is dat de toediening van exogeen EPO leidt tot hogere SVR (25), is met de bevinding van een suboptimale EPO respons gedurende antivirale therapie wel een

theoretische basis gelegd voor het gebruik van EPO om anemie tijdens antivirale therapie te voorkomen.

De relatie tussen CHC infectie en alpha-1 antitrypsine (A1AT) deficiëntie is niet volledig duidelijk. A1AT deficiëntie zou mogelijk vaker voorkomen bij CHC patiënten met voortgeschreden leverziekte (26;27) dan bij gezonde controles. Bovendien lijkt A1AT deficiëntie geassocieerd te zijn met een verslechtering van het klinische beloop van CHC en daardoor een hogere kans op levertransplantatie (28;29). A1AT is een acute fase eiwit dat proteolytische enzymen als elastases en andere, door neutrofielen uitgescheiden, proteinases remt en daarmee een centrale rol in ontstekingsprocessen speelt. Hierdoor zou een A1AT deficiëntie geassocieerd kunnen zijn met een verminderde klaring van het HCV zowel voor als tijdens antivirale therapie. Wij onderzochten daarom retrospectief de prevalentie van A1AT allel heterozygositeit (Pi MS en Pi MZ) en het mogelijke effect hiervan op de uitkomst van antivirale therapie in twee verschillende cohorten van in totaal 1048 CHC patiënten. De resultaten van dit onderzoek werden beschreven in **hoofdstuk 6**. A1AT heterozygositeit werd aangetoond bij 6% van de patiënten en had geen invloed op SVR percentages. Hoewel de gemiddelde serum A1AT waarden lager waren in A1AT heterozygote patiënten, werd hiervan geen effect gezien op de mate van fibrose.

De effectiviteit, veiligheid en haalbaarheid van extracorporele totale lichaamshyperthermie (extracorporeal whole body hyperthermia (EWBH)) werd onderzocht bij 13 CHC patiënten met een eerdere non-respons op antivirale therapie. De rationele onderbouwing van deze studie was afkomstig uit studies bij patiënten met HIV/Acquired ImmunoDeficiency Syndrome (AIDS). In één van deze studies werd bij 2 van de 6 patiënten die met EWBH werden behandeld een initiële stijging van het HIV RNA gevolgd door een daling van het HIV RNA tot aan ondetecteerbaar niveau gezien (30). In een tweede EWBH studie was er sprake van een tijdelijke reductie van het HIV RNA en werd een positief effect op de verschijnselen van AIDS, de Karnofsky score en op het handhaven van het lichaamsgewicht waargenomen (31). In 1999 werd een fase 2 studie met EWBH verricht bij patiënten met HIV/AIDS die eerder geen respons hadden op medicamenteuze therapie. Eén van de patiënten in deze studie had een co-infectie met HCV en was een eerdere non-responder op interferon alfa therapie. De behandeling met EWBH leidde tot een daling van het HCV RNA en het HCV RNA was 12 weken na EWBH ondetecteerbaar (<0.2 Meq/L (31.746 IU/ml)) (32). Deze bevindingen leidden tot de hypothese dat hyperthermie een therapeutische rol zou kunnen hebben in de behandeling van CHC. De resultaten van deze experimentele therapie werden beschreven in **hoofdstuk 7**. De primaire en secundaire eindpunten ten aanzien van effectiviteit van EWBH waren respectievelijk een reductie van HCV RNA met 90% ten opzichte van de waarde voor starten van de therapie en het niet detecteerbaar zijn van HCV RNA aan het einde van de follow-up. Acht van de 13 behandelde patiënten haalden

het primaire eindpunt en hadden vanaf het einde van de plateaufase een reductie van de "viral load" van tenminste 90%. Echter, 1 dag na EWBH waren de HCV RNA waarden weer gestegen naar het uitgangsniveau. De daling in HCV RNA bleek bij geen enkele patiënt van blijvende duur.

