Response in Hepatitis C Virus Non-Responders

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Chapter 1

General introduction

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1.1 The virus

The identification of hepatitis A and hepatitis B virus in the sixties and seventies of the last century (1, 2) led to the recognition that one or more other viruses were capable of causing acute liver inflammation or *hepatitis* after blood transfusion (3). The then not yet identified virus was subsequently named post-transfusion non-A, non-B hepatitis. In 1989 a RNA virus could be identified by molecular cloning techniques, consequently named hepatitis C virus (HCV) (4). This virus proved to be responsible for most cases of post-transfusion non-A, non-B hepatitis.

The hepatitis C virus is an enveloped virus, approximately 50 nm in diameter that belongs to the family of flaviviruses. Its genome is a positive sense, single-stranded RNA with an average length of 9.6 kb, including 2 untranslated regions at the 5' and 3' ends, and an open-reading frame encoding a large polyprotein that is processed into structural and nonstructural proteins (5). HCV has high rates of replication (10^{12} virions per day) and mutation that promote chronicity and the development of resistance to antiviral therapy. Within an individual, viral mutations produce closely related strains called quasi-species. Genetic heterogeneity within the population is categorized in 6 major genotypes that show geographic and patient communities dependant variation. In Western Europe genotypes 1a and 1b are most common, followed by genotype 2 and 3. Genotype 3 is mainly found in (former) drug addict communities; genotype 1 in people having received blood transfusion.

HCV is spread almost exclusively by direct blood contact. Transmission through blood (product) transfusions that are not screened for HCV infection, through the reuse of unsterilized injection equipment (needles, syringes) or other medical equipment, or through needle sharing among drug-users, is well documented. In addition, people who undergo traditional scarification, body piercing, tattoo and circumcision are at risk if they use or reuse unsterilized tools. Sexual and vertical transmission does occur, but is unusual.

Humans and chimpanzees are the only known species susceptible to infection, with both species developing similar disease. HCV preferentially infects hepatocytes but evidence of extrahepatic replication sites has accumulated during the past decade.

Worldwide, it is estimated that 3% of the world population, about 170 million people, have been infected with HCV and 3 to 4 million persons are newly infected each year. The prevalence varies throughout the world (fig. 1), with the highest number of infections reported in Egypt and the lowest in Europe and North America. In Europe, HCV prevalence is not clearly established. It could be at least 1.03% of the general population, which represents 8.9 million infected people (6). Prevalence in Northern Europe is lower than in Southern Europe. For the Netherlands the estimated prevalence is 0,1% (7). However, this figure is based on a limited survey in only 7373 persons in whom 6 had antibodies against HCV. In patient communities like hemophiliacs, drug addicts or people transfused with blood before 1990 the prevalence can be as high as 25 to 60 %.

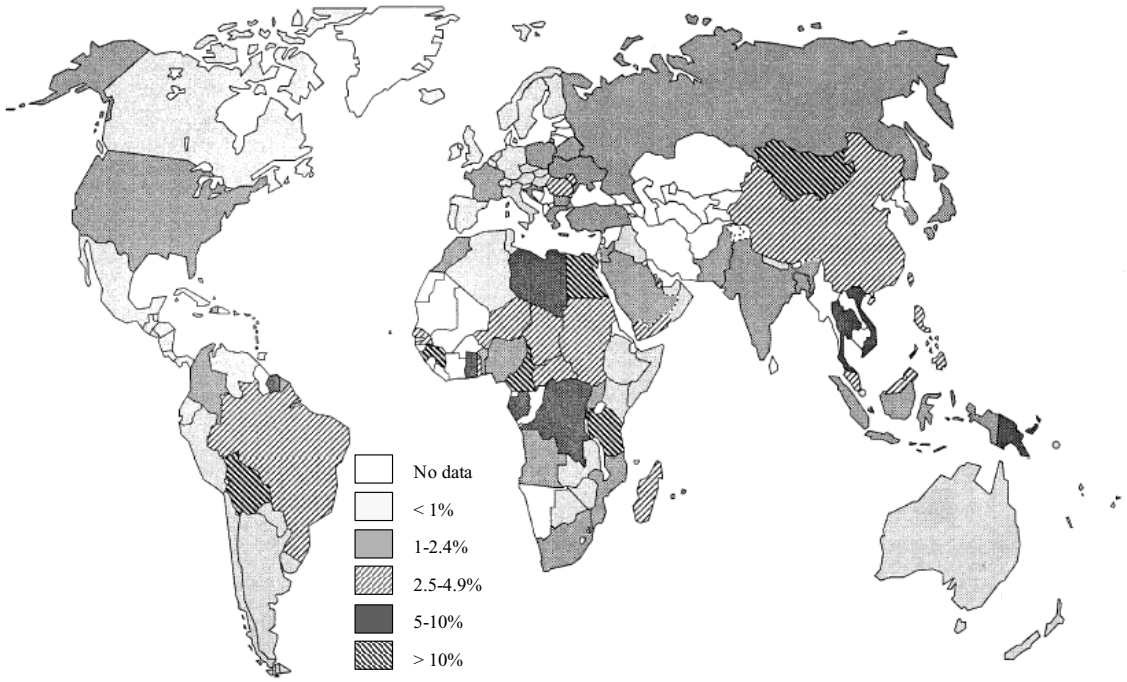

Figure 1. Global prevalence of hepatitis C virus (6).

It is estimated that 3% of the world population have been infected with the hepatitis C virus. The prevalence of HCV in the Netherlands is among the lowest in the world.

1.2 The disease

Hepatitis C is a dichotomous disease in which only a subset of patients will die from liver-related causes (fig. 2). Acute infection can be severe but is rarely fulminant (8); in fact the majority of persons who develop acute HCV is unaware of it, so that disease onset is rarely identified other than through assumption based on potential circumstances of exposure. In ±85% the initial acute infection leads to a chronic phase; defined as persistence of HCV RNA in the blood for 6 months of more. ±15% of the infected persons is able to clear the virus after an acute infection. Factors associated with spontaneous clearance of HCV infection appear to include female gender, younger age and some major histocompatibility complex genes (9).
Chronic viral hepatitis leads to slow but progressive increasing degrees of fibrosis but is often asymptomatic or associated with non-specific symptoms such as fatigue. Most patients are diagnosed in a presymptomatic stage, because of an identifiable risk factor or because of abnormal liver biochemistry. Of those infected, ± 20% will eventually develop cirrhosis and its related complications within a period of 10-20 years; about 1 to 5% will eventually develop liver decompensation or liver cancer over a period of 20 to 30 years. Fibrosis progression is not necessarily linear and seems to be highly variable among persons. The rate of fibrosis progression is affected by some host-related characteristics such as the age of infection and alcohol abuse and factors like co-infection with other hepatitis viruses or HIV.

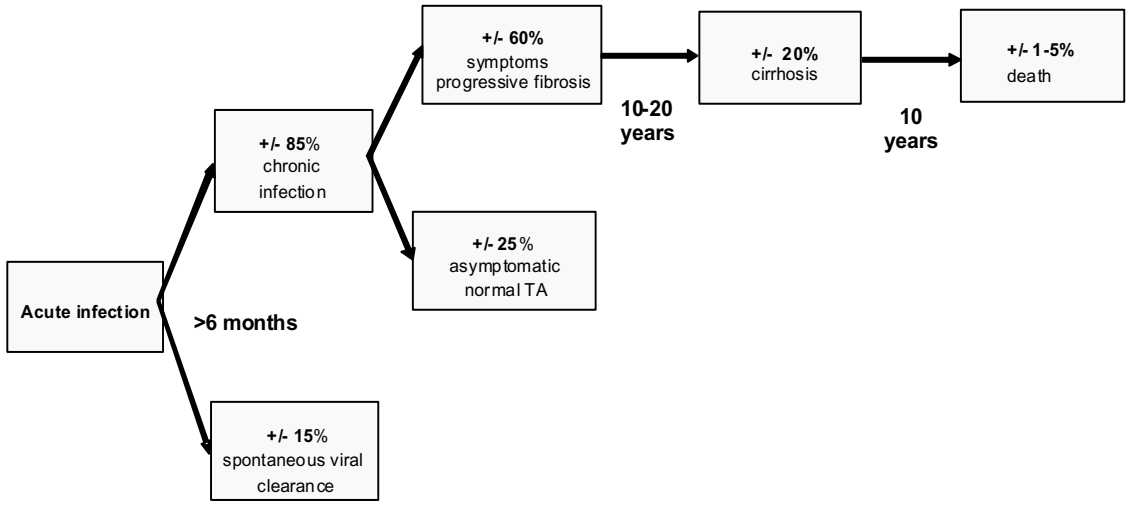

Figure 2. Natural history**.**

Data from the EASL consensus 1999; adapted from Orlent et al.(10)

1.3 The problem in the treatment of chronic hepatitis C

In 1986, three years before the identification of the hepatitis C virus Hoofnagle et al. introduced “long-term, low-dose” interferon-α (IFN) for the treatment of chronic non-A non-B hepatitis (11). Since then, the treatment of chronic HCV has made remarkable progress. Originally, the response to antiviral therapy in patients with HCV was expressed in terms of biochemical response (normalization of serum alanine aminotransferase (ALT) levels). However, since the introduction of sensitive PCR assays for the detection of viral RNA, expression in terms of virological response is preferred. Consequently, the success of an antiviral treatment modality is expressed in terms of sustained virological response, defined as a negative result of a sensitive PCR assay for HCV-RNA after a 24-week treatment-free follow-up period. A sustained virological response should be regarded as a surrogate marker for cure of HCV; it does not completely exclude late virological relapse (1-3% after 4 years for patients treated with interferon alfa-2b and ribavirin for 24-48 week (12)).

For interferon-α monotherapy, sustained virological response rates were between 2-9% in genotype 1 and between 16-23% in genotype 2 and 3 (13, 14). By adjusting treatment duration up to 48 weeks for genotype 1 and combining regular interferon-α with ribavirin, sustained response rates could be improved to 28-31% in genotype 1 and 65% in genotype 2 and 3 (13, 15). With the introduction of long acting pegylated interferon-α (PEG-IFN-α) in combination with ribavirin sustained virological response rates of 80% can be obtained in genotype 2 and 3 (16, 17).

Genotype 1 is the predominant genotype and patients infected with it are still cured in less then 50%. Another group in need for better treatment options are patients with cirrhosis in whom sustained response rates are also considerably lower. Moreover, as increasing numbers of patients are treated, the number of patients failing to respond will also increase.

1.4 The hypothesis

A strong decline and early clearance of serum HCV RNA within the first weeks of treatment, is a strong predictor for SVR (18, 19). The rate of decline appeared to be dose dependent for interferon (20). With 4 weeks of daily high dosed interferon-α an early virological response could be obtained in 80% of difficult-to-treat patients eventually leading to a high rate of SVR (67%)(21). However, whether high-dosed

Peginterferon-α has comparable or even improved efficacy remains to be established. Moreover, whether the individual Peginterferon pharmacokinetics affect the rate HCV RNA decline in that individual and its chance to achieve SVR has not yet been revealed.

Major pre-treatment factors influencing the response rates to therapy are HCV genotype and the degree of fibrosis (14-16, 22, 23). These characteristics are however, not useful for the prediction of the chance to respond to therapy in the individual patient. Immune markers at the site of disease, i.e. in the liver might have a more causal relation to response.

Aims of the study

A) To evaluate the effects of high-dose (Peg)interferon therapy in non-responders and “difficult to treat” patients.
B) To identify immunological factors in peripheral blood and the liver associated with (non-)response.
C) To describe (peg)interferon pharmacokinetics and its relation to (non-)response.

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Part I

Treatment of non-responders and “difficult to treat” HCV patients

Chapter 2

The Treatment of Hepatitis C: History, Presence and Future

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ABSTRACT

The treatment of chronic hepatitis C has made remarkable progress over the past two decades. For interferon-α monotherapy, sustained virological response rates were between 2-9% in genotype 1 and between 16-23% in genotype 2 and 3. By adjusting treatment duration up to 48 weeks for genotype 1 and combining regular interferon-α with ribavirin, sustained response rates could be improved to 28-31% in genotype 1 and around 65% in genotype 2 and 3. Attempts to further increase efficacy included the addition of amantadine without conclusive evidence up till now. With the recent introduction of long acting pegylated interferon-α in combination with ribavirin sustained virological response rates of 80% can be obtained in genotype 2 and 3. However, sustained virological response rates for patients with either genotype 1, non-response to prior treatment, cirrhosis or a combination of these characteristics are still less than 50%. In view of results with daily high dose interferon-α induction in combination with prolongation of treatment duration up to 18 months such patients might benefit from induction and prolonged PEG-IFN-α treatment and should be treated in an experimental setting.

2.2 INTRODUCTION

The *Netherlands Journal of Medicine* is an offspring from the *Folia Medica Neerlandica* (1958). The very first publication in this magazine was entitled: “Viruses as a cause of disease”, a topic which has not lost its relevance (1).

Hepatitis C virus (HCV) is such a cause and currently leading to a global public health problem. Worldwide, 150 - 170 million people are estimated to be chronically infected with HCV of whom an estimated 5 million are living in Western Europe. Twenty percent of those chronically infected will eventually develop cirrhosis of the liver and its sequelae within 10-20 years in the absence of treatment (2). Antiviral therapy has now been used for nearly two decades to slow down and prevent this progression. In the past years, the response rate to antiviral therapy has made remarkable progress.

Originally, the response to antiviral therapy in patients with HCV was expressed in terms of biochemical response (normalisation of serum alanine aminotransferase (ALT) levels). However, since the introduction of sensitive PCR assays for the detection of viral RNA, expression in terms of virological response is preferred. Consequently, the success of an antiviral treatment modality is expressed in terms of sustained virological response, defined as a negative result of a sensitive PCR assay for HCV-RNA after a 24-week treatment-free follow-up period. A sustained virological response can be regarded as a surrogate marker for cure of HCV, although it does not completely exclude late virological relapse.

The focus of this review is to summarize past treatments of chronic hepatitis C, to describe the present standard of care and to give recommendations how to improve therapy in the future.

2.3 TREATMENT HISTORY: INTERFERON AND RIBAVIRIN

2.3.1 Interferon

In 1986, three years before the identification of the hepatitis C virus by molecular cloning techniques, Hoofnagle et al. introduced long-term, “low-dose” interferon-α (IFN-α) for the treatment of chronic non-A and non-B hepatitis (3). Based on these preliminary data, large randomised placebo-controlled trials were performed confirming the effectiveness of IFN-α (4, 5). IFN-α given as a single agent (monotherapy) dosed 3 million units (MU) thrice a week (tiw) subcutaneously for 24 weeks induced end of treatment responses (normalisation of ALT levels) in about 50% of patients. However, in about half of the responding patients a relapse was seen within 6 months after discontinuation of treatment. Further experience indicated that the sustained virological response was even lower; only 6-18%. Patients with genotype 2-3 had higher sustained virological responses (16-23%) than patients with the predominant genotype 1 (2-9%, table 1) (6, 7).

In the years following the introduction of IFN-α, strategies to increase its antiviral activity included the optimisation of dose, treatment duration and the combination with other antiviral agents. One of the first improvements was made by prolongation the treatment duration to 48 weeks. Hereby, post-treatment relapse in HCV-RNA could be reduced significantly; resulting in an increased sustained virological response rate of 7%-11% in genotype 1 and 29%-33% in genotype non-1 (table 1) (6, 8). By therapy prolongation beyond 12 months, the ALT relapse rate in IFN-α monotherapy could even further be reduced (9, 10).

2.3.2 Ribavirin

From all the efforts to increase efficacy, the combination of IFN-α with ribavirin has been the most fruitful. Ribavirin, a nucleoside analogue in use for the treatment of respiratory syncytial virus, lowers ALT levels in many patients with chronic hepatitis C; it has however no significant effect on serum HCV-RNA levels (11-13). When used in combination with IFN-α, it increases the end of treatment response and reduces post treatment relapse (7, 14). Large randomised placebo-controlled trials in the U.S.

and other countries have confirmed the enhanced efficacy of combined interferon-ribavirin therapy. Among patients with genotype 1 a sustained virological response

was found in 16-23% of those treated for 6 months and in 28-31% of patients treated for 12 months. Results in genotype non-1 were around 65% for both 6 and 12 months (table 1)(6-8). These findings led to the recommendation that patients infected with genotype 1 require a course of 48 weeks IFN-α plus ribavirin therapy whereas 24 weeks combination therapy was sufficient for patients with genotype 2 and 3 (2).

Table 1. Effect of ribavirin and treatment duration.

	Interferon-α2b[#]		**Interferon-α2b[#] and ribavirin[$]**	
	24 weeks	48 weeks	24 weeks	48 weeks
Genotype 1	2- 9	7-11	16-23	28-31
Genotype non-1*	16-23	29-33	50-69	64-66

Sustained virological response rates in percentages in genotype 1 and genotype non-1 for interferon-α (IFN) monotherapy and IFN plus ribavirin for 24 and 48 weeks.

*Reflects data on genotype 2 and 3, data on genotype 4-6 are too limited for inclusion in the table.

[#] Interferon-α2b subcutaneously; dosed 3 million units MU three times a week.

[$] Ribavirin orally; dosed 1.0-1.2g in two divided doses according to weight.

2.3.3 Amantadine

Amantadine has been used for many years to prevent infection with influenza A virus. Most randomised controlled trials were unable to demonstrate a significant beneficial effect of amantadine-IFN-α over IFN-α monotherapy, largely because of small sample sizes (15-19). The trials are comparable in design and when the data are pooled –a total of 920 patients has been included- a modest improvement can be demonstrated (table 2). Health related quality of life analysis showed an improvement of fatigue and vigour scores in patients receiving combined IFN-α amantadine treatment compared with those treated with IFN-α alone (15, 20). Two trials (one randomised) in IFN-α non-responders demonstrated significant benefits of the addition of amantadine to the combination of IFN-α and ribavirin (21, 22). The mechanism with respect to this improvement in sustained response remains unclear. Currently a Dutch placebo-controlled multicentre trial on "triple" therapy in naive patients is in progress in more than 30 hospitals (CIRA study). This study will need a large sample size to demonstrate beneficial effects of amantadine.

Table 2: Effect of amantadine when added to interferon-α.

	Interferon-α2b	**Interferon-α2b and amantadine** [$]	***P****
Zeuzem et al. (15)	13/60 (22)	6/59 (10)	0.087
Mangia et al. (16)	17/101 (17)	29/99 (29)	0.036
Caronia et al. (17)	15/89 (17)	21/90 (23)	0.280
Tabone et al. (18)	15/90 (17)	22/90 (24)	0.197
Helbling et al. (19)	17/121 (14)	25/121 (21)	0.175
ALL	77/461 (17)	103/459 (22)	0.028

Number of patients out of whole group (and percentages between brackets) with sustained virological response for Interferon-α2b (IFN) monotherapy and IFN plus amantadine for 48 weeks.

[$] Amantadine orally; dosed 200mg in two divided doses.