Afwezigheid van graad 4 en 3 bijwerkingen was in deze studie het eindpunt met betrekking tot veiligheid van EWBH. Gedurende EWBH ontstonden er bij de 13 patiënten, 359 matige en 42 ernstige bijwerkingen. De meest frequent gerapporteerde bijwerkingen waren milde tot matig ernstige klachten van moeheid, hoofdpijn, misselijkheid, diarree, spierpijn m.n. in de bovenarmen en huidafwijkingen zoals blaren en schaafwonden op de drukpunten op het achterhoofd, ellebogen en neus, oedeem in het gelaat en extremiteiten en hematomen op de plekken van de infusen. Bij de meeste patiënten waren deze bijwerkingen tijdelijk en verdwenen ze zonder dat er sprake was van blijvende schade. Bij 4 patiënten ontstond neuropathie in aansluiting op EWBH. Deze neuropathie was waarschijnlijk te wijten aan oedeem en ischemie van de vascularisatie van de zenuwen. Bij alle 4 patiënten bleven de gevoelsstoornissen en tintelingen bestaan gedurende de follow-up van 24 weken. De meeste ernstige bijwerkingen (serious adverse events) hadden te maken met spier- en levercelverval waarbij er verhoogde waarden van ALAT en/of ASAT, creatinekinase (CK) en lactaat dehydrogenase (LDH) aantoonbaar waren in het serum. Bij alle patiënten waren deze waarden binnen 2 tot 3 weken genormaliseerd. Eén patiënt ontwikkelde acuut leverfalen met ASAT en ALAT waarden van respectievelijk 8221 en 8752 U/L met daarbij een protrombine tijd van 35 seconden en een bilirubine van 179 µmol/L. De transaminasen normaliseerden binnen 3 weken en het bilirubine was na 8 weken weer op de uitgangswaarde.

EWBH onder sedatie met propofol bleek goed mogelijk; na een mediane opwarmingsfase van 89 ± 27 minuten konden de kernlichaamstemperaturen gedurende 2 uur tussen de 41.3^{0}C en 42^{0}C gehouden worden.

Op basis van deze studie concludeerden wij dat EWBH, hoewel technisch goed mogelijk, geen verbetering geeft van de behandeling van CHC gezien het ontbreken van een antiviraal effect. Daarnaast ging behandeling met EWBH gepaard met veel bijwerkingen, waarvan sommige een ernstig beloop hadden.

CONCLUSIE: THE "RISE AND FALL" OF NEW TREATMENT OPTIONS FOR CHRONIC HEPATITIS C

In dit proefschrift beschrijven wij een 10 jaar durende zoektocht naar nieuwe behandelingen voor CHC met als doel het verhogen van de SVR.

- Uitgebreid onderzoek naar de virale kinetiek en dynamiek van het HCV zoals dit verricht is in de jaren negentig van de vorige eeuw, heeft duidelijk gemaakt dat de replicatie van

het HCV een dynamisch proces is met een hoge virus turnover en de vorming van quasi-species. Deze observatie heeft geleid tot het concept van hoge dosis inductie therapie met interferon alfa voor CHC. Hoewel deze inductie therapie in staat is een dosisafhankelijke daling in het HCV RNA te bewerkstelligen, resulteert dit niet in het verhogen van de SVR. Momenteel is het onderzoeksveld van de virale kinetiek en dynamiek opnieuw van groot belang geworden in het tijdperk van de nieuwe protease- en polymeraseremmers die recentelijk zijn geïntroduceerd.