***** Chi-square test

2.3.4 High dose induction and prolonged treatment

Increasing the IFN-α dose or administrating IFN-α daily could be beneficial, because of the high viral replication rate and the short half-life of IFN-α (only hours) (23). Indeed, high daily dosed IFN-α for several weeks, i.e. induction therapy, has been used for years in Japan, resulting in high (more than 80%) initial virological response rates (24). High daily dose IFN-α therapy with or without ribavirin in Western Europe and the USA, however, had no lasting beneficial effect on sustained virological response rates (25, 26). In those early reports, a significant increase of virological response rate (% HCV-RNA negative patients) was found during the high daily dosing period as compared to IFN-α 3 MU t.i.w. However, this effect did not result in an increased sustained virological response.

This failure to maintain the initial response might be explained by the premature lowering of the IFN-α dose within the first week (27) or due to a short duration of treatment which was stopped after 24-26 weeks (24). In a later randomised controlled trial in 373 patients (28) two experimental induction schedules were compared to a regular schedule of 38 weeks IFN-α 5 MU every other day. In genotype 1 patients, the sustained virological response was nearly twice as high (SVR: 42% vs. 27%) when treated with induction therapy (10 MU IFN-α daily for 2 weeks, followed by 10 MU every other day for 12 weeks, followed by 5 MU every other day for 24 weeks). Sustained virological response rates in the non-1 genotypes varied between 56-76% in the three treatment arms but did not differ significantly. These data suggest beneficial effects of high dose induction and prolonged daily IFN-α dosing in genotype 1 patients in whom success is limited when treated with standard therapy.

In an effort to analyse the above mentioned treatment strategies combining high dose induction IFN-α and prolonged daily IFN-α plus ribavirin we performed a meta-analysis on individual treatment data of an exploratory study performed in our centre (29). In total, fifty-four consecutive chronic HCV patients selected for unfavourable base-line characteristics associated with therapy resistance such as genotype 1 infection, cirrhosis, previous non-response to IFN-α or combinations of these, were treated intensively for 76 weeks. The first 24 patients were treated with a very intensive schedule which resulted in an overall sustained response rate of 67% (95% confidence interval: 45-84%) (30). In the following patients, attempts were made to decrease morbidity and cost without losing efficacy. However, by shortening the

induction period adverse effects decreased somewhat as did the effectiveness (27). Thus no clear advantage was found over the original intensive treatment schedule.
The induction phase of 10 MU IFN-α daily for 2-4 weeks was followed by daily 3-5 MU IFN-α injections until week 52 and 3 MU daily or thrice weekly until week 76. Throughout the study all patients received 1,000 - 1,200 mg ribavirin.
The overall sustained virological response rate was 57% (95%-CI: 43-71%). Sustained virological response varied between 75-83% for patients with 1 unfavorable characteristic; between 25-60% for patients with 2 unfavorable characteristics, but only 17% for those with 3 unfavorable factors. Ten patients had detectable HCV RNA at week 12; in whom treatment was discontinued (19%). Two patients experienced a virological breakthrough (4%) and 1 patient was HCV RNA negative at 72 weeks but relapsed in the 24 weeks of untreated follow-up (2%). Four patients were not compliant: they stopped in the first four weeks after discharge from the hospital. In six patients (11%), therapy was stopped because of adverse effects (hepatic decompensation (n=2), depression (n=2), cardiac complaints (n=1) and Staphylococcus sepsis (n=1)). These data indicate that patients with either genotype 1, cirrhosis or prior non-response can have sustained virological response rates approaching those in genotype 2/3 when treated with high-dosed and prolonged IFN-α based therapy. However, in patients with combinations of these unfavorable criteria, sustained virological response rates were low.

2.4 TREATMENT TODAY: PEG-INTERFERON-α AND RIBAVIRIN

2.4.1 Peg-interferon-α monotherapy

The covalent attachment of polyethylene glycol (PEG) to IFN-α extends the half-life and duration of therapeutic activity of IFN-α in-vivo, allowing less frequent dosing.
Four randomised controlled trials have compared the efficacy and safety of PEG-IFN-α monotherapy with standard IFN-α monotherapy (31-34). One of these studies was designed to investigate treatment efficacy and safety in patients with bridging fibrosis or cirrhosis (32). Administration of PEG-IFN-α once weekly has an increased antiviral effect compared to IFN-α 3MU thrice a week in naive patients resulting in a reduced breakthrough rate and an increased end of treatment and sustained virological response rate. The optimal dose of PEG-IFN-α was found to be 180 ug per week for PEG-IFN-α2a and 1.5ug/kg body weight for PEG-IFN-α2b. Sustained response rates

in genotype 1 were between 12-31% for PEG-IFN-α and between 2-6% for standard IFN-α. In genotype 2 and 3 sustained virological response rates were around 50% and 28% respectively.

2.4.2 Peg-interferon-α and ribavirin

When PEG-IFN-α is combined with ribavirin the sustained response rate is further improved. Sustained virological response rates in genotype non-1 were 78% for 6 and 77% for 12 months PEG-IFN-α2a plus ribavirin therapy. Like the treatment with regular IFN-α plus ribavirin, there is no benefit in prolonging treatment till 48 weeks in patients with genotypes 2 and 3 (table 3). A reduced ribavirin dosage of 800 mg daily was found to be as effective as 1000-1200 mg daily in patients with genotype 2 and 3, but the standard dosage of 1000-1200 mg yielded better sustained response rates in patients with genotype 1 in whom sustained virological response rates were 42-51% (table 4). These outcomes are improved over standard IFN-α ribavirin therapy although still about 50% of genotype 1 patients do not respond (35-37). During treatment, a 2 log or more decrease in viral load in the first 12 weeks is predictive for sustained virological response. Patients who fail to achieve a 2 log decrease in viral load have a limited chance to achieve sustained virological response and should stop therapy.

One of the major differences between the two PEG-IFNs is fixed dosing for PEG-IFN-α2a and dosing according to weight for PEG-IFN-α2b. Trials comparing efficacy and safety of PEG-IFN-α2a to PEG-IFN-α2b with or without ribavirin have not (yet) been conducted. However, both PEG-IFNs have been compared to standard IFN-α and the increased sustained response rates, safety profile and side effects seem similar.

Table 3: Effect of pegylation of interferon-α.

	Interferon- α2b[#] and ribavirin[¥] 48 weeks	**Peg-interferon-α2a/b and ribavirin[¥]** 48 weeks
Genotype 1	33-36%	42[$] -46%
Genotype non-1*	61-79%	76-82[$] %

Sustained virological response rates in genotype 1 and genotype non-1 for interferon-α2b and Peg-interferon-α2a or Peg-interferon-α2b plus ribavirin for 48 weeks.

*Reflects data on genotype 2 and 3, data on genotype 4-6 are too limited for inclusion in the table.

[#] Interferon-α2b dosed 3 million units three times a week. Peg-interferon-α2a dosed 180mcg per week. Peg-interferon-α2b dosed 1.5ug/kg body weight per week.

[¥]Ribavirin orally; dosed 1.0-1.2g in two divided doses according to weight.

[$] Ribavirin orally: dosed 800mg/ day in two divided doses.

Table 4: Effect of ribavirin dose and treatment duration.

	Peg-interferon-α2a 180 ug per week			
	Ribavirin 800 mg		**Ribavirin 1-1,2 g**	
	24 weeks	48 weeks	24 weeks	48 weeks
Genotype 1	29%	40%	41%	51%
Genotype 2-3	78%	73%	78%	77%

Sustained virological response rates in genotype 1 and genotype 2 & 3 for Peg-interferon-α2a 180 ug per week plus 800 mg vs 1.0-1.2g ribavirin for 24 and 48 weeks. (based on literature reference 36)

2.4.2 Consensus guidelines

The improvement in treatment results necessitated the need of updating the available guidelines. Two consensus guidelines, both funded from the public sector to assure independence from the pharmaceutical industry, were provided in 2002 (38, 39). According to the NIH and French consensus guidelines all patients with chronic hepatitis C and an increased risk of developing cirrhosis are potential candidates for antiviral therapy. Patients with genotype 2 and 3 should be treated for 24 weeks. The NIH guidelines state that sustained virological response for patients with genotypes 2 or 3 are similar to PEG-IFN-α and ribavirin or standard IFN-α and ribavirin therapy. Thus standard IFN-α and ribavirin can still be used in treating patients with genotypes 2 or 3. Since low dosed ribavirin was found to be as effective as 1000-1200 mg daily, 24 weeks of treatment and an 800 mg dose of ribavirin is the new standard for patients with genotype 2 and 3. According to the consensus statements, patients with genotype 1 should be treated with PEG-IFN-α in combination with 1-1.2 g ribavirin for 48 weeks.

Currently, much attention is focused on patient and virus characteristics to enable identification of patients that will or will not benefit from treatment. Major pre-treatment factors influencing response rates to combination therapy are HCV genotypes and the degree of fibrosis. Viremic level, age and gender are of less importance in pegylated IFN-α therapy (7, 31, 33, 35, 36, 40).

Since genotype 1 is the predominant genotype in many parts of the world and the improved sustained response rate with PEG-IFN-α -ribavirin therapy is only about 50%, more effective treatment for this large group is desirable.

Patients with cirrhosis form another group that currently is in need for better treatment options. In view of the fact that morbidity and mortality of chronic hepatitis C is predominantly in this category of patients it is of note that most of the large trials of interferon-ribavirin combination as well as those assessing PEG-IFN-α with or without ribavirin comprised only a minority of patients with cirrhosis. Responses in cirrhotic patients are generally less than in non-cirrhotic patients although treatment with PEG-IFN-α has diminished these differences. Heathcote et al. treated 271 patients with bridging fibrosis or cirrhosis with IFN-α2a versus two schedules of PEG-IFN-α2a.

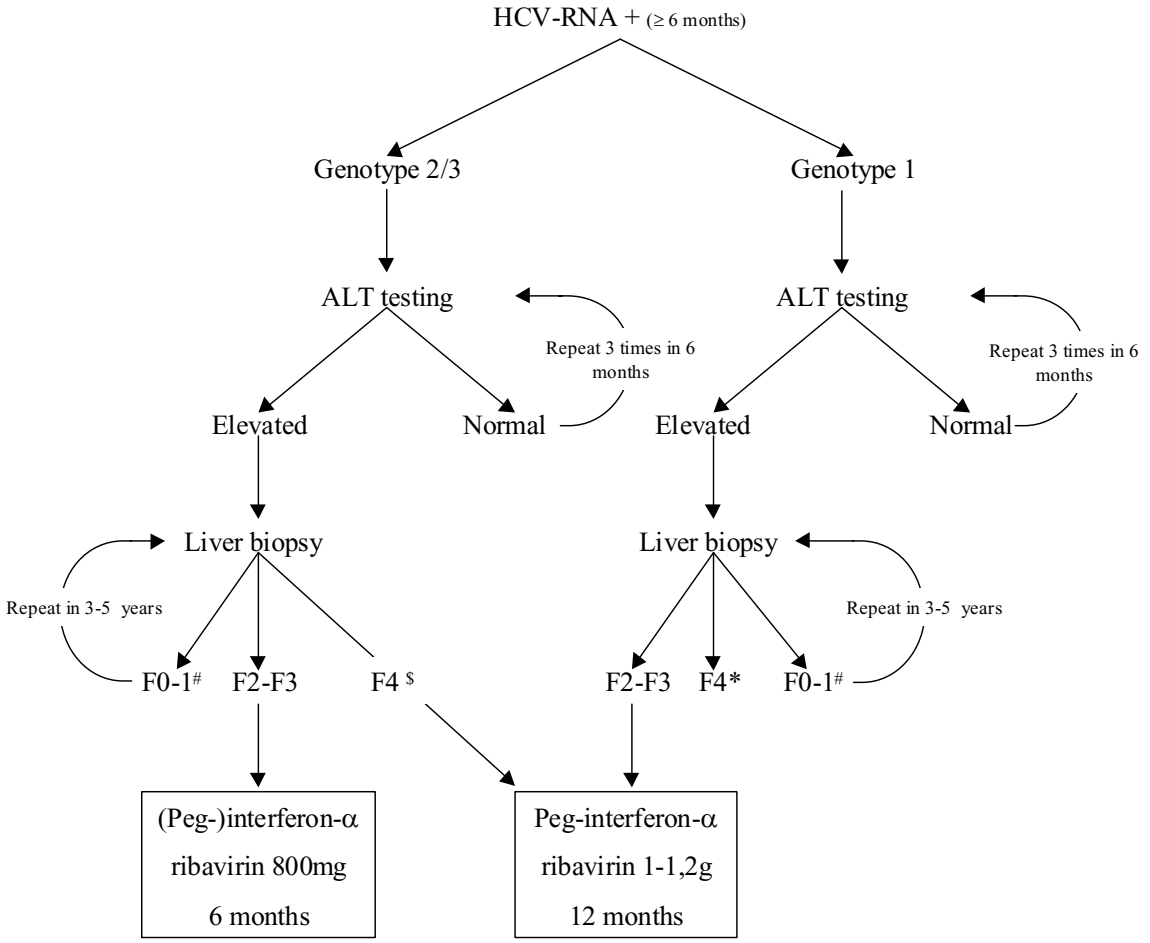

Figure: Individualised approach to treatment of naive patients with chronic hepatitis C according to the authors' interpretation of consensus statements (38, 39). Treatment is recommended for patients with an increased risk of developing cirrhosis. # Patients with F0-1 (no or minor fibrosis) have a limited risk of developing cirrhosis and should only be treated when highly motivated after complete information about side effects. Re-evaluation within 3 to 5 years is recommended. *Patients with genotype 1 and cirrhosis (F4) should in view of the limited chance for sustained virological response be treated in an experimental setting. $Patients with genotype 2 or 3 in whom cirrhosis is likely should be treated for 1 year with PEG-IFN-α and standard dosed ribavirin.

Sustained response rates of 12% in genotype 1 and 51% in genotype non-1 infection were found (32).

2.5 TREATMENT TOMORROW: FURTHER THERAPEUTIC OPTIONS FOR GENOTYPE 1 AND CIRRHOSIS

As described above, treatment with high dose induction IFN-α and prolonged daily IFN-α plus ribavirin was successful in the majority of patients selected for treatment resistant characteristics. Given the increased effectiveness of PEG-IFN-α, a schedule of high dose PEG-IFN-α combined with ribavirin for a prolonged period might increase response rates. For PEG-IFN-α induction, the optimal PEG-IFN-α dosage has to be determined by measuring both the levels of IFN-α and the anti-viral effects in vivo during therapy. Currently, a Dutch multicentre randomised trial (PIT study) comparing PEG-IFN-α induction and prolonged PEG-IFN-α - ribavirin combination treatment to standard therapy in previous non-responders is under way. Until now, randomised studies comparing PEG-IFN-α induction to standard PEG-IFN-α therapy have not been published yet.

In naive patients, interferon based treatment strategies can possibly be further tailored to each individual patient according to early response dynamics. By measuring the decline in viral load in each patient in the first weeks of treatment, dose and treatment duration can possibly be optimised.

Completely different forms of medications, the so-called proteinase-inhibitors, being investigated for their (additional) anti-HCV effect. These drugs can be taken orally and appear highly effective. Phase II - III clinical trials are currently underway; results are pending. Still it is unlikely that such new therapy will be available for routine clinical use within the next 3 to 5 years.

2.6 CONCLUSIONS AND RECOMMENDATIONS

In the figure our opinion on how to treat naive patients with chronic hepatitis C is shown. Treatment is recommended for patients with an increased risk of developing cirrhosis. Therefore patients with F0-1 (no or minor fibrosis) should only be treated when highly motivated after complete information about side effects Genotype 2 and 3 patients without cirrhosis are optimally treated for 24 weeks with (PEG)-IFN-α in

combination with a low dose (800 mg/d) of ribavirin resulting in an 80% sustained response rate. Patients with cirrhosis and genotype 2 and 3 have a limited chance for sustained response when treated for 24 weeks and should therefore be treated with PEG-IFN-α2a/2b for 48 weeks in combination with 1000-1200 mg/d of ribavirin. Genotype 1 patients are preferably treated with PEG-IFN-α2a/2b in combination with 1000-1200 mg/d of ribavirin for 48 weeks. “Difficult to treat patients” with either genotype 1, non-response to prior treatment, cirrhosis or a combination of these characteristics who, despite notable advances, still have a chance of less than 50% for sustained virological response might benefit from induction and prolonged PEG-IFN-α treatment and should preferably be treated in an experimental setting. Further research is needed to optimise treatment schedules and to investigate new antiviral drugs in clinical practice; Dutch co-operative studies have the potential to solve some of these outstanding issues.

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Chapter 3

High sustained virological response in chronic hepatitis C by combining induction and prolonged maintenance therapy

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3.1 ABSTRACT

Background: Chronic hepatitis C patients with genotype 1 infection, liver cirrhosis, high viral load, or those who have not responded to anti-viral treatment in the past have limited chances of clearing the virus, even with pegylated interferon-ribavirin therapy. In this study we treated such patients with a treatment schedule that combines high dose induction Interferon (IFN), prolonged daily IFN and ribavirin treatment.

Methods: Twenty-four consecutive patients were included in this study with either genotype 1 infection, cirrhosis, previous non-response to IFN or a combination of these poor-response characteristics. Patients were treated with 10 million units (MU) of IFN daily for 4 weeks followed by 5 MU/d until week 24, 3 MU/d until week 52 and 3 MU thrice weekly until week 76 in combination with 1-1,2 gram ribavirin daily. HCV RNA levels were assessed weekly until week 4 and at least once every 3 months thereafter, by a validated assay with a detection limit below 500 copies/ml.

Findings: Both intention to treat (ITT) and per protocol (PP) analysis showed a high sustained virological response (ITT 67%, PP 80%). A virological response occurred rapidly (before 8 weeks of treatment) in all patients with a sustained response. Relapse after stopping therapy was observed in only 5%. Side-effects were observed frequently, 6 patients had to be hospitalised.

Interpretation: With this new treatment regimen that combines induction-and prolonged daily interferon treatment with ribavirin it seems possible to eliminate hepatitis C virus in the majority of patients that have an *a priori* limited chance of sustained response. Further clinical evaluation of intensive interferon and ribavirin combination therapy (now also including PEG-interferon) is recommended in centres that can provide close patient monitoring and experienced hepatological support.

3.2 INTRODUCTION

In the first decade after the licensure of interferon as therapy for chronic hepatitis C, results of treatment have shown stepwise improvement, firstly by prolongation of treatment from 6 to 12 months, secondly by combination of interferon with ribavirin (1, 2), and thirdly by the current replacement of standard interferon by pegylated interferon (3). With current pegylated interferon-ribavirin combination therapy , a sustained clearance of the virus is observed up to 65% of patients (4, 5).

However, for patients that have unfavourable patient or virus characteristics (cirrhosis, genotype 1 or 4, non-response to previous antiviral therapy) the sustained response rate is much lower and ranges around 40% (4, 5).

Failure to clear the virus can be observed at 3 different phases: during the initial (4-12 weeks) treatment period (non-response, 40-50% of patients), during maintenance treatment after an initial response (breakthrough, about 10-20% of patients), and after treatment discontinuation (relapse, 25-50% of patients) (6, 7).

This study focuses on patients with a limited chance to response to standard combination therapy (2, 4, 5). In 1998 we altered the standard treatment schedule in order to reduce the failure to respond at each of the three different phases. High-dose daily induction therapy was given to optimise the initial response (8, 9). Interferon was administrated daily in order to obtain a more constant drug level that would optimise viral inhibition and minimise breakthrough. The importance of constant antiviral pressure (10) has become clearer since the introduction of pegylated interferons resulting in a marked increase in sustained response rates (3-5). Lastly: in order to prevent relapse, all patients were advised to prolong the standard treatment period of 12 months to a total duration of 18 months (11-13).

3.3 MATERIALS AND METHODS

3.3.1 Study population

Twenty-four consecutive patients with documented chronic hepatitis C were included in this study. Patients were eligible if their maximal chance of a sustained response with 1 year of interferon-ribavirin combination therapy was less than 30% according to major studies (6, 7, 14). Such patients had genotype 1, cirrhosis, non-response to IFN or combinations of these criteria. Non-response to prior therapy was defined as HCV-RNA PCR positivity after at least 12 weeks of IFN therapy or 26 weeks of IFN-Ribavirin combination treatment. Other clinical relevant concomitant diseases were excluded as previously described (15).

3.3.2 Treatment schedule

All patients were admitted for the first 7 days of treatment and thereafter intensively monitored at the outpatient clinic. Patients received 10 million units (MU) of alpha-interferon (Intron A, Schering-Plough, Kenilworth, NJ, USA) daily for 4 weeks followed by 5 MU daily until week 24, 3 MU daily until week 52 and 3 MU thrice weekly until week 76. Ribavirin was given twice daily orally in divided doses of 1,000 mg (weight <75 kg) or 1,200 mg (weight >75 kg).

Dose reduction was based on clinical intolerance or granulocytopenia below 0.5 x $10^6/mm^3$. In such cases interferon was reduced to the next level of the treatment schedule, ribavirin was reduced by steps of 200 mg/day. Combination therapy was discontinued in case of virological non-response, defined as detectable HCV RNA at week 12 and 16.

3.3.3 Detection of HCV RNA

Blood was drawn at baseline and at week 1, 2, 3, 4, 8, 12, 16, 24, 32, 48, 52, 76, 88, 100 and 104. Blood samples were collected in plasma preparation tubes (Becton-Dickenson, Plymouth, UK) which were spun directly after collection in order to avoid RNA breakdown. A qualitative HCV RNA assay was used to asses viremia (Cobas Amplicor HCV test, Roche Diagnostics, Almere, The Netherlands). The test was

found to have a comparable sensitivity to the NGI assay (less than 500 copies/ml when tested in the Eurohep panel).

3.3.4 Descriptive analysis

The percentage of HCV RNA negativity at various time points was calculated for the total population (Intention To Treat analysis) and for those who completed the treatment and follow-up according to protocol (Per Protocol analysis). The initial response rate was calculated at the end of the induction phase (4 weeks). The breakthrough rate was calculated at the end of treatment for those reaching HCV RNA negativity during treatment, and the relapse rate at 6 months of follow-up for those who were HCV RNA negative at the end of treatment.

3.4 RESULTS

Seventeen patients were males, seven were females; the mean age was 47 years. Fifteen patients were of European descent, and 9 were born outside Europe (Surinam: 3, Pakistan: 2, and Far East: 4).

All patients were estimated to have less than 30% chance of a sustained response of standard interferon-ribavirin therapy, based on their baseline characteristics (fig 1). Of the total group 11 (46%) had genotype 1, 11 (46%) had cirrhosis, of whom 7 could be classified as advanced cirrhosis (serum bilirubin >17 μmol/l or platelets <130x10^6/mm^3) (16), and 15 were non-responders; 10 patients had 2 and 3 patients had 3 poor-response criteria.

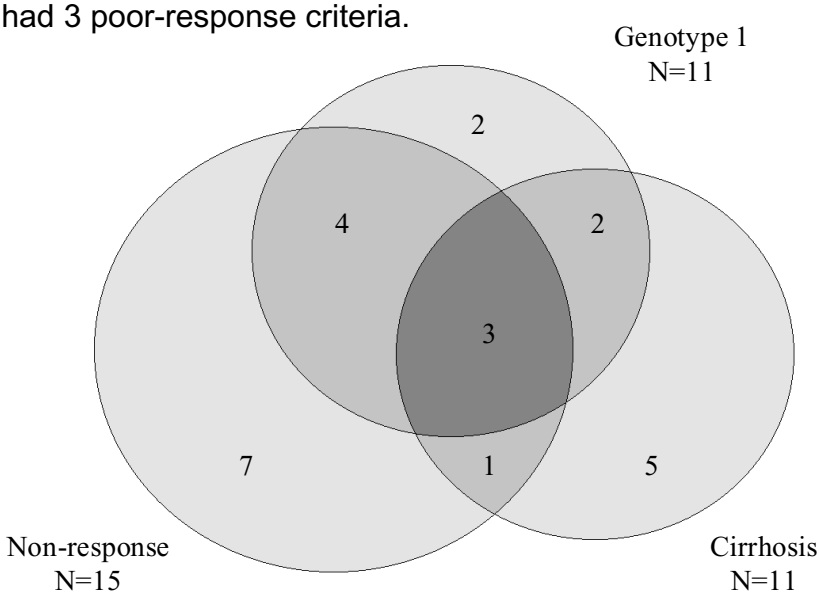

Figure 1: Van diagram dysplaying the study population by characteristics associated with poor response.

In addition, 17 patients had a high viral load (> 2.0 x 10^6 copies/ml (2)) at baseline. The median viral load was 7.4 x 10^6 copies/ml. The intention to treat population (ITT) consisted of 24 patients, 20 completed the study according to protocol (PP) (Fig 2). The initial response rate at 4 weeks (HCV-RNA below the threshold of sensitivity) was 67% (16/24) by intention to treat analysis and 75% (15/20) by per protocol analysis. This high initial response rate coupled to an additional response of 10% during treatment and a very low breakthrough of 5%, led to an end of treatment response of 75% (18/24,ITT) to 85% (17/20,PP). Due to the very low relapse rate (5%) the sustained response rate was 67% (16/24,ITT) to 80% (16/20,PP) (Fig 3).

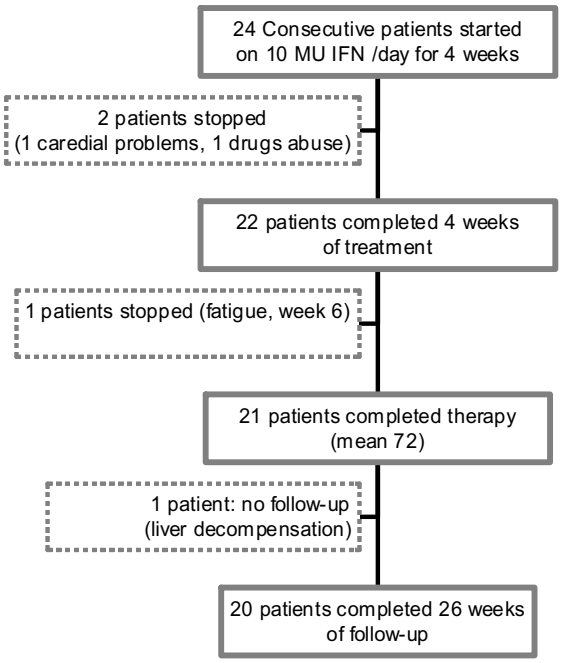

Figure 2: Trial profile. The Intention To Treat population included all 24 patients who started treatment. The Per Protocol population comprised the 20 patients who completed treatment and follow-up according to the protocol.

Table I shows the results of treatment according to the baseline characteristics. According to the per protocol analysis all predefined sub-groups had a sustained response rate of 50% or more. For this intensive treatment schedule a sustained virological response was observed in almost all patients without cirrhosis (ITT: 92%; PP: 100%); only the patient characteristic, cirrhosis, was a significant predictor for not reaching a sustained viral response ($p<0,05$ fisher-exact test);

Adverse events led to hospitalisation in 6/24 patients (hepatic decompensation, de novo diabetes mellitus in association with jaundice and ascites, cardiac complaints, weight loss of more than 10%, infected injection site and decreased vision); 5 out of

these 6 patients had cirrhosis. Combination treatment was discontinued in one of the hospitalised patients prior to week 52; discontinuation of therapy occurred in 3 non-hospitalised patients due to drug abuse, cardiac complaints and fatigue with depression in the first 8 weeks.

The dose of Interferon was reduced in 8/24 and a reduction of ribavirin was observed in 5/24 patients; in the group of cirrhosis treatment was discontinued or reduced in 7/11 versus 4/13 in the non-cirrhotic group.

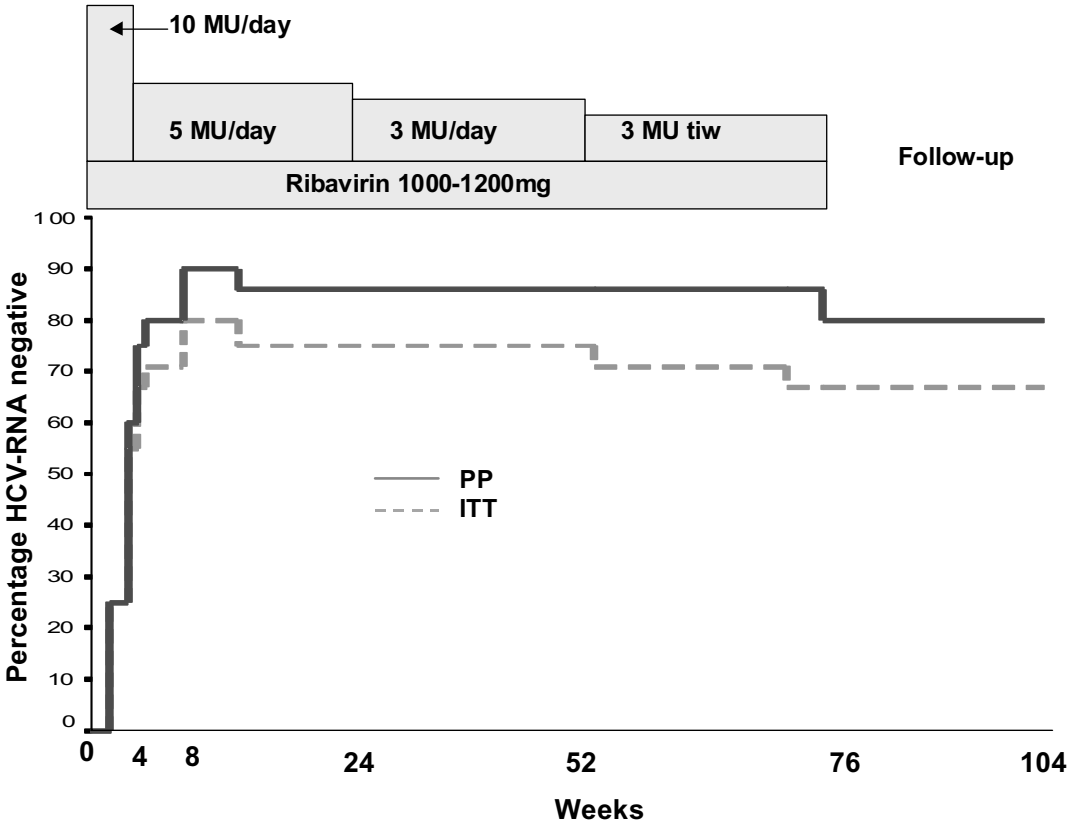

Figure 3. Study profile and percentage of patients with HCV-RNA below detection during treatment and follow-up by intention to treat analysis (ITT) and by per protocol analysis (PP).

Tabel I **Response rates at different key points by baseline characteristics.**

		Percentage HCV RNA negativity (Intention To Treat analysis)				*Percentage HCV RNA negativity (Per Protocol analysis)*		
	N=	**Week 4**	**End of Treatment**	**End of Follow-up**	**N=**	**Week 4**	**End of Treatment**	**End of Follow-up**
ALL	**24**	**16 (67%)**	**18 (75%)**	**16 (67%)**	**20**	**15 (75%)**	**17 (85%)**	**16 (80%)**
Genotype:								
Non-1	13	11 (85%)	11 (85%)	10 (77%)	11	10 (91%)	10 (91%)	10 (91%)
1	11	5 (45%)	7 (64%)	6 (55%)	9	5 (55%)	7 (78%)	6 (67%)
Cirrhosis:								
Absent	13	11 (85%)	12 (92%)	12 (92%)	12	11 (92%)	12 (100%)	12 (100%)
Present	11	5 (45%)	6 (55%)	4 (36%)	8	4 (50%)	5 (68%)	4 (50%)
Response to previous Rx:								
Nonresponder	15	9 (60%)	10 (66%)	10 (66%)	13	9 (69%)	10 (77%)	10 (77%)
Relapser/naïve	9	7 (77%)	8 (88%)	6 (67%)	7	6 (86%)	7 (100%)	6 (86%)

3.5 DISCUSSION

This exploratory study shows that in patients with chronic hepatitis C and a low *a priori* chance of a sustained response with 1 year of standard interferon-ribavirin therapy a high rate of sustained response can be obtained. Our favourable results (sustained virological response in 67% (ITT)) contrast with the 28% sustained virological response rate in patients with unfavourable characteristics as reported in the so-called pivotal studies (6, 7) and with the approximately 40% sustained virological response rate reported for pegylated interferon-ribavirin combination therapy (4, 5). Therefore our results remain also of interest for the immediate future now pegylated interferon is replacing standard interferon in many schedules.

The basis for the high-sustained response rate was the 70% serum negativity for HCV RNA by PCR at 4-12 weeks of treatment associated with daily high dose induction therapy. High initial response rates have been described previously by Japanese investigators (8). The median duration of induction therapy needed to reach viral negativity using daily 5-10 MU of interferon combined with ribavirin is estimated to be 6-9 weeks in previously untreated genotype 1 patients (17). Induction therapy was applied in our study for 24 weeks in contrast to many other studies that changed the daily high dose of induction therapy to maintenance therapy after 2-8 weeks. According to viral kinetics, the effect of induction therapy can be lost by early lowering of the dose; not only is the initial drop in viral load partly reversed, the slope of the second phase can also become more flat due to the diminished inhibitory effect of interferon on viral production (18).

During maintenance therapy breakthrough is apparently better prevented by daily interferon than by interferon thrice weekly. This effect of the more constant presence of interferon is also clearly demonstrated by the use of pegylated interferon, leading to increased end of treatment responses (3, 10, 19).

The duration of the maintenance therapy is likely a third independent factor affecting the sustained response rate (12). Relapse rates for patient with undetectable HCV-RNA are about 50% for 6 months therapy, 25% for 12 months and preliminary data indicates less

than 10% for 18 months of combination interferon-ribavirin therapy (15).

Our study is one of the first in which daily high dose induction for more than 12 weeks was followed by daily interferon therapy to prevent breakthrough till 52 weeks and prolonged till 72 weeks to prevent relapse. In these three aspects it differs from major studies assessing induction therapies. In two European multicenter studies (20, 21) improvement in virological response of high dose daily induction interferon therapy plus ribavirin could only be found in patient subgroups. Both studies were conducted in treatment naive patients. Daily high induction therapy was changed after 2-4 weeks to thrice-weekly interferon administration. The total treatment period was limited to only 26-38 weeks. In an international multicenter study conducted in North America and Europe (22) daily induction therapy was given for 8 weeks and maintenance therapy with daily interferon was reduced at 24 weeks to interferon thrice weekly for an additional 24 weeks. In these studies the initial higher response rates observed with induction therapy faded at the end of treatment and disappeared at the end of follow-up. Our exploratory study strongly suggest that daily induction therapy might be beneficial for patients with a low chance of a sustained response but only when combined with adequate maintenance therapy of long duration. This hypothesis needs further testing in a large controlled trial.

The intensive treatment regiment in our study was expected to be associated with clinical significant non-compliance and/or adverse events. The incidence of non-compliance and intolerability could be kept low by intensive monitoring. Clinically relevant adverse events occurred mainly in patients with advanced cirrhosis.

This early report, in a relatively small number of patients, suggests that the large majority of patients with chronic hepatitis C can be cured. Further clinical evaluation of intensive (peg-) interferon/ribavirin combination therapy is recommended in centres which can provide close monitoring and experienced hepatologic support.

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Chapter 4

High dose induction and prolonged daily interferon plus ribavirin therapy improves effectiveness in HCV patients with unfavourable baseline characteristics

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4.1 ABSTRACT

Background and aims: Results of interferon-based treatments of chronic hepatitis C have markedly improved except for patients with unfavourable characteristics. To assess whether such patients can respond to modified interferon regimens we performed a meta-analysis on data of individual patients treated in our center with high-dose induction and prolonged daily interferon plus ribavirin.

Methods: Fifty-four patients selected for poor-response characteristics such as genotype 1, cirrhosis or non-response to previous therapy were enrolled sequentially to one of three therapy schedules. Treatment consisted of 10 MU interferon-a2b (IFN) daily for 2-4 weeks followed by daily 3-5 MU IFN until week 52 and 3 MU daily or thrice weekly until week 76 in combination with ribavirin.

Results: Sustained virological response rate varied between 75-83% for patients with 1 unfavorable characteristic (n=23). In patients with combination of such characteristics, sustained virological response rate were 60%, but only and 21% for genotype 1 and cirrhosis (n=14). Six patients (11%) stopped therapy because of adverse effects; clinically serious adverse events occurred in 2/7 patients with advanced cirrhosis.

Conclusion: High-dose induction and prolonged daily interferon-ribavirin therapy is an acceptable and potentially highly effective treatment modality for selected patients with unfavourable characteristics. Patients with either genotype 1, cirrhosis or prior non-response can have sustained virological response rates approaching those of pegylated-interferon ribavirin combination therapy in genotype 2/3 when treated with high-dosed and prolonged interferon in combination with ribavirin. However, in patients with combinations of these unfavorable criteria, sustained virological response rates progressively decrease.

4.2 INTRODUCTION

The interferon-based treatment of chronic hepatitis C has made remarkable progress over the past ten years. Since the introduction of pegylated interferon (PEG-IFN-α) ribavirin combination therapy, results of large international trials indicate cure-rates of 77-82% in genotype 2 and 3 infection. However, the predominant genotype in many parts of the world is genotype 1 and the sustained response rate in this type of infection is still only 42-46% (1, 2).

Morbidity and mortality of chronic hepatitis C is predominantly in patients with cirrhosis (3). Responses in patients with cirrhosis are generally less than in those without cirrhosis although treatment with pegylated interferon diminishes the difference (4). It is therefore of note that the large trials of interferon-ribavirin combination as well as those assessing PEG-IFN-α with or without ribavirin comprised only a small number of patients with cirrhosis (1, 2, 5, 6).

Earlier, we reported an improved efficacy with high induction dose (10 MU daily) and prolonged (12-18 months) interferon therapy in combination with ribavirin in a small pilot study (7). To assess whether patients with unfavorable characteristics might benefit from such a modified treatment regimen in subsequent experience, we analyzed individual data of all patients with unfavourable baseline characteristics such as genotype 1, cirrhosis, non-response to IFN-α or combinations of these criteria treated in our centre with high-dose induction, daily and prolonged daily interferon-ribavirin combination therapy.

Three treatment schedules of high dose induction and prolonged daily IFN-α in combination with ribavirin were used. The initial study (8) was designed to explore by viral kinetics the IFN-α induction dose leading to a maximal initial response. The patients were treated with a relative intensive and long induction regimen of IFN-α. Subsequently, two other regimens were designed to preserve the favourable results in terms of viral kinetics but to reduce the intensity in terms of side effects and costs. In all three protocols the administration of IFN was given daily during at least 12 months in order to maintain a constant drug level and thereby maximise viral inhibition and to minimise virological breakthrough. Thereafter, treatment was continued up to 76 weeks in an attempt to reduce viral relapse (9, 10).