- Hoewel het gebruik van amantadine veelbelovend leek in het begin van dit millennium, laten we in dit proefschrift zien dat er geen rol van betekenis is weggelegd voor dit middel bij de behandeling van eerder onbehandelde CHC patiënten. De vraag of amantadine effectief is bij non-responders zal waarschijnlijk binnenkort niet meer actueel zijn gezien de recente introductie van de veelbelovende direct werkende antivirale middelen.
- De exacte onderliggende pathofysiologische ontstaansmechanismen van (hemolytische) anemie tijdens anti-HCV behandeling zijn nog steeds niet geheel duidelijk en zijn waarschijnlijk multifactorieel bepaald. Onze bevinding van een suboptimale endogene erythropoëtine productie bij CHC patiënten tijdens PEG-interferon en ribavirine therapie zou van groot belang kunnen zijn nu, nieuwe direct werkende antivirale middelen beschikbaar zijn en deze als één van de bijwerkingen een verergering van de reeds door PEG-interferon en ribavirine geïnduceerde anemie hebben. Een hogere leeftijd van de patiënt, een lagere uitgangswaarde van de creatinine klaring, een hogere uitgangswaarde van het Hb gehalte, een meer uitgesproken daling van het Hb gehalte 2 weken na het starten van de therapie en een hogere serum ribavirine concentratie op week 24 van de antivirale therapie zijn daarnaast risicofactoren die de ernst van de anemie mede bepalen en kunnen van pas komen bij het selecteren van kandidaten voor de "nieuwe" behandeling met proteaseremmers gecombineerd met de "oude"standaard behandeling bestaande uit PEG-interferon en ribavirine.
- De combinatie A1AT allel heterozygositeit (Pi MS en Pi MZ) en CHC, doet de ernst van de leverziekte niet toenemen. Bovendien heeft A1AT deficiëntie geen invloed op de uitkomst van de behandeling van CHC. Dit betekent dat het testen van CHC patiënten op A1AT deficiëntie niet geïndiceerd is.
- Het toepassen van EWBH bij CHC geeft slechts een voorbijgaande daling van het aantal virusdeeltjes en is niet in staat een SVR te bewerkstelligen. Bovendien werd duidelijk, mede gezien de vele (ernstige) bijwerkingen van de behandeling, dat studies met hyperthermie niet langer dienen te worden uitgevoerd bij CHC. Deze studie laat zien dat dit soort experimentele studies slechts kunnen en mogen worden uitgevoerd als er d.m.v. pre-klinisch onderzoek voldoende overtuigend bewijs is voor een eventueel positief effect. Zelfs indien dat het geval is, is het van het grootste belang dat in dit soort fase 1 studies, maximale zorg en veiligheidsmaatregelen toegepast worden teneinde patiënten niet aan ongewenste (ernstige) bijwerkingen bloot te stellen.

Dit afgelopen decennium gaf ons richting om de behandeling van CHC patiënten te verbeteren. Het was teleurstellend dat de nieuwe behandelingen niet effectief bleken (amantadine en EWBH) en/of gepaard gingen met ernstige, levensbedreigende bijwerkingen (EWBH).

Hopelijk zal de introductie van de nieuwe direct werkende protease- en polymeraseremmers leiden tot een effectieve anti-HCV behandeling met een acceptabel veiligheidsprofiel. Hierbij moet echter altijd in gedachten worden gehouden dat de verwachting van toekomstige effectieve behandelingen gebaseerd is op de lessen die in het verleden zijn geleerd.

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Curriculum Vitae

Hanneke van Soest werd geboren op 17 juni 1973 in Schiedam. In 1991 behaalde zij het gymnasium diploma aan het Johan van Oldenbarnevelt gymnasium te Amersfoort waarna zij begon aan de studie Geneeskunde aan de Universiteit van Amsterdam. Na het behalen van de het propedeuse heeft zij de doctoraal fase van de studie voortgezet aan de Rijksuniversiteit Groningen. Tijdens deze periode deed zij, onder supervisie van Dr. R.J. Odink en Dr. Ch. Bijleveld, onderzoek op de afdeling kindergeneeskunde van het UMCG naar de groei bij kinderen met een levertransplantatie. In 1999 behaalde zij, cum laude, het artsexamen. Hierna werkte zij 1 jaar als AGNIO Interne Geneeskunde in het Meander MC in Amersfoort. Van 2000-2002 werkte zij als arts-onderzoeker op de afdeling Maag-, Darm- en Leverziekten van het UMC Utrecht onder begeleiding van Dr. K.J. van Erpecum, Prof. dr. G.P. van Berge Henegouwen, Dr. J. van Hattum en Prof. dr. M. Samsom. In april 2002 begon zij met de vooropleiding Interne Geneeskunde in het UMC Utrecht (opleider Prof. dr. D.W. Erkelens). In 2004 werd de opleiding tot MDL arts voortgezet in de Isala klinieken te Zwolle (opleider Dr. F. Nelis en Dr. J. Vecht) en deze werd in 2008 afgerond in het UMC Utrecht (opleider Prof. dr. P.D. Siersema). Sindsdien is zij werkzaam als MDL arts in het Medisch Centrum Haaglanden, te Den Haag.

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