4.3 PATIENTS AND METHODS

4.3.1 Study population

Between February 1998 and March 2001, 55 consecutive patients with documented chronic hepatitis C were enrolled in a program of high dose induction and prolonged daily IFN-α in combination with ribavirin therapy. The ethical committee approved the study and patients gave written informed consent for inclusion. Patients were eligible if their maximal chance of reaching a sustained response with standard therapy according to the 1998 EASL consensus statement (11) was considered less than 30%. Such patients had genotype 1 or 4, cirrhosis, non-sustained response to prior treatment or combinations of these criteria. In this study non-sustained response was defined as a positive HCV-RNA serum test after at least 6 months of IFN-α monotherapy (3 MU t.i.w.) or a relapse after 12 months of IFN-α ribavirin combination treatment. Patients with clinical relevant concomitant diseases were excluded according to criteria previously described (10, 12).

4.3.2 Study design

Patients were allocated successively to receive one of the following treatment regimens (figure 1). The first 24 consecutive patients received 10 million units (MU) IFN-α2b (Intron A, Schering-Plough, Kenilworth, NJ, USA) daily for 4 weeks followed by 5 MU daily until week 24, 3 MU daily until week 52 and 3 MU thrice weekly until week 76 (group A). The next fifteen patients were assigned to receive 10 MU IFN-α daily for 10 days followed by 3 MU daily until week 52 and 3 MU thrice weekly until week 76 (group B). The following 16 patients were assigned to receive 10 MU IFN-α daily for 2 weeks followed by 5 MU daily until week 4 and 3 MU per day until week 76 (group C). Ribavirin was supplied to all patients as 200-mg capsules given orally in 2 divided daily doses of 1,000 mg (weight <75 kg) or 1,200 mg (weight >75 kg) throughout the entire treatment period.

All patients were admitted to the ward for the first 7 days of treatment and thereafter intensively monitored on an outpatient basis. Clinical and biochemical assessments were conducted every 4 to 6 weeks during therapy and during a 6-month follow-up period.

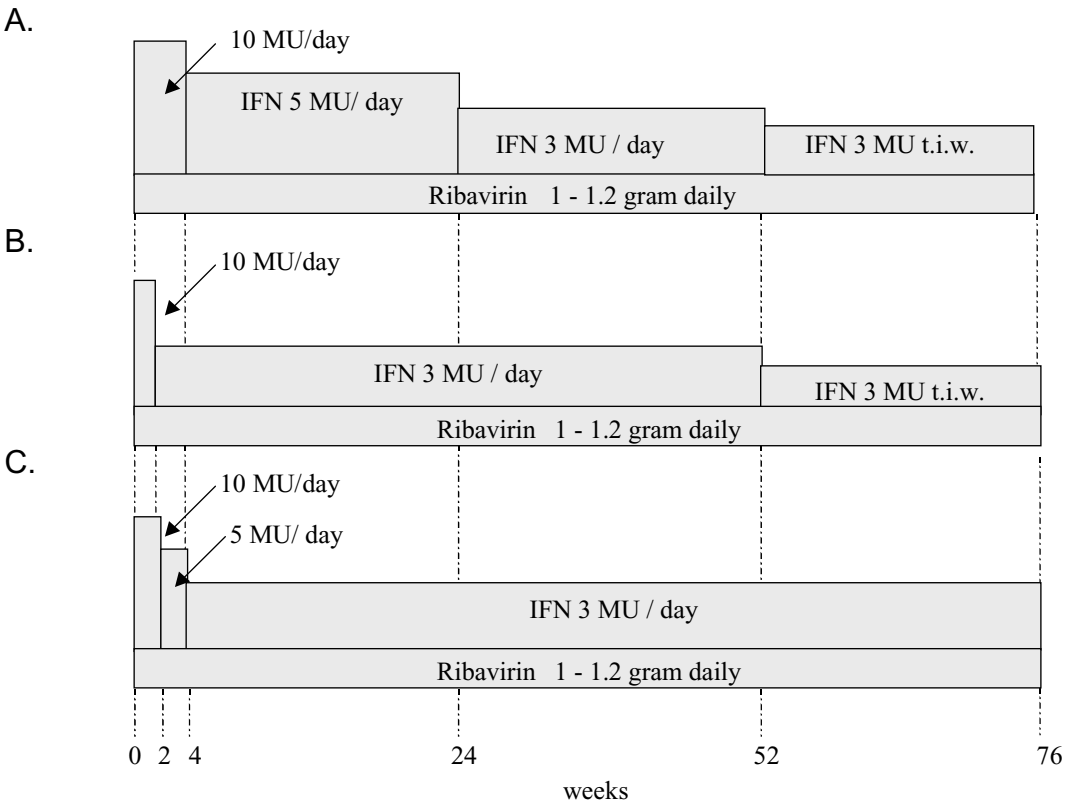

Figure 1. Schematic overview of 3 treatment arms.

The first 24 consecutive patients were included in group A, the next fifteen patients were assigned to group B and the last 15 patients were assigned to group C.

Dose adjustment was based on clinical intolerance, granulocytopenia below 0.5 x $10^6/mm^3$ (IFN-α) or hemoglobin below 6 mmol/l (ribavirin). In such cases interferon was reduced to the next level of the treatment schedule, ribavirin was reduced by steps of 200 mg/day. Combination therapy was discontinued in case of virological non-response, defined as detectable HCV RNA at week 12.

4.3.3 Histology

All patients underwent liver biopsy before the start of therapy. An experienced pathologist who was unaware of the clinical outcome evaluated each liver biopsy for the presence of cirrhosis, comparable to Metavir F4.

4.3.4 Virology

Blood was drawn at baseline and at week 4, 12, 16, 24, 48, 76, 88 and 100. Blood samples were collected in plasma preparation tubes (Becton-Dickenson, Plymouth,

UK), which were spun directly after collection in order to avoid RNA breakdown. A qualitative HCV RNA assay was performed in a single local laboratory to assess viremia (Cobas Amplicor HCV test, Roche Diagnostics, Almere, The Netherlands). Genotypes were identified before the start of treatment by in-house sequence analysis.

4.3.5 Descriptive analysis

The primary end point of this analysis was the rate of sustained response after a 24-week treatment free follow-up period. Results were calculated for the total population (Intention -to-treat analysis). The breakthrough rate was calculated at the end of treatment for those reaching HCV RNA negativity during treatment and the relapse rate at 24 weeks of follow-up for those who were HCV RNA negative at the end of treatment.

4.3.6 Statistics

Data were analysed using SPSS for Windows (Version 10.1 SPSS Inc, Chicago, IL, USA). Percentages are given with 95% confidence intervals (CI).

4.4 RESULTS

4.4.1 Patient characteristics

In table 1, baseline characteristics of the study population are shown per treatment schedule and for the whole group. One patient, who was assigned to treatment regimen C and who had a sustained response, did on retrospect not comply with the inclusion criteria associated with poor response and was excluded from the analysis. The intention to treat population (ITT) consisted of 54 patients of whom 44 completed the study (81%) according to the protocol.

The proportion of cases with cirrhosis was 46%. Genotype 1 was the predominant genotype (69%). More than half of the patients (54%) had a failure to prior IFN treatment. Fourteen patients (26%) had both a genotype 1 and cirrhosis. The majority of patients (81%) had a high viral load ($\geq 2 \times 10^6$ copies/ml).

4.4.2 Outcome

In table 2, intention-to-treat results are shown per treatment arm and for the total study population. The ITT sustained response rates were 67% (95% CI: 45-84%), 53% (95% CI: 27-79%), 47% (95% CI: 21-73%) for respectively treatment regimen A, B, C. The outcomes for the three treatment regimens were not significantly different according to multivariate logistic regression analysis performed to correct for differences in baseline characteristics and study groups.

Table 1. Baseline characteristics of the total study population and by treatment regimen.

	Treatment regimen			
	Group A	Group B	Group C	All
Patients	24	15	15	54
Mean age (years ± SD)	47 ± 10	45 ± 7	45 ± 10	46 ± 9
Sex (M / F)	17 / 7	11 / 4	12 / 3	40 / 14
ALT (mean IU ± SD)	103 ± 83	125 ± 78	117 ± 51	113 ± 73
Histology				
Cirrhosis (%)	12 (50%)	1 (7%)	12 (80%)	25 (46%)
Serum HCV-RNA				
Median (copies/ml)	$7.4x10^6$	$3.2x10^7$	$7.9x10^6$	$1.4x10^7$
≥ 2 x 10^6 (%)	17 (71%)	14 (93%)	13 (87%)	44 (81%)
Genotype				
1 and 4* (%)	12 (50%)	15 (100%)	10 (67%)	37 (69%)
2 & 3 (%)	12 (50%)	0 (0%)	5 (33%)	17 (32%)
Non-responders	15 (63%)	11 (73%)	3 (20%)	29 (54%)

Plus-minus values are means ± SD. For details of the treatment regimen see figure 1. For details of "overlap in unfavourable baseline characteristics" see figure 2.* only one patient had genotype 4.

The overall sustained virological response rate was 57% (95%-CI: 43-71%) and varied between 75-83% for patients with 1 unfavorable characteristic; 25-60% for patients with 2 unfavorable characteristics, and 17% for those with 3 unfavorable factors (figure 2).

Ten patients had detectable HCV RNA at week 12; in whom treatment was discontinued prematurely (19%; 95% CI: 9-31%). Two patients experienced a virological breakthrough (4%) and 1 patient was HCV RNA negative at 72 weeks but relapsed in the 24 weeks of untreated follow-up (2%). Four patients were not compliant: they stopped in the first four weeks after discharge from the hospital. In six patients (11%; 95% CI: 4-23%), therapy was stopped because of adverse effects (hepatic decompensation (n=2), depression (n=2), cardiac complaints (n=1) and Staphylococcus sepsis (n=1)). Both patients that developed decompensated liver disease, had advanced cirrhosis: according to the Child-Pugh classification their baseline scores were 6, but both had elevated bilirubin and low platelets (3). All other adverse effects occurred within the first 8 weeks of treatment.

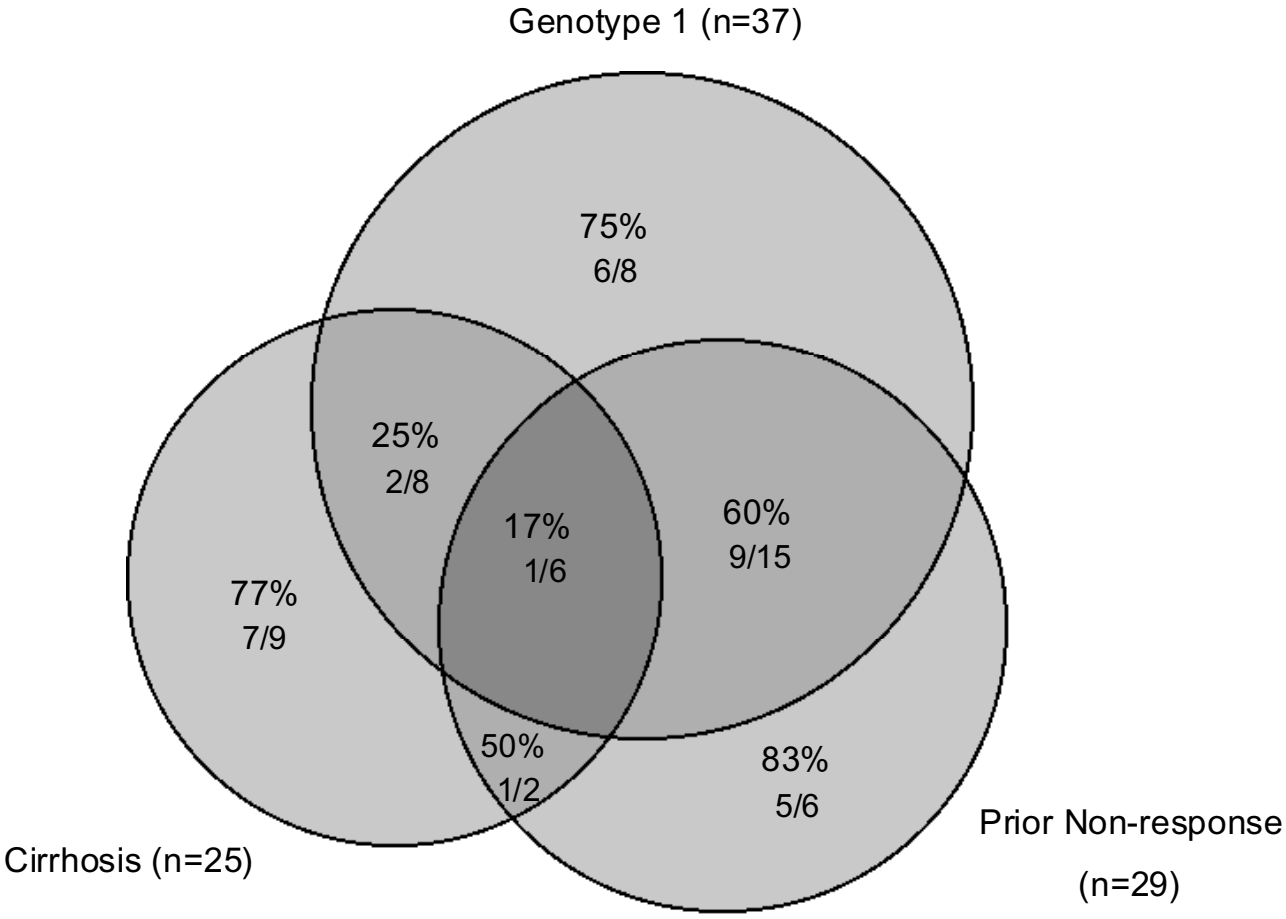

Figure 2. "Van diagram" displaying the study population: number of unfavorable baseline characteristics and accompanying sustained response rates (%).

For example, the study population consisted of thirty-seven patients infected with genotype 1; eight of them also had cirrhosis, fifteen were also prior non-responders. Six patients had three unfavorable characteristics: genotype 1, cirrhosis and prior non-response.

The sustained response rate varied between 75-83% for patients with 1 unfavorable characteristic; genotype 1: 75%, cirrhosis: 77%, prior non-response: 83%. For patients with 2 unfavorable characteristics sustained response rates were between 25-60%: genotype 1 and cirrhosis: 25%, genotype 1 and prior non-response: 60%. In the 6 patients with 3 unfavorable characteristics the sustained response was only 17%.

4.5 DISCUSSION

This study documents the efficacy rates of high dose induction and prolonged daily interferon plus ribavirin treatment in patients with baseline characteristics associated with poor response. The overall sustained virological response rate was 57% (95%-CI: 43-71%) and varied between 75-83% for patients with 1 unfavorable characteristic. Results with modern schedules of PEG-IFN-α and ribavirin yield similar percentages, but the baseline characteristics in our study population were vastly more unfavourable. In patients with combinations of these unfavourable criteria, sustained virological response rates were considerably decreased: 25-60% for patients with 2 unfavorable characteristics, and 17% for those with 3 unfavorable factors.

The studies were originally designed to explore the optimal IFN-α induction dose by viral kinetics (7, 8, 13). The first group of patients was treated with an intensive schedule resulting in a sustained response rate of 67% (7). The next two strategies were attempts to decrease morbidity and cost without loosing efficacy. However, by shortening the induction period adverse effects were somewhat decreased but also effectiveness was reduced in some patients (13). No clear advantage of either treatment schedules B or C was found over treatment schedules A.

How can these favourable results be explained? First, in all three treatment groups, therapy was started with a daily high-dose interferon “induction” period in order to reach early HCV-RNA negativity. Early viral response is a strong predictor for sustained response (1, 7, 14). In this study, 17 patients were HCV-RNA negative after 4 weeks of treatment. Fourteen of them had a sustained response (82%) as compared to 17 out of 37 patients (46%) who had their HCV RNA response after week 4. Second, given the short half-live of IFN-α, daily IFN-α injections were given to obtain a more constant drug level in order to optimise viral inhibition and minimise viral breakthrough. The importance of constant antiviral pressure has become more clear since the introduction of PEG-IFN-α with its prolonged half-life (1, 2). Third, we prolonged the treatment duration in order to prevent relapse (9, 10). As a result, the relapse rate could be reduced to less than 5% in this study.

The intensive treatment schedules were expected to be associated with an increased rate of intolerance and side effects. Due to intensive monitoring the rate of non-compliance could be kept low. Clinically serious adverse effects occurred in 2 patients with impaired liver function due to advanced cirrhosis. Since the sustained response rate in patients with advanced cirrhosis was limited to 1 out of 7, we conclude that risks outweigh benefits in this category of patients.

How should these results be implemented into future treatment schedules? The results of this study show that patients with either genotype 1, cirrhosis or non-response to previous therapy can have sustained virological response rates approaching those of pegylated-interferon ribavirin combination therapy in genotype 2/3 when treated with high-dosed and prolonged interferon in combination with ribavirin. However, in patients with combinations of these unfavorable criteria, sustained virological response rates progressively decrease. Given the augmented efficacy of peginterferon, we think our results should stimulate further research on initial high-dose peginterferon with and without therapy prolongation in combination with ribavirin for the treatment of “difficult-to-treat” hepatitis C patients.

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Chapter 5

High dose vs. standard dose Peginterferon therapy in patients with chronic hepatitis C and interferon non-response

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5.1 ABSTRACT

Background: Standard dosed peginterferon-ribavirin therapy has a limited success in the retreatment of hepatitis C patients who failed to achieve a sustained response with previous therapy. Since daily high dosed interferon induced early clearance of hepatitis C virus RNA in a small number of patients, we investigated high-dose peginterferon in comparison to standard dose peginterferon with regard to early viral clearance and its impact on health related quality of life.

Methods: 40 patients who previously failed interferon therapy were randomly assigned to high-dose Peginterferon-α2b (3.0 µg/kg) or standard dose Peginterferon-α2b (1.5 µg/kg) during the study period of 28 days. Both groups received 1-1.2 gram ribavirin daily. HCV RNA levels were assessed at baseline, t=2, 7, 14, 28 days and 12 weeks. Health related quality of life was assed using the SF-36 at baseline and t=28 days.

Results: 19 patients were randomized to high-dose and 21 to standard dose therapy. At the t=7 the decrease in HCV-RNA compared to baseline for high-dose peginterferon vs. standard dose peginterferon was 1.73 ± 0.42 Log vs. 1.06 ± 0.35 Log (p=0.12) and at 28 days 3.10 ± 0.51 Log vs. 2.78 ± 0.57 Log (p=0.65). Most patients (25/40; 63%); 13/19 high-dose peginterferon treated vs. 12/21 standard dose peginterferon (p=0.53) showed peginterferon sensitivity. Nine high-dose treated vs. 8 standard treated patients reached HCV-RNA levels below the limit of detection (rapid response). Four patients in both groups showed a partial response (p=1.00). Fifteen patients, 6 treated with high-dose peginterferon and 9 with standard dose peginterferon (p=0.53) were non-responders. Quality of life measured decreased considerably in both groups; a dose dependant effect could not be observed.

Conclusions: High-dose peginterferon appears approximately 10% more effective in initially suppressing viral replication, predominantly due to a differential effect in genotype 1 and 4.

5.2 INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a major cause of liver cirrhosis and the most common indication for liver transplantation in Europe and the United States (1). Currently, the most effective available therapy for HCV is the combination of pegylated interferon-α (peginterferon) and ribavirin. With its introduction, sustained virological response (SVR) rates were significantly improved with roughly 10% compared to (non-pegylated) interferon therapy (2, 3). As a consequence, there is interest in re-treating patients who did not achieve SVR with previous non-pegylated therapy. However, preliminary reports on peginterferon-ribavirin re-treatment mention SVR rates of only 7-15% (4, 5).

One of the major causes of treatment failure is incomplete blockage of viral replication resulting in suboptimal decline of HCV-RNA during the first weeks. It is therefore notable that with 4 weeks of daily high dosed interferon therapy, HCV-RNA had become undetectable in 80% of “difficult-to-treat” patients eventually leading to a SVR of 65% (6).

The rate of serum HCV-RNA decline proved to be dose dependant (7, 8). However, initial efficient blockage of viral replication by daily high dosed interferon can be lost later in treatment by premature dose reduction (9) and the same appears to be true for Peginterferon-α2b (8). Whether high-dose peginterferon for a prolonged period has improved efficacy over standard peginterferon therapy remains to be established. In the current study the efficacy of prolonged high-dose Peginterferon-α2b on the early virological response was compared to standard therapy; the safety and impact on “health related quality of life” were also compared.

5.3 METHODS

5.3.1 Participants

Outpatients with chronic hepatitis C infection, elevated serum ALT activity documented on at least 2 occasions, liver biopsy findings consistent with active fibrosis (not obligatory in hemophiliacs) and a history of non-response or relapse to at least 3 months of interferon therapy were potentially eligible for the study. Patients were excluded if they had evidence of severe concomitant illness, if they had used any investigational drug or any systemic anti-viral, anti-neoplastic or immunomodulatory treatment within 12 weeks prior to the first dose of the study, if they were pregnant or breast feeding, if they were under 18 or over 70 years of age, if they had decompensated liver disease or if they refused or were unable to give consent.

5.3.2 Setting and Locations

The patients were recruited from the departments of Gastroenterology and Hepatology of six Dutch health centers. The Central Committee on Research Involving Human Subjects (known by its Dutch initials, CCMO) assessed the study in terms of medical ethics, the ethics committee of each institution evaluated the local feasibility, and all participants gave written informed consent.

5.3.3 Study design and Interventions

This is a randomized controlled investigator-initiated multi center study. Patients were randomly assigned to receive standard combination therapy or an experimental schedule. Standard therapy consisted of Peginterferon-α2b 1.5 μg per kg body weight (PEG-Intron; Schering-Plough, Kenilworth, NJ) once weekly (QW). The experimental schedule started with high-dose Peginterferon-α2b 3.0 μg/kg QW for 12 weeks. Therapy was continued thereafter at a lower dosage for at least 36 weeks. During treatment, ribavirin (Rebetol, Schering-Plough) was administered orally twice daily at a total daily dosage of 1-1.2 gram according to weight.

During the study period, patients were seen every week as outpatients. At each visit blood was drawn to monitor possible hematological side effects. Dose adjustment was based on clinical intolerance, hemoglobin below 5 mmol/l (ribavirin), white blood cell count below 1.5 x 10^9/l (peginterferon), granulocytopenia below 0.75 x 10^9/l

(peginterferon) or platelets below 50 x 10^9/l (peginterferon). In such cases the peginterferon dose was reduced by 50%, ribavirin was reduced by steps of 200 mg/day. If a laboratory adverse event necessitated dose reduction the patient returned for assessment at intervals of every 2 weeks.

5.3.4 Randomization

Participants had an equal probability of assignment to the groups. Randomization was performed in computer-generated blocks, with block sizes of 4 and with stratification according to center, genotype (1 or 4 vs. other) and previous response (responder-relapsers versus non-responders). The randomization assignments were concealed in opaque envelopes. A centrally located data manager opened the envelopes sequentially after the patient's consent form had been approved eligible and signed by the treating physician and the study coordinator. The treating physician was informed of the randomization result by fax-message.

5.3.5 Virology

To assess the effects of treatment, samples for quantitative HCV RNA measurements were drawn during screening, at baseline and during treatment at days 2, 7, 14 and 28. The assay (Cobas Amplicor HCV test, Roche Diagnostics, Almere, The Netherlands) was performed in a single central laboratory (Institute of Virology, Erasmus MC, Rotterdam, The Netherlands). Genotypes were identified before the start of treatment by in-house sequence analysis.

5.3.6 Health related Quality of life

The impact of both treatment arms on the patients' health related quality of life (HRQoL) was measured by the generic Short Form 36 (SF-36) questionnaire during screening (before randomization) and at 28 days. The SF-36 questionnaire measures eight multi-items: physical functioning (PF), role physical (RP), role emotional (RE), bodily pain (BP), mental health (MH), vitality (V), social functioning (SF), and general health (GH) and was analyzed as recommended by the originators (10).

5.3.7 Statistical Analysis

The primary analysis compared the effect of high-dose peginterferon to standard dose treatment on the decline in HCV RNA over 28 days and the number of patients

who were peginterferon sensitive or became HCV RNA negative for every time point using the non parametric Mann-Whitney t-test and the Pearson's Chi-square test respectively. Baseline characteristics were compared using the 2-sided Fisher's exact test or the 2-tailed Mann-Whitney U test when appropriate. SF-36 scores were compared using the 2-tailed Mann-Whitney U test (between treatment arms) and the Wilcoxon signed rank test (between baseline and t=4 weeks). Viral load data were log 10 base transformed; viral loads below the limit of detection were transformed to 1 log. Missing viral load data were substituted by the average of the preceding and the following data with correction for time. Statistical significance was inferred if *P* was <0.05 (2-tailed/2-sided).

Table 5.1. Baseline characteristics

	Standard therapy (1.5ug/kg)	High dose therapy (3.0ug/kg)	P
Sex; male/female	18/3	12/7	
Age in years ± SD	48 ± 8	47 ± 8	.68#
Length in cm ± SD	174 ± 6	174 ± 8	.77#
Weight in kg ± SD	81 ± 18	78 ± 14	.67#
BMI (kg/m^2) ± SD	26 ± 4	26 ± 4	.81#
Cirrhosis (%) ± SD	7 (26)	5 (33)	.53*
ALT in U/l ± SD	128 ± 113	91 ± 45	.63#
Viral load (copies /ml;) ± SE	6.87 Log ± 1.31	6.61 Log ± 1.82	.38#
Genotype 1 (%)	9 (43)	11 (58)	.53*

* Fisher's exact test (2-sided); # Mann-Whitney U test (2-tailed)

5.4 RESULTS

5.4.1 Patients

Forty patients (10 women; 30 man) with chronic hepatitis C (median age: 47 years; range 26-68 years) who previously failed to achieve a sustained virological response (including 9 IFN-α -monotherapy non-responders, 5 IFN-α -monotherapy responder-relapsers, 23 IFN-α ribavirin non-responders and 3 IFN-α ribavirin responder-relapsers) were included. Of these patients, 21 were randomized and assigned to receive the standard dose Peginterferon-α2b (1.5 µg/kg body mass per week) and 19 to receive the experimental Peginterferon-α2b schedule (3.0 µg/kg QW). Genotypes were equally divided among the treatment arms. In total 20 patients (50%) were HCV genotype 1, 9 patients (22%) genotype 3, 6 patients (15%) genotype 4, 4 patients (10%) genotype 2 and 1 patient (3%) genotype 6 infected. No differences were found between the treatment groups with regard to previous treatment and type of response failure, sex, age, length, weight, BMI, histology, ALT and viral load levels. Demographic and baseline characteristics of the patients per study arm are shown in table 5.1.

5.4.2 Descriptive response

During the study period of 28 days, twenty five patients (13/19 high-dose peginterferon vs. 12/21 standard dose peginterferon; p=0.53) showed peginterferon sensitivity. Seventeen patients, 9 treated with high-dose and 8 with standard dose peginterferon reached HCV RNA levels below the limit of detection and were considered “rapid-responders” (p=0.75; table 5.2). Rapid responders (n=17) were predominantly infected with genotypes other than 1/4 (n=12, p=0.00). The majority of rapid genotype 1/4 responders were treated with high-dose peginterferon (4 high-dose peginterferon vs. 1 standard dose peginterferon; p=0.33).

Tabel 5.2. Response pattern throughout the study period relative to treatment arm for all patients and genotype 1 and 4 patients between brackets.

	High dose therapy (3.0ug/kg)	Standard therapy (1.5ug/kg)	P*
Rapid response	9 (4)	8 (1)	0.75 (0.33)
Partial response	4 (4)	4 (2)	1.00 (0.65)
Non response	6 (6)	9 (9)	0.53 (0.13)
	19 (14)	21 (12)	

Rapid response = HCV RNA below the limit of detection (10^3 copies/ml), Non response = HCV RNA decline less then 1 Log, Partial response = Between Rapid response and Non response

* Fisher's exact test (2-sided)

Fifteen patients, 6 treated with high-dose peginterferon and 9 with standard dose peginterferon (p=0.53) were "non-responders"; they showed a decline in HCV RNA less than 1 Log. All non-responders (n=15) were infected with genotype 1/4 (p=0.00).
A partial response (between "rapid-" and "non response") was seen in 4 patients of both groups (p=1.0) (table 5.2). Among the 8 partial responders, 6 were genotype 1/4 infected (p=0.69).

5.4.3 Quantitative HCV RNA kinetics

The average decline in HCV RNA during the study period was 2.94 ± 0.38 Log (Mean ± Std. Error of the mean). Both treatment groups showed an initial response between day 0-2 with a rapid decline in HCV RNA (first phase), followed by a distinct phase of HCV RNA increase between day 2 and 7 (rebound) where after the viral load decreased steadily (second phase) (figure 5.1).
The initial response or first phase was more pronounced in the high-dose peginterferon group than in the standard dose peginterferon; at 2 days a mean reduction in HCV RNA of 2.01 ± 0.37 Log and 1.66 ± 0.33 Log was observed in the high-dose peginterferon group and standard dose peginterferon group respectively (p=0.23).
Between day 2 and day 7, a period of HCV RNA increase or "rebound" could be observed. In the high-dose peginterferon group HCV RNA increased slightly: 0.36 ± 0.27 Log. In the standard dose peginterferon group the increase in HCV RNA was more pronounced: 0.60 ± 0.27 Log (p=0.52).

Subsequently, HCV RNA declined steadily in both treatment groups. At the end of the first (t=7), second (t=14) and fourth (t=28) week a decrease in HCV RNA compared to baseline could be seen in both groups; 1.73 ± 0.42 Log vs. 1.06 ± 0.35 Log (p=0.12); 2.37 ± 0.51 Log vs. 2.05 ± 0.48 Log (p=0.67) and 3.10 ± 0.51 Log vs. 2.78 ± 0.57 Log (p=0.65) in the high-dose peginterferon group and standard dose peginterferon group respectively. The average second phase slope between day 7 and 28 were comparable; 0.46 ± 0.1 Log and 0.58 ± 0.1 Log decline per week for high-dose peginterferon and standard dose peginterferon.

Within genotype 1/4 infected patients, high-dose peginterferon induced a stronger suppression of HCV RNA replication than standard dose peginterferon. At t=2 the decrease in HCV RNA was 1.64 ± 0.33 Log vs. 0.78 ± 0.21 Log (p=0.03) for high-dose peginterferon vs. standard dose peginterferon and 1.36 ± 0.48 Log vs. 0.21 ± 0.16 Log (p=0.02), 1.79 ± 0.54 Log vs. 0.82 ± 0.31 Log (p=0.25), 2.36 ± 0.57 Log vs. 1.20 ± 0.55 Log (p=0.08) at t=7, 14 and 28 respectively (table 5.3).

Tabel 5.3. HCV RNA decrease in Logs throughout the study period relative to treatment arm for genotype 1 and 4.

	High dose therapy (3.0ug/kg)	Standard therapy (1.5ug/kg)	**P[#]**
Baseline	0	0	1.00
Day 2	1.64 ± 0.33	0.78 ± 0.21	0.03
Day 7	1.36 ± 0.48	0.21 ± 0.16	0.02
Day 14	1.79 ± 0.54	0.82 ± 0.31	0.25
Day 28	2.36 ± 0.57	1.20 ± 0.55	0.08

[#] Mann-Whitney U test (2-tailed)

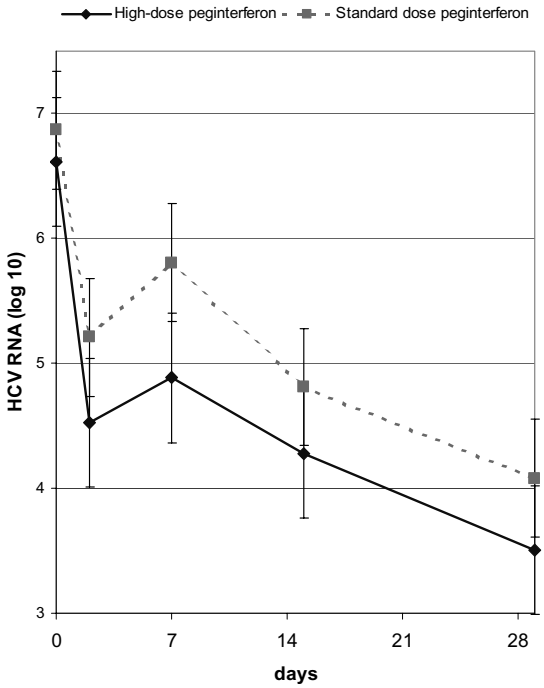

Figure 5.1.

Viral load in log over time in days for the two study arms: High dose peginterferon therapy (3.0ug/kg body weight; straight line) and standard dosed peginterferon therapy (1.5ug/kg body weight; dashed line). Patients treated with high-dose peginterferon showed a stronger response at all time points. Significance could however not be reached because beneficial responses in patients infected with other genotypes then 1 or 4.

5.4.4 Health related Quality of life

HRQoL scores were comparable between the two treatment regimens at baseline and t=28 days, except for vitality (VT) which scored lower in the high-dose peginterferon group at baseline (p=0.05) and at t=28 days (p=0.03). Scores for general health (GH), physical functioning (PF), role physical (RP), role emotional (RE), and bodily pain (BP) decreased significantly during the study period (figure 5.2). Mental health (MH), vitality (VT) and social functioning (SF) scores were unchanged.

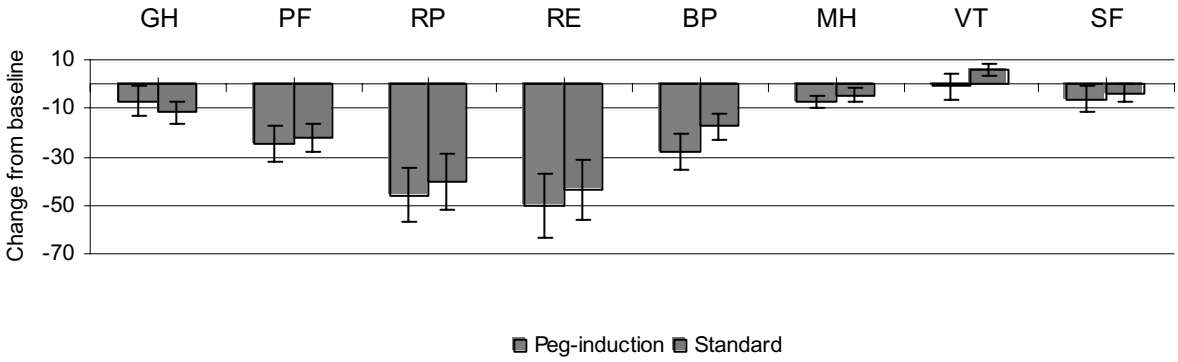

Figure 5.2.

Change in health related quality between baseline and four weeks of treatment relative to treatment arm. General health (GH), physical functioning (PF), role physical (RP), role emotional (RE) and bodily pain (BP) were decreased considerably during the study period. Mental health (MH), vitality (VT), social functioning (SF), and scores were unchanged. Scores were comparable between the two treatment regimens except for vitality (p=0,05 at baseline and p=0,03 at t=28 days).

5.5 DISCUSSION

With the introduction of peginterferon, SVR rates improved with roughly 10% over non-pegylated interferon (2, 3). As a consequence, there is interest for re-treating patients who did not achieve SVR with preceding non-pegylated interferon. However, expectations were tempered by reports revealing SVR estimates of only about 7-15% for standard dosed peginterferon (4, 5). We assessed whether high-dose peginterferon has beneficial effects in the early suppression of HCV RNA replication because of its high predictability for SVR (11, 12).

The effect of high dosed therapy has been controversial since the beginning of interferon therapy. Most studies, with a short period of high dosed interferon have shown high initial virological response rates (13). However, without lasting beneficial effects on SVR rates (14, 15). The initial beneficial effects were lost to premature lowering the interferon dose (virological breakthrough) (9) or due to the short duration of treatment of only 24-26 weeks leading to an end of treatment relapse (13). Indeed, we found that with 4 weeks of daily high dosed interferon therapy, HCV RNA had become undetectable in 80% of “difficult-to-treat” patients eventually leading to a SVR of 65% (6). Results that were confirmed by a randomized controlled trial in 373 patients showing beneficial effects of high dosed interferon when continued for a longer period in genotype 1 patients (16).

Our study is the first with high-dose peginterferon therapy in interferon non-responders revealing that in most previous non-responders a peginterferon response can be obtained (25/40; 63%). High-dosed peginterferon showed an averaged beneficial effect over standard dosed peginterferon of 10%; 13 out of 19 high-dosed peginterferon treated patients vs. 12/21 standard dose treated patients responded (68% vs. 57%, difference 9%) of whom 9/19 vs. 8/21 were even able to reach HCV RNA negativity within the treatment period (rapid responders; 47% vs. 38%, difference 11%). Especially genotype 1/4 patients had benefit from high-dosed peginterferon. A stronger suppression of HCV RNA replication could be measured at all time points.

By responding to therapy, most high-dose treated patients (13/19; 68%) obtained a fair chance to achieve an end-of-therapy sustained response. After all, non-response is the most common reason for failure of clearing the virus and even with a high breakthrough or end-of-treatment relapse rate, the SVR will be considerably higher

than the SVR rates of 7-15% reported for standard therapy by other groups (4, 5). Due to the constant anti viral pressure of peginterferon ribavirin therapy virological breakthrough has become a rarity and even if the end of treatment relapse will be as high as 10%, the end of follow-up results of this study will still be above 50%. We are the first to report such favorable results and we think the results indicate than in a selected subset of patients, who non-responded to prior therapy, high dosed peginterferon therapy should be considered.

High-dosed peginterferon was expected to be associated with an impaired quality of life. Indeed, quality of life, measured at baseline and during treatment, considerably decreased following treatment. A dose dependant effect could however not be observed.

In conclusion, the results of our study show that in the majority of patients who previously failed to achieve a sustained response, an initial response could be obtained when retreated with Peginterferon-ribavirin therapy. High-dosed peginterferon is more beneficial than standard dosed peginterferon with an average extended effect of 10%. Especially genotype 1 infected patients might benefit from high-dose peginterferon. Ten percent beneficial effect can be compared to effect of pegylating interferon. Therefore we believe that, since due to the small sample size of this study significance could not be reached, a prospective randomized study with an estimated sample size of 300-350 patients, needs to be conducted.

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Part II

Host related immunological factors correlating with (non-) response

Introduction to part II

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The role of T-cells in hepatitis C virus infection

The components of the antiviral immune response are schematically displayed for hepatitis C infection in figure 1. T-helper cells (Th) play a central role in the coordination of the anti-viral immune response. Naïve Th are not activated by infected tissue cells, but only by professional Antigen-Presenting Cells (APC), i.e. macrophages and dendritic cells. The APC recognize the virus by specific Pattern Recognition Receptors (PRR), endocytose it, and process the viral proteins to peptides which are displayed on their MHC class II molecules. Upon recognition of the viral peptides by their T cell receptor (TCR), the naïve Th are stimulated to differentiate to effector Th. Depending on the virus Pathogen Associated Molecular Pattern (PAMP) as recognized by PRR, the APC produce either interleukin 10 (IL-10) or IL-12. IL-10 and IL-12 stimulate the differentiation of naïve CD4+ Th to effector helper type 2 T-cells (Th2) or effector helper type 1 T-cells (Th1), respectively. This interaction between APC and Th takes place in secondary lymphoid organs, like lymph nodes and spleen.

CD4+ Th2 effector-cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 that stimulate B-cells (humoral response) and down-regulate the Th1 response. B-cells need stimulatory signals from Th2 cells for differentiation into antibody-producing cells. The antibody response serves to opsonize circulating virus particles for elimination by macrophages, neutralization of viral proteins and prevention of virus entry into host cells.

CD4+ Th1 effector-cells secrete IL-2, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) which stimulate CD8+ cytotoxic T-cells and macrophages. Subsequently, the CD8+ cytotoxic T-cells migrate through the blood stream to the infected organ. Parenchymal cells (such as liver cells) infected with a virus can be recognized by CD8+ cytotoxic T-cells upon recognition of viral peptides presented on MHC class I molecules by their TCR CD8 complex (cellular response). For this purpose, infected cells cleave viral proteins proteolytically, and insert the resulting peptides in the groove of MHC class I molecules. Upon recognition, the cytotoxic T-cells either lyse the infected cells, or inhibit the viral replication within these cells by local secretion of antiviral cytokines like TNF-α.

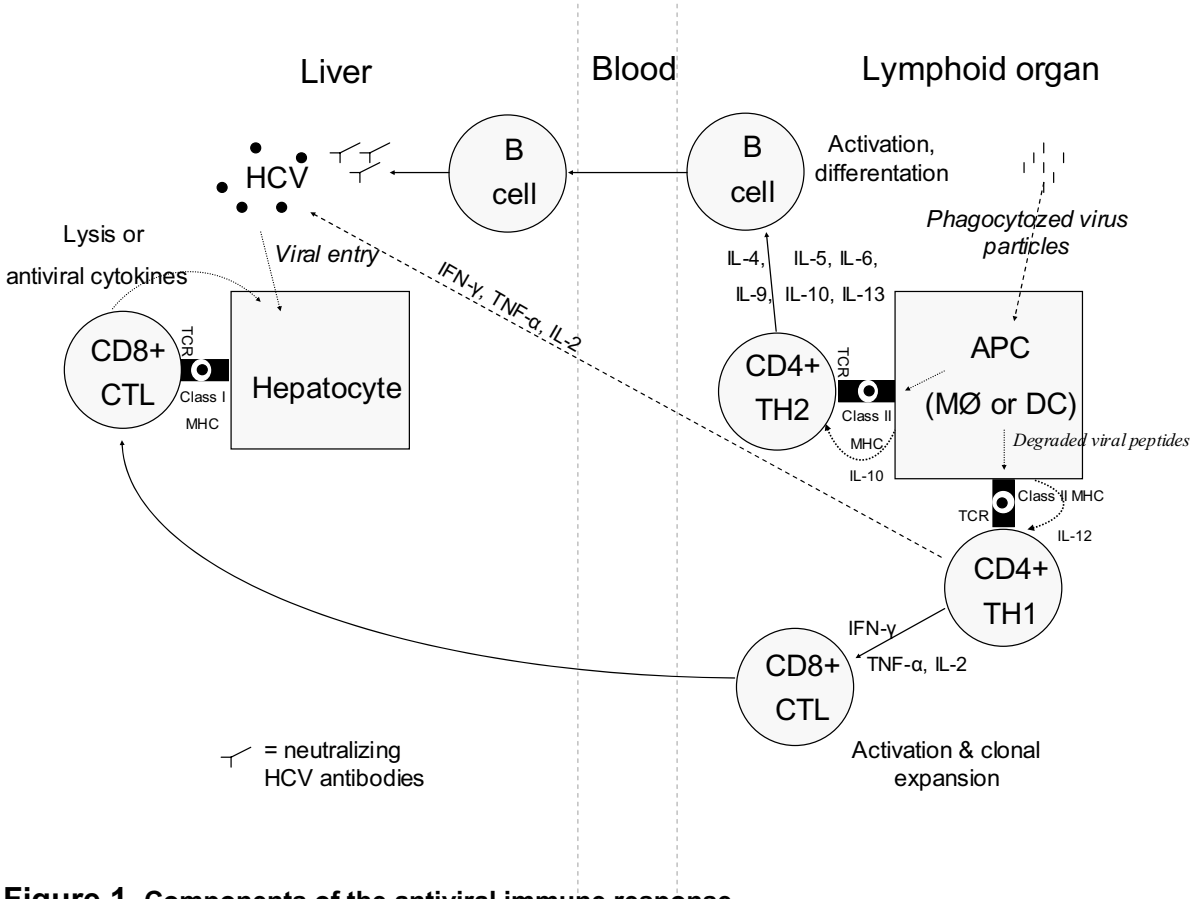

Figure 1. Components of the antiviral immune response.

APC = Antigen Presenting Cell, DC = dendritic cell, MØ = macrophage, CD8+ CTL=cytotoxic T cell; CD4+ Th cell = T helper cell; Th1 = helper T cells with a type 1 cytokine profile; Th2 = helper T cells with a type 2 cytokine profile; IL = interleukin; IFN = interferon, TNF = tumor necrosis factor; MHC = major histocompatibility complex; TCR = T cell receptor.

HCV infection elicits an active humoral as well as a cellular immune response. It is however, not precisely understood which role the immune system plays and why approximately 85% of infected persons who are otherwise considered immunocompetent is unable to clear the virus.

Most studies have concentrated on the virus-specific CD4+ Th and CD8+ cytotoxic T-cells in peripheral blood (1). Studies with chimpanzees, who successfully had recovered from acute hepatitis C, demonstrated an early and strong cytotoxic T-cell response against multiple HCV epitopes in blood. In contrast, chimpanzees that develop a chronic HCV infection showed a weak peripheral cytotoxic T-cell response in the early stages of the disease in peripheral blood (2). The same has been seen in humans who clear HCV during acute infection and in those who develop chronic

infection, and a relation between IFN-therapy induced control of hepatitis C viremia and HCV-specific T-cells responses in blood has been suggested (3, 4).

However, within the liver, HCV-specific CD8+ cytotoxic T-cells have been detected during chronic HCV infection (4, 5), in a significantly higher frequency than in peripheral blood (6, 7); suggesting a specific compartmentalization at the site of infection. It is thought that in the infected liver, there is a balance between viral replication and the host immune response. This balance may be of importance for the outcome of anti-viral treatment. We therefore quantified diverse subsets of intrahepatic immune cells ($CD4^+$, $CD8^+$ and $CD68^+$ cells) by immunohistochemistry in pre- and end of treatment liver biopsies and established their association to therapy response for chronic HCV-infection. For comparison also peripheral immune parameters such as plasma interleukin (IL)-10, IL-12, IFN-γ levels and circulating HCV-specific T cells were determined (chapter 7). Because of the patient discomfort accompanying the liver biopsy procedure we also explored the feasibility of a less invasive and traumatic fine-needle aspiration biopsy technique (FNAB) as a tool for monitoring the cellular immune status in the liver during chronic HCV-infection (chapter 8).

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Chapter 6

Pretreatment Intrahepatic CD8$^+$ Cell Number Correlates with Virological Response to Antiviral Therapy in Chronic Hepatitis C

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6.1 ABSTRACT

Interferon-ribavirin therapy is successful in only half of the chronically infected hepatitis C patients. We analyzed the relationship between virological response and baseline immune factors in 17 chronically infected patients who completed 26 weeks of interferon-ribavirin therapy. Numbers of intrahepatic $CD8^+$ cells located in the portal tracts of pre-treatment liver biopsies were found to be significantly higher in patients responding to therapy than in non-responders ($p=0.002$). The relation between portal $CD8^+$ cells and chance of response could be described by a logistic curve (univariate logistic regression analysis); its prognostic value was superior to that derived from genotype and other baseline factors (multivariate analysis). In contrast, neither peripheral cytokine levels nor HCV specific T cell reactivity in peripheral blood mononuclear cells did show a relationship to therapy response. These findings suggest that significant prognostic immune markers are to be found in the liver.

6.2 INTRODUCTION

The potency of interferon (IFN) to inhibit hepatitis C virus (HCV) replication and to stimulate the clearance of infected cells is the basis of current standard antiviral therapy using IFN-α and ribavirin. The majority of patients show an initial response with loss of circulating HCV-RNA. Unfortunately, complete viral clearance is not observed in all patients (8, 9). Although the mechanism responsible for the eradication of HCV-infected hepatocytes is not well understood, there is consensus that this process is immune-mediated. Revealing mechanistic studies have been hampered by the lack of representative laboratory models and by difficulty of studying the intrahepatic compartment.

In peripheral blood, HCV specific T lymphocytes are easily detected during acute HCV infection, mostly exhibiting a broad reactivity towards different HCV antigens (2, 10). In chronic hepatitis HCV-specific T cell frequencies are low (11), and response to subsequent IFN-α therapy is difficult to predict on the basis of this parameter. Higher frequencies of HCV-specific T cells may be found in the liver (6, 7). Accordingly, liver biopsies may prove to be instrumental to study immune factors associated with response to subsequent IFN-α therapy. Not only the frequency of virus specific intrahepatic T cells (5, 12) but also absolute numbers of these cells present in the liver can possibly mirror the ability of the immune system to generate a sufficient anti-viral response after start of antiviral therapy. We therefore examined localization of diverse subsets of intrahepatic immune cells by quantitative immunohistochemistry ($CD4^+$, $CD8^+$ and $CD68^+$ cells) in pre- and end of treatment liver biopsies and causality to response to therapy for chronic HCV-infection. For comparison also peripheral immune parameters such as plasma interleukin (IL)-10, IL-12, IFN-γ levels and circulating HCV-specific T cells were determined.

6.3 PATIENTS, MATERIALS AND METHODS

Seventeen patients with chronic HCV infection who were assigned to 26 weeks of therapy combining IFN-α2b 3MU thrice a week with ribavirin 1000-1200mg/d (Schering-Plough, Kenilworth, USA) in our center and from whom a pretreatment biopsy was available were subject of the current study.

All were treatment naive patients, HCV-RNA positive, had biopsy proven chronic HCV infection, and were seronegative for HBV and HIV. None had clinical or biochemical evidence of other liver diseases. Blood samples for HCV-RNA quantification were taken at 0, 4, 12 and 26 weeks of therapy. Response to treatment was defined as HCV-RNA levels below 100 copies/ml at the end of therapy, determined using the Superquant^{tm} assay (National Genetics Institute, USA; detection limit 100 copies/ml).

Before and in the last week of treatment, biopsies were obtained percutaneously with a 14 Gauge tru-cut needle. Immunohistochemistry was performed on formalin-fixed paraffin-embedded liver biopsy sections. After an over-night incubation at 37 ^{o}C, 4 µm thick sections were dewaxed in xylene and rehydrated in graded alcohols. Epitope retrieval was achieved by temperature-controlled incubation for 20 minutes at 98^{o}C in citrate-buffer (pH 6.0). The primary antibodies against CD8 (clone cd8/144B, DAKO), CD68 (clone PG-M1, DAKO) and CD4 (clone NCL-CD4-1F6, NovaCastra) were applied and visualised as described previously (13).

The number of cells staining positive within the portal tract and lobular area was determined by counting the number of positive cells per equivalent microscopic field at a magnification of 400x using a Zeiss light microscope. In every slide, 10 fields for the lobular region and 3 equivalent fields for the portal tracts were examined and the mean count of these fields was expressed as cell number per microscopic field. The cell counting was performed on slides blinded by codes, by two investigators in an independent fashion (inter observer correlation was 0.996). HCV-hepatitis activity, interface hepatitis, intralobular degeneration, fibrosis and the number of apoptotic bodies were histologically assessed and graded routinely in all specimens according to the Knodell classification by an experienced liver pathologist blinded to the clinical status of the patient.

Peripheral blood mononuclear cells (PBMC), obtained at start of the therapy, were isolated from heparinized blood by density gradient centrifugation after which cells

were frozen in liquid nitrogen. After thawing, PBMC were cultured in 96-well microtiter plates (NUNC, Breda, The Netherlands) using 6 replicates for each condition with 2 x 10^5 cells/well. Cells were cultured in RPMI 1640, supplemented with 10 % heat inactivated human serum at 37 ^oC with 5% CO_2 and 100% humidity for 6 days in the presence or absence of HCV proteins at a concentration of 3 μg/ml. Recombinant HCV proteins used included HCV core (amino acid [aa] 2-120), and NS3-NS4 (aa 1192-1931), which were kindly provided by dr. M. Houghton (Chiron, Emeryville, USA). All antigens were expressed as COOH-terminal fusion proteins with human superoxide dismutase (SOD) in yeast. Controls included phytohemagglutinin-L, inactivated influenza virus (Duphar, Weesp, The Netherlands) and SOD. After 6 days cultures were labeled by incubation for 16 h with 1μCi 3H-thymidine (Amersham, Breda, The Netherlands). Cells were harvested and radioactive incorporation was estimated using a beta counter (Wallac, Helsinki, Finland). Results were expressed as mean counts per minute (cpm). The stimulation index was calculated as the ratio between cpm obtained in the presence of antigen and that obtained without antigen; a stimulation index >3 was considered significant.

Plasma obtained from EDTA blood, taken before the start of treatment, was stored at –70^o C. Plasma cytokine concentrations were measured using commercially available ELISAs for IFN-γ, IL-10 and IL-12-p40 (CLB, Amsterdam, The Netherlands).

Data were analyzed using SPSS for Windows (Version 10.1, Chicago, USA). Mann-Whitney non-parametric rank sum tests and Pearson correlation analyses were applied. Logistic-regression models with backward selection procedures were used to explore baseline factors (viral load, genotype, sex, lobular and portal $CD8^+$ cell number) predicting response. P-values of <0.05 were considered statistically significant.

6.4 RESULTS

Baseline patient characteristics as well as outcome and immunohistochemistry results are presented in table 1. The median duration of infection was 14.5 years (range 5 to 30 years); in three patients the duration of infection could not be estimated. Median baseline viral load was 9.0 x 10^5 copies/ml (range 8.0 x 10^4 to 3.5 x 10^7, mean 5.3 x 10^6 copies/ml); eleven patients were infected with genotype 1a/b. All patients completed 26 weeks of IFN-ribavirin therapy. Nine patients responded by the end of therapy (HCV-RNA < 100 copies/ml), whereas in eight HCV-RNA remained detectable during the entire treatment period. No significant differences were observed with respect to pretreatment viral load or genotype between responders and non-responders. The average rate of viral load decline within the first 4 treatment weeks was 2.32 log; 1.45 log in non-responders compared to 3.32 log in responders (p=0.015). The rate of decline in viral load was not different between genotypes 1 and non-1. In four patients, the quantitative PCR assay for HCV-RNA was negative after a 24 week treatment-free follow-up period.

No significant difference was observed between responders and non-responders in baseline ALT levels (p=0.321). HCV specific lymphoproliferative responses were detected in 6/17 patients. However, baseline HCV specific lymphocyte proliferation did not differ significantly between responders and non-responders (table 1). Additionally, other pre-treatment peripheral markers of immune activity like numbers of circulating $CD8^+$ T-cells (p=0.535), plasma IL-12-p40 (p=0.336), IL-10 (p=0.321) and IFN-γ (p=0.236) levels did not differ significantly.

Liver biopsies were evaluated histologically by determination of the Knodell score and the presence of $CD4^+$, $CD8^+$ and $CD68^+$ cells. Knodell scores were similar in responders and non-responders (table 1). $CD8^+$ and $CD68^+$ cells were present both in the portal tract and intralobular, but $CD4^+$ cells were observed less frequently; mostly in the portal tracts. Numbers of $CD68^+$ or $CD4^+$ cells did not differ significantly between responders and non-responders. Numbers of lobular $CD8^+$ cells in pre-treatment liver biopsies were similar in both groups (p=0.815), but the numbers of $CD8^+$ cells within the portal tracts were higher in responders compared to

Table 1. Patient and virological characteristics at baseline and end of treatment response.

Patient subject	Sex	Age	race	genotype	viral load	transmission	known duration of exposure	ALT	Knodell score	Pre-treatment CD8+ cells portal tracts[#]	Pre-treatment CD8+ cells lobular tracts[#]	LST	LST SI	End-of-treatment CD8+ cells portal tracts[#]	End-of-treatment CD8+ cells lobular tracts[#]	R
1	F	41	Asian	2a	$4.8x10^5$	sporadic	16	41	6	65.7	19.7	ND	ND	29.2	2.10	R
2	F	53	Caucasian	10a	$1.2x10^6$	sporadic	Unknown	159	10	62.8	13.7	ND	ND	27.2	3.5	R*
3	F	60	Asian	2a	$4.6x10^5$	transfusion	14	179	12	51.3	30.0	ND	ND	52.2	10.9	R
4	M	64	Caucasian	2b	$2.8x10^6$	transfusion	7	40	9	62.0	28.2	ND	ND	n.p.	8.3	R*
5	M	39	Caucasian	3a	$4.5x10^5$	parenteral	8	75	2	n.p.	5.0	Core	3.8	47.0	5.7	R*
6	M	50	Caucasian	1a/1b	$9.0x10^5$	parenteral	30	88	7	57.5	14.2	ND	ND	32.5	12.4	R*
7	F	55	Asian	1b	$1.4x10^7$	unknown	Unknown	55	9	45.0	24.9	ND	ND	27.0	4.8	R
8	M	30	Caucasian	1a	$1.3x10^6$	parenteral	15	48	6	n.p.	4.3	Core	4.9	15.7	5.3	R
9	F	41	Caucasian	1b	$3.5x10^7$	transfusion	18	58	4	75.5	15.3	ND	ND	26.2	8.3	R
10	F	32	Caucasian	1a/1b	$8.8x10^5$	parenteral	16	39	4	23.5	15.2	Ns3-4	10.1	23.7	14.4	NR
11	M	45	Caucasian	1a/1b	$9.8x10^6$	parenteral	11	49	8	37.3	5.6	ND	ND	23.8	2.0	NR
12	F	53	Caucasian	1b	$4.4x10^5$	sporadic	Unknown	179	12	24.0	25.1	ND	ND	84.5	7.2	NR
13	F	40	Caucasian	3a	$5.1x10^5$	parenteral	12	36	3	34.0	9.9	Ns3-4	11.7	27.2	6.4	NR
14	F	44	Caucasian	1b	$8.0x10^4$	parenteral	5	22	6	27.0	17.8	ND	ND	n.a.	n.a.	NR
15	M	55	Caucasian	1a	$1.2x10^7$	transfusion	13	46	6	53.2	18.7	Core	4.6	68.0	3.0	NR
16	M	33	Caucasian	1a/1b	$8.8x10^6$	parenteral	23	103	8	46.5	27.1	ND	ND	48.0	12.7	NR
17	F	61	Caucasian	1b	$8.1x10^5$	transfusion	25	73	7	39.7	4.7	Core	3.3	35.8	8.1	NR
Median		**45.0**			**$9.0x10^5$**		**14.5**	**55.0**	**7**							

Abbreviations: F= female, M = male, NR = non-response, R = response, n.p.= no portal tracts, n.a.= not available, LST= lymphocyte stimulation test (shown is the antigen to which a specific response could be detected), ND = not detected (SI<3.0).

[#] 10 fields for the lobular region and 3 equivalent fields for the portal tracts were examined and the mean count of these fields was expressed as cell number per microscopic field.* Experienced a sustained virological response.

non-responders (p=0.002) (figure 1a). Based on univariate logistic regression modeling a scatterplot displaying the predicted probability of response to following therapy as a function of the number of $CD8^+$ cells in the portal tract in biopsies taken before treatment could be made (figure 1b). The number of pre-treatment $CD8^+$ cells in the portal tract alone predicted response better than viral or patient characteristics (multivariate logistic regression analysis including sex, age, genotype, stage of fibrosis and viral load). Numbers of intrahepatic $CD8^+$ cells were not significantly different in responders and non-responders in end of treatment biopsies (figure 1a).

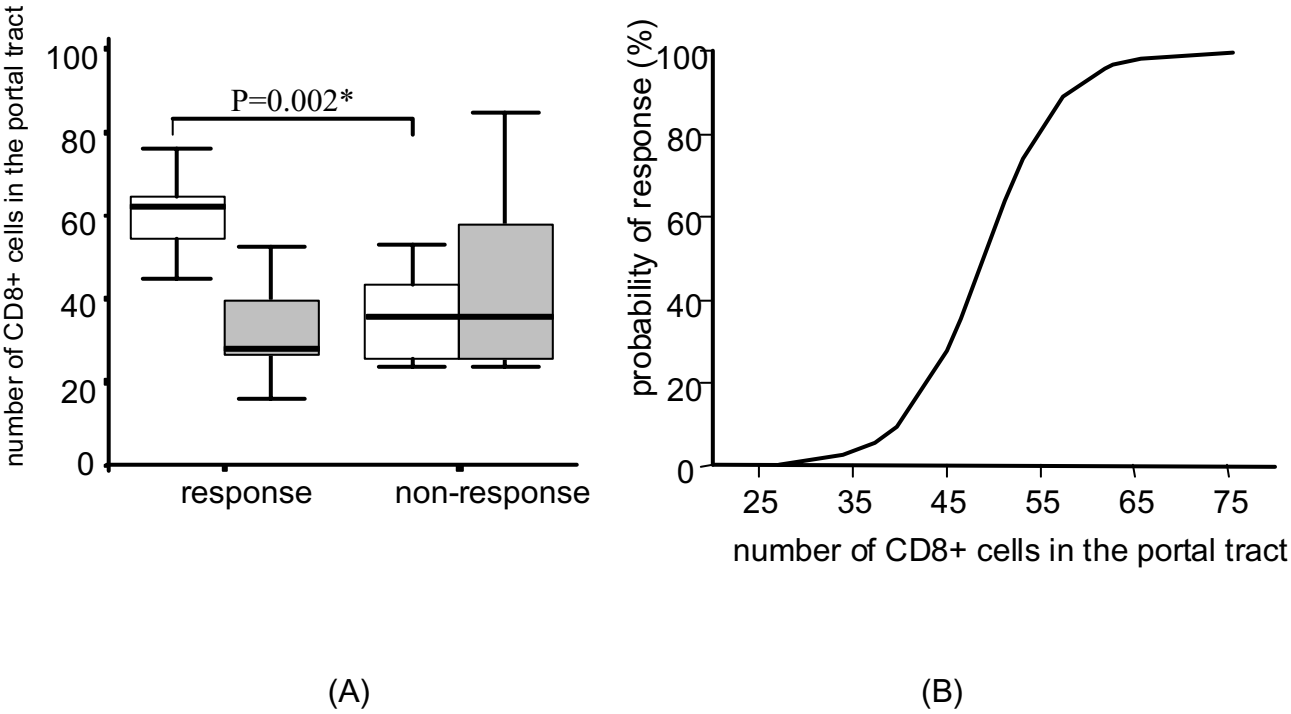

Figure 1. Numbers of intrahepatic $CD8^+$ cells in the portal tract of liver biopsies in relation to interferon response. (A) Boxplots of intrahepatic $CD8^+$ cells in the portal tract of pre-treatment (open boxes) and end of treatment (closed boxes) biopsies in relation to interferon response. The $CD8^+$ cell count in pre-treatment portal tracts is significantly higher in responders than in non-responders (p=0.002*). The upper and lower limits of the boxes and the middle line across the boxes indicate the 75th and 25th percentiles and the median (the 50th percentile), respectively. The length of the box is thus the interquartile range; the box represents 50% of the data. The upper and lower horizontal bars indicate the 90th and 10th percentiles, respectively. Comparison of end-of-treatment non-response and response data was performed by Mann-Whitney non-parametric rank sum tests. (B) The predicted probability of response to following therapy displayed as a function of the number of $CD8^+$ cells in the portal tract in biopsies taken before treatment. The curve is based on univariate logistic regression modeling (log likelihood: p<0.001; score test 0.002).

6.5 DISCUSSION

Intrahepatic localization of HCV specific T cells may be crucial to control acute hepatitis C (2, 10), but also augment inhibition of viral replication and clearance of infected hepatocytes when chronic hepatitis C patients are treated with IFN-α and ribavirin (3, 4). In this report we demonstrate that, when analyzed by quantitative immunohistochemistry, the number of intrahepatic $CD8^+$ cells present in the portal tract before the start of IFN-ribavirin therapy is significantly higher in patients responding to treatment. Based on univariate logistic regression modeling an S-shaped logistic curve displays the predicted probability of response to following therapy as a function of the number of portal $CD8^+$ cells in biopsies taken before treatment. The number of $CD8^+$ cells in the portal tract alone predicts response better than viral characteristics or patient features. From our immunohistochemical analysis we did not observe any correlation of other subsets analyzed ($CD4^+$ and $CD68^+$ cells) with response to antiviral treatment.

The patients described in this study were assigned to an experimental treatment schedule of 26 weeks of therapy combining Interferon-α2b 3MU thrice a week with ribavirin 1000-1200mg/d. Only 4 out of 17 patients were sustained responders, making the analysis with respect to the end of follow up status difficult. The results suggest that the number of pre-treatment intrahepatic $CD8^+$ cells in the portal tract is a significant and independent predictor for the impairment of viral replication leading to undetectable HCV RNA levels at the end of treatment. However, in the end of treatment biopsies portal $CD8^+$ cell numbers were comparable in responders and non-responders (figure 1a). Reduced intrahepatic $CD8^+$ cell numbers in responders at the end of therapy might relate to the high relapse rate observed (5 out of 9 patients showed a virological relapse).

IFN-α most likely has a pronounced direct antiviral activity in patients with HCV. The first dose dependent phase of the HCV decay curve which lasts approximately 24 hours, can be explained assuming IFN-mediated direct antiviral activity (14). The slower subsequent second phase is thought to be partly immune-mediated. Indeed we observed a significant correlation between pre-treatment $CD8^+$ cell numbers and the decay of viral titers during the first month of therapy ($r=0.514$, $p=0.05$). Both innate and virus specific immune responses may further amplify HCV clearance

during antiviral therapy either because of their presence already at the start of therapy or by IFN-ribavirin mediated activation. Several reports have demonstrated the appearance of HCV-specific T cell activity after treatment with IFN-α (3, 4, 15), even when baseline HCV-specific T cell reactivity was undetectable (11). We could not differentiate responders based on LST responses measured at start of therapy. Rather, actual numbers of infiltrating $CD8^+$ cells may have to be taken into account. Our observations are consistent with a study by Nelson et. al., who demonstrated that the presence of HCV specific cytotoxic T cells in the liver was associated with biochemical response to IFN-α (5). $CD8^+$ cells could be directly involved in the clearance of HCV from the liver during antiviral treatment through production of antiviral cytokines or killing of infected hepatocytes. Alternatively $CD8^+$ cell number may correlate with other antiviral mechanisms implicated in the clearance of HCV during therapy.

The portal localization of $CD8^+$ cells is of interest. As demonstrated in HBV infection, patients with high viral load were characterized by the presence of high numbers of $CD8^+$ cells located in the portal tracts, whereas relatively more $CD8^+$ cells were found intralobular in patients controlling HBV (12). Possibly, high numbers of $CD8^+$ cells in the portal tract may benefit patients at later stages during therapy when viral load is diminished and the migration of $CD8^+$ cells into the lobular area may have been facilitated. Future studies need to further analyze frequency and localization of HCV specific $CD8^+$ cells in the liver.

In conclusion, the pretreatment number of $CD8^+$ lymphocytes in the liver, but not in peripheral blood is higher in patients who will respond to IFN-ribavirin therapy than in patients who don't. We suggest that immunohistological evaluation of biopsies may be considered with respect to factors reflecting a patient's immune system capacity to respond adequately to therapy. Moreover, these findings suggest that significant prognostic immune markers are to be found in the liver, and should encourage further study of hepatic immune cells as important predictive factors.

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Chapter 7

Monitoring intrahepatic CD8$^+$ T-cells by fine-needle aspiration cytology in chronic hepatitis C infection

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7.1 ABSTRACT

Infection of the liver with hepatitis C virus causes compartmentalization of $CD8^+$ cytotoxic T-cells to the site of disease. These cells are thought to be involved in viral clearance during interferon therapy. The repetitive analysis of the intrahepatic immune response is hampered by the difficulty to obtain the intrahepatic T-cells. The fine needle aspiration biopsy (FNAB) technique was evaluated for its use to obtain liver-derived $CD8^+$ T-cells in a minimally invasive way. In 26 chronic HCV-patients who were evaluated for Peg-interferon and ribavirin combination therapy, pre-treatment FNABs and peripheral blood specimens were obtained simultaneously with liver tissue biopsies, and $CD3^+$ and $CD8^+$ T-cells were quantified by immunocytochemistry. The $CD8^+/CD3^+$ ratio was significantly higher in the FNABs than in peripheral blood ($p<0.01$), and similar to those in portal areas in the tissue biopsies. A significant correlation was observed between numbers of $CD3^+CD8^+$ T-lymphocytes in the FNABs and the numbers of $CD8^+$ cells in the lobular fields or in the portal tracts of the liver tissue biopsies, but not with $CD3^+CD8^+$ T-lymphocytes in peripheral blood. Finally, the ratio of $CD8^+/CD3^+$ T-lymphocytes in FNABs was significantly higher in those patients who responded rapidly to therapy as compared to slow responders at 4 weeks of treatment ($p=0.02$). These findings demonstrate that the intrahepatic T-cell composition is reflected in FNABs, and that the FNAB-technique can be used for predicting early virological response to therapy of patients chronically infected with HCV.

7.2 INTRODUCTION

In chronic hepatitis C infection, virus-specific T-cells are predominantly found in the liver and are almost absent in peripheral blood (1, 2). Intrahepatic localization of $CD8^+$ hepatitis C virus (HCV) specific cytotoxic T-cells is thought to be crucial to control acute hepatitis C infection (3), and also augment inhibition of viral replication and clearance of infected hepatocytes when chronic hepatitis C patients are treated with interferon-α and ribavirin (4-6). Both total as well as virus-specific intrahepatic $CD8^+$ T-cell numbers in pretreatment liver tissue biopsies as determined by respectively quantitative immunohistochemistry (7) or tested in vitro against target cells (4) are associated with virological response to interferon-α therapy. To study intrahepatic T-cell kinetics during acute or therapy of chronic infection, the cellular immune status in the liver should be monitored on a repetitive basis. The liver tissue biopsy technique is the golden standard for obtaining liver tissue. Unfortunately, the biopsy procedure is accompanied with considerable patient discomfort. Therefore we investigated the feasibility of a less invasive and traumatic fine-needle aspiration biopsy (FNAB) technique as a tool for monitoring the cellular immune status in the liver during chronic HCV-infection. Since the early eighties, the FNAB has been used for monitoring rejection activity after liver transplantation (8, 9). The FNAB is performed with a 25 Gauge needle (diameter 0.5mm) and therefore hardly traumatic. Our aim was to investigate whether intrahepatic T-cells as determined by quantitative immunohistochemistry ($CD3^+$ and $CD8^+$ cells) in liver tissue biopsies are reflected in FNABs, and whether intrahepatic T-cells assessed by FNAB could be used to predict response to interferon-α therapy.

7.3 METHODS

7.3.1 Patients

The study group consisted of patients from a single hospital, who were screened for participation in a multicenter clinical study in which the efficacy of treatment with Peg-interferon-α2a 180mcg per week (Pegasys, Roche, Basel, Switzerland) in combination with 1000-1200mg ribavirin (Copegus, Roche) was evaluated. A liver biopsy was part of the evaluation. Standard inclusion and exclusion criteria were used. In brief, all patients were interferon-naive, anti-HCV antibody positive and had detectable HCV RNA by PCR and elevated serum alanine aminotransferase levels (>30 U/l) for at least 6 months on at least two occasions. Patients with decompensated liver disease, co-infection with hepatitis B or human immunodeficiency virus, alcohol abuse or any other relevant comborbidity were excluded. Within two months after the liver biopsy, treatment was started when indicated according to the current treatment consensus (10). The study was in accordance with the Helsinki Declaration of 1975 and approved by the in-house medical-ethical committee. All patients gave written informed consent for participation.

7.3.2 Fine-needle aspiration and liver tissue biopsies

Pre-treatment two FNABs were obtained. The full details of this procedure are described elsewhere (11, 12). Briefly, a mandarin containing 25-gauge needle (diameter 0.5 mm) is punctured in the 8th or 9th right intercostal space. After removal of the mandarin, liver cells are aspirated by negative pressure into a syringe filled with culture medium. Blood was collected in 4 mL EDTA plasma tubes (Vacutainer Systems, Plymouth, England). FNABs and blood cells were centrifuged on to glass slides using a cytocentrifuge. One cytospin preparation of each specimen was stained according to the May-Grünwald-Giemsa procedure. FNABs were considered to be representative if the aspirate contained at least seven hepatocytes per 100 leukocytes. If both FNABs were representative, the specimen with the highest hepatocyte/leukocyte ratio was used in the study.

Directly after the FNAB procedure, a liver tissue biopsy was obtained percutaneously with a 14 Gauge Tru-cut needle. The liver specimen was fixed in phosphate-buffered formalin and embedded in paraffin.

7.3.3 Immunocytochemistry fine-needle aspiration biopsies

$CD3^+$ and $CD8^+$ cells present on cytospin slides were visualized by an immunocytological double stain technique. The slides were fixed in 4% paraformaldehyde in phosphate-buffered saline pH 7.3. Subsequently, endogenous peroxidase was blocked by incubation with citric acid buffer pH 5.8 supplemented with 0.03% hydrogen peroxide containing 0.2% (w/v) sodium azide for 15 seconds. To reduce aspecific antibody binding, slides were incubated with TBS supplemented with 10% normal rabbit serum (Life Technologies, Inc, Gaithersburg, USA) and 10% normal human plasma (CLB). Subsequentially, the slides were incubated overnight at 4°C with CD8 mAb (clone C8/144B, DAKO, Glostrup, Denmark). Detection of primary antibody was performed with rabbit-anti-mouse immunoglobulins (RAM, DAKO) for one hour followed by alkaline-phosphatase-anti-alkaline phosphatase complex (APAAP, Serotec, Oxford, UK).

At the end of day 2, the slides were incubated overnight with the second primary mAb CD3-FITC (clone UCHT1, Immunotech, Marseille, France) at 4°C. At day 3, one hour incubation of rabbit-anti FITC-HRP (DAKO) was used to detect CD3. Visualization was performed by incubation of the slides firstly in Fast Blue salt / naphtol AS-BI followed by 3-amino-9-ethylcarbazole (AEC). Slides were counterstained with methylgreen 1% for 10 seconds and mounted in glycerol. Negative controls were either performed by replacement of the primary mAb by an isotype matched control mAb or by leaving out the primary antibody.

7.3.4 Immunohistochemistry liver tissue biopsies

The distribution of $CD3^+$ and $CD8^+$ cells in the liver was visualized in consecutive sections of formalin-fixed, paraffin-embedded liver specimens. The liver sections were firstly deparaffinized with xylene (Lab Scan Ltd, Dublin, Ireland) and ethanol (Merck, Darmstadt, Germany). Thereafter antigen retrieval was performed by a 15 minutes incubation at 99°C in citrate buffer (pH 6.0) under intermittent microwave exposure. Overnight, the paraffin slides were incubated with CD3mAb (clone

F7.2.38, DAKO) and CD8mAb (clone C8/14415, DAKO) at 4°C. Slides were washed with Tris-buffered saline (TBS) pH 7.3 supplemented with 0.1% Tween-20. CD3 expression was demonstrated by a goat anti-mouse Ig conjugated to a peroxidase labeled polymer (En Vision HRP system, DAKO) and CD8 antigen was detected with the RAM-APAAP procedure. Histochemical revelation was performed by incubation with diaminobenzidine (DAB) or Fast Blue salt / naphtol AS-BI phosphate. Negative controls were performed by replacement of the primary mAb by an isotype-matched control mAb. Slides were counterstained in case of DAB staining with Mayer's solution (Merck) or in case of Fast Blue staining with nuclear Fast Red (Fluka Chemie, Buchs, Switserland).

7.3.5 Quantification of Immunohisto- and Immunocytochemistry positive cells

Two investigators independently examined and counted $CD3^+$ and $CD8^+$ cells in all consecutive liver tissue biopsy slides, and $CD3^+CD8^+$ and $CD3^+CD8^-$ cells in FNAB and peripheral blood cytospin preparations microscopically at a magnification of 400x. FNAB cytospin preparations and tissue biopsy sections were examined independently and in separate sessions. The investigators were blinded to the virological and clinical biochemical conditions of the patients. In every paraffin biopsy slide 8 microscopic fields within the liver lobuli were randomly selected to count $CD3^+$ or $CD8^+$ T-lymphocytes. In addition at least 3 portal tracts were counted.

Since the numbers of leukocytes on individual cytospin preparations may vary, the numbers of positive cells counted were normalized to the number of leukocytes. For that purpose, in every FNAB cytospin preparation, 6 microscopic fields were randomly selected to count the number of leukocytes. The count was multiplied with 104 (total number of high power fields in a cytopsin slide) to obtain the total number of leukocytes per cytospin slide. For analysis, the numbers of positive cells were expressed per 1000 leukocytes.

7.3.6 Virological assessments

A quantitative HCV RNA assay was performed to assess viremia (Cobas Amplicor HCV test, Roche Diagnostics, Almere, The Netherlands) within one week before or after the liver biopsy. In addition these tests were performed at the start of treatment and at 4 and 12 weeks during treatment.

7.3.7 Statistical analysis

Data are expressed as mean ± standard deviation, unless otherwise indicated. Pearson's correlation coefficients (r) were used to investigate the correlation between liver, peripheral blood and FNAB cell counts. Mann-Whitney tests were performed to investigate differences in $CD3^+/CD8^+$ ratios between FNAB, peripheral blood and liver tissue biopsies, and between treatment response groups. P-values (p) of < 0.05 (two sided) were considered to be statistically significant.

7.4 RESULTS

7.4.1 Patients

Twenty-six patients with chronic hepatitis C who underwent a FNAB and a liver tissue biopsy in the evaluation for the necessity of antiviral treatment were included in this study. According to the most recent treatment consensus (10) therapy was indicated and sequentially started in 23 patients. There was no indication for treatment in 2 patients, 1 patient was lost prior to therapy for further follow-up.

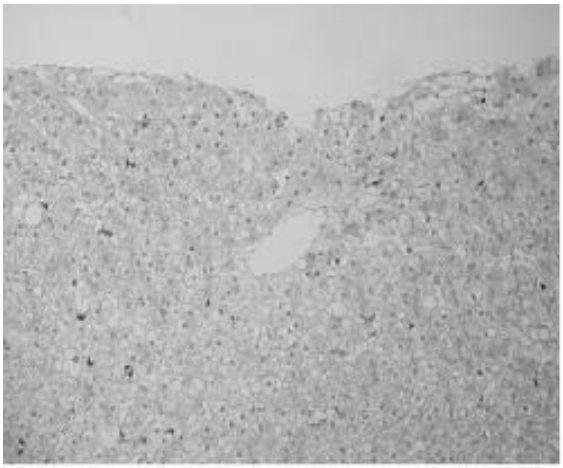
7.4.2 Relation between T-cells in liver FNAB, liver tissue biopsy and peripheral blood

$CD8^+$ and $CD3^+$ T-cells were immunocytochemically detected in FNABs, liver tissue biopsies, and peripheral blood specimens (Figure 1). In peripheral blood, about forty percent of the $CD3^+$ T-cells were cytotoxic T-cells bearing the CD8 marker. In contrast in the liver, both in the portal tracts as well as intralobular, the $CD8^+/CD3^+$ ratio's were significantly higher (both $p<0.01$; figure 2).

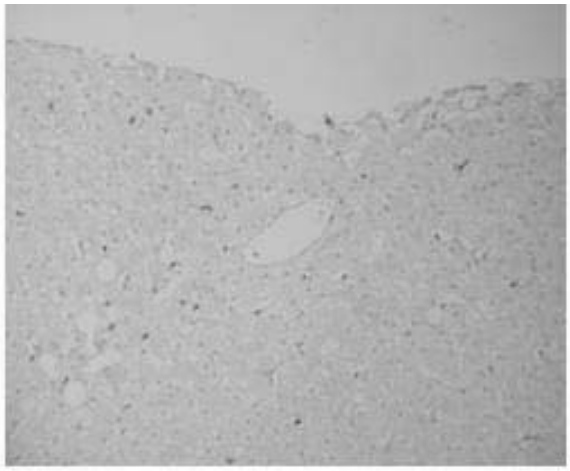
FNABs are cytological aspirates and will therefore contain liver-derived as well as blood-derived leukocytes. To investigate whether the leukocytes in FNABs reflect intrahepatic inflammatory cells, we first compared the $CD8^+/CD3^+$ ratios found in FNAB, liver tissue biopsies, and blood. The $CD8^+/CD3^+$ ratio in the FNAB was significantly higher than that in peripheral blood ($p<0.01$), significantly lower than that in the lobular fields ($p<0.01$), but not different from that in portal tracts of the liver tissue biopsies ($p=0.46$), indicating that T-lymphocytes in FNAB originate primarily from the liver (Figure 2). The difference in $CD8^+/CD3^+$ between lobular fields and FNAB may be due to the fact that in the liver the majority of both the $CD8^+$ and $CD3^+$ cells were present in the portal tracts (Figure 1).

Figure 1.

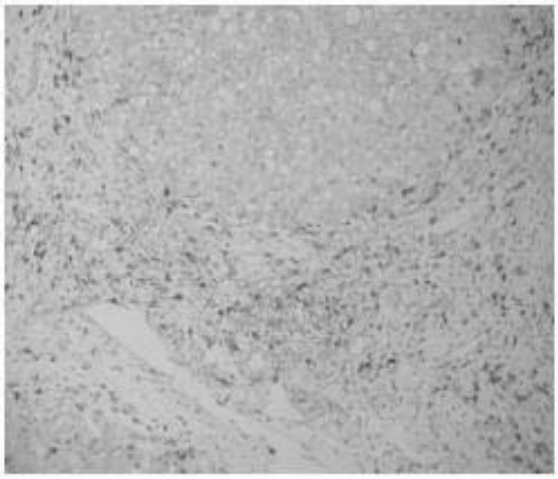
a)



b)



c)



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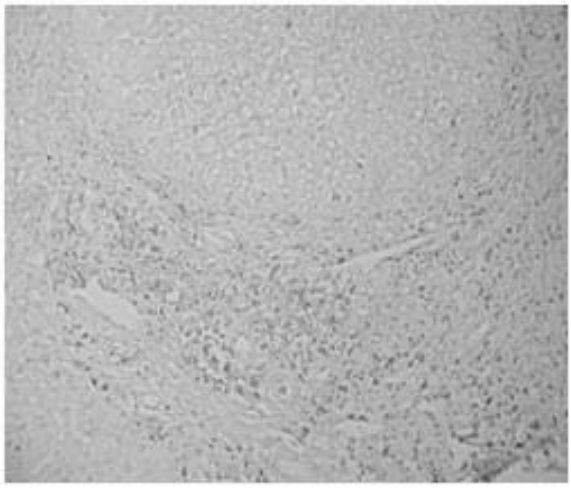
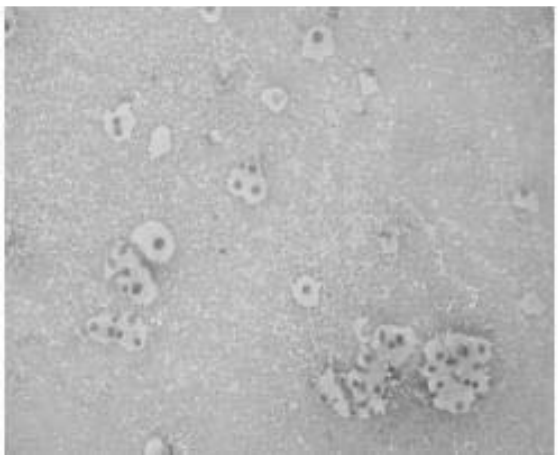
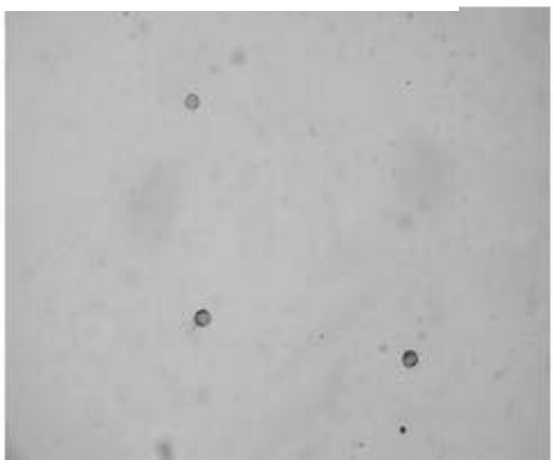


e)



f)



Next, we investigated whether variations in numbers of $CD8^+$ T-lymphocytes in the liver were reflected in the FNAB. Significant correlations between the numbers of $CD8^+$ cells in the lobular fields (r=0.52, p=0.02; figure 3a) or in the portal tracts (r=0.58, p=0.01; figure 3b) with the numbers of $CD8^+CD3^+$ T-lymphocytes in the FNAB (expressed as number per thousand leukocytes) were found. $CD3^+CD8^+$ T-lymphocytes in the FNAB did not correlate with $CD3^+CD8^+$ T-lymphocytes from peripheral blood (r=0.37, p=0.11; figure 3c).

The numbers of $CD3^+$ T-lymphocytes in the FNAB were also correlated with $CD3^+$ T-cells in the portal tracts (r=0.47, p=0.04), but in addition these were weakly associated with those in lobular fields (r=0.40, p=0.08) and peripheral blood (r=0.38, p=0.06), indicating that $CD3^+$ T-lymphocytes in FNAB originate both from liver and blood.

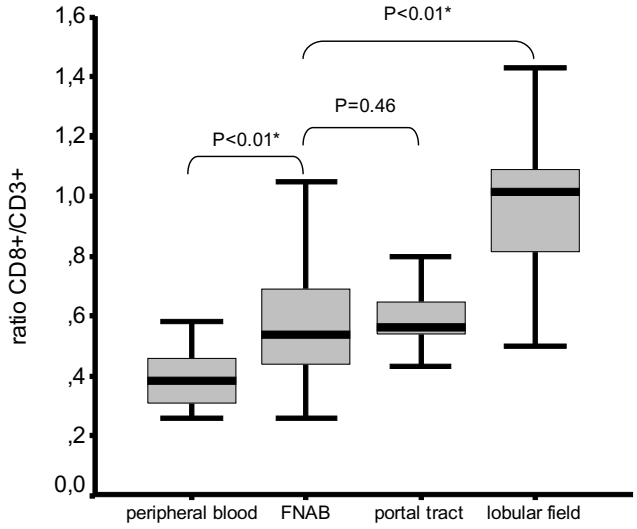
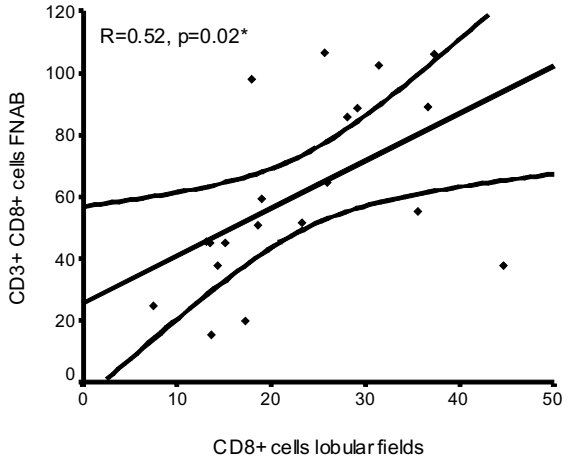


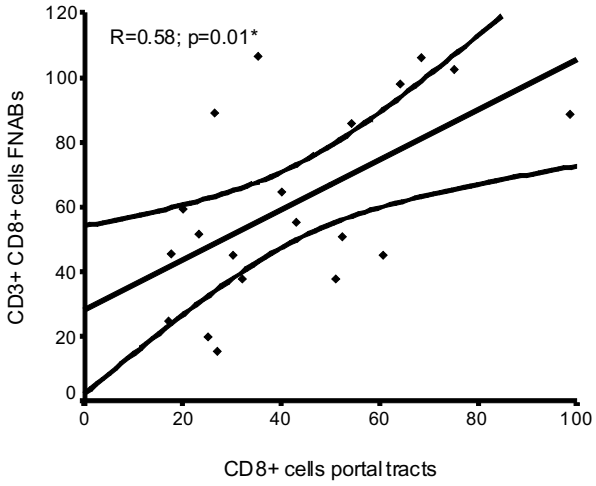
Figure 2. The median $CD8^+/CD3^+$ ratio in peripheral blood, FNABs, portal tract and lobular fields. The upper and lower limits of the boxes and the middle line across the boxes indicate the 75th and 25th percentiles and the median (the 50th percentile), respectively. The length of the box is thus the interquartile range; the box represents 50% of the data. The upper and lower horizontal bars indicate the 90th and 10th percentiles, respectively.

Legend to Figure 1 (opposite page). Immunohistochemical CD8 (A) and CD3 (B) stainings of lobular fields and CD8 (C) and CD3 (D) stainings of portal tracts in consecutive slides of a formalin-fixed and paraffin-embedded liver tissue biopsy. Fine-needle aspiration biopsy cytospin preparations stained with May-Grunwald-Giemsa solution (E), or immunocytochemically for CD3 (red) and CD8 (blue) (F).

a)



b)



c)

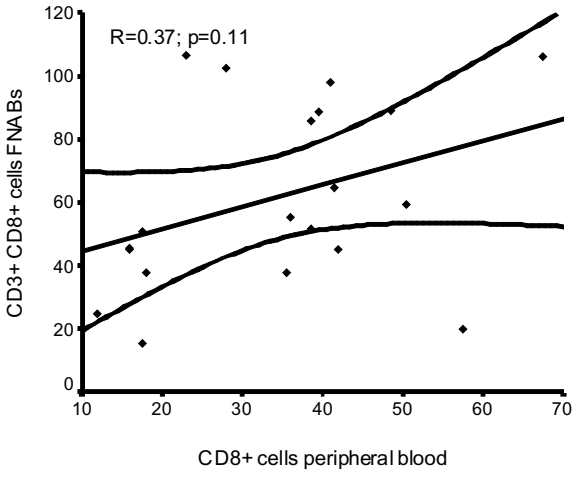


Figure 3. Variations in numbers of $CD8^+$ T-lymphocytes in the liver tissue biopsies were reflected in the FNAB.

7.4.2 T-cells in pretreatment FNAB in relation to response

T-cell parameters in FNAB, liver tissue biopsy and peripheral blood as well as treatment data such as HCV RNA levels at baseline and at 4 and 12 weeks of treatment and were available in 20 out of 23 treated patients. In order to investigate differences between treatment response groups we divided the patients on treatment into two groups according to the decline in HCV RNA at 4 and 12 weeks of therapy. The average decline in HCV RNA within the first month of treatment was 2.7-log (range: 0.45-5.27). Patients who had a serum HCV RNA decline of less than 2.7-log within this period were considered “slow responders” (n=7) and those with a decline of more than 2.7-log as “rapid responders” (n=13). The relative numbers of CD8+ T-cells were not associated to response (p=0.94), but the ratio of $CD8^+/CD3^+$ T-lymphocytes in FNABs was significantly higher in responders than in non-responders (p=0.02; figure 4a). At 12 weeks of therapy 4 patients did not have at least a 2-log drop of HCV RNA. All 4 were “slow responders” at week 4. In these patients the $CD8^+/CD3^+$ T-lymphocytes ratio in FNABs was lower compared to the group with more then 2-log decline, although with borderline significance (p=0.06; figure 4b).

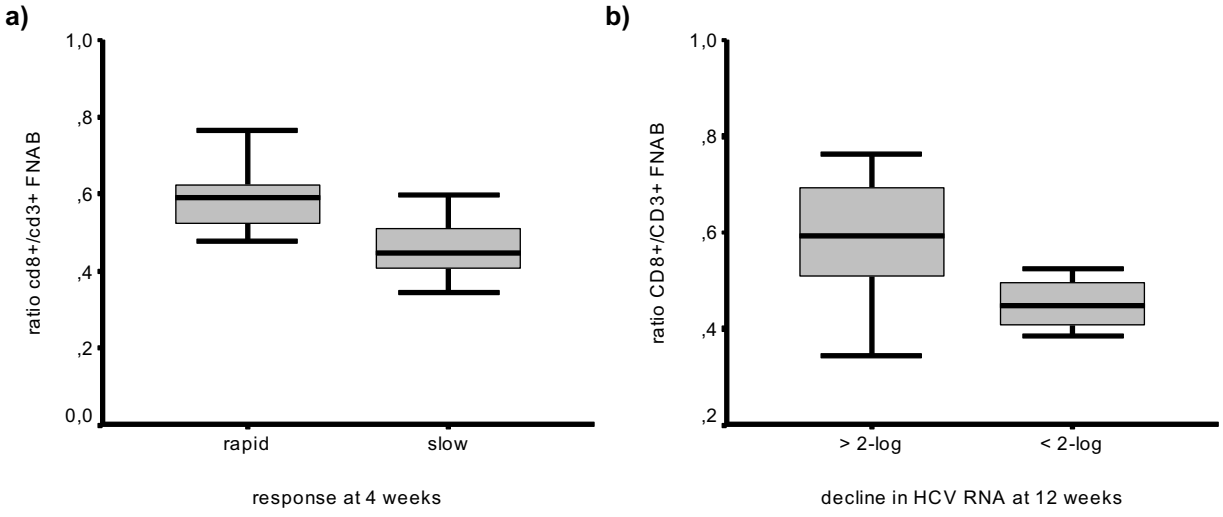


Figure 4. In FNABs a significantly higher median $CD8^+/CD3^+$ T-lymphocyte ratio was found in “rapid responders” than in “slow responders” at 4 weeks of treatment (p=0.02)(A). At 12 weeks the $CD8^+/CD3^+$ T-lymphocyte ratio was lower in patients who did not have a 2-log drop with borderline significance (B). For explanation on boxes and lines see figure 2.

7.5 DISCUSSION

In the present study we investigated whether the minimal invasive fine needle aspiration biopsy (FNAB) technique could be used as a tool to monitor $CD8^+$ T-lymphocytes in the liver during chronic HCV-infection. Comparison of the ratio's and numbers of $CD8^+$ and $CD3^+$ T-cells in simultaneously obtained FNABs, liver tissue biopsies and peripheral blood specimens showed that $CD8^+$ T-cells in FNABs were primarily liver-derived. Since, in contrast to circulating T-cells, the majority of intrahepatic T-cells are $CD8^+$ T-cells (13, 14), first the $CD8^+/CD3^+$ ratio's in the three sources were compared. In the FNABs these were similar to those in portal tract, where the majority of the intrahepatic $CD8^+$ T-cells reside, but different from that in the blood samples. Second, the relative numbers of $CD3^+CD8^+$ T-cells in the FNABs correlated significantly with the absolute $CD8^+$ T-cell numbers in the lobular fields and the portal tracts of simultaneously obtained liver tissue biopsies, while there was no correlation with numbers in peripheral blood. On the other hand, variations in numbers of $CD3^+$ T-lymphocytes in the FNABs were associated with variations in both peripheral blood and liver tissue biopsies, indicating that $CD3^+$ cells in the FNABs originated from the liver and blood. Recently we found that numbers of $CD8^+$ T-cells in portal tracts of pre-treatment liver tissue biopsies were associated to the occurrence of response of HCV-infection on interferon-α therapy (7). To investigate whether quantification of T-cells in FNABs showed association with early virological response during therapy, the patients were divided into two response groups according to their individual decline in HCV RNA within the first 4 treatment weeks. We choose the average decline of HCV-RNA within the first month as value to divide the patients into "rapid" and "slow" responders. According to this limit, a significantly higher FNAB $CD8^+/CD3^+$ T-lymphocyte ratio was found in “rapid responders” than in “slow responders”.

For 12 weeks of therapy, a drop in HCV RNA of at least 2-log recently proved to be essential for obtaining a sustained virological response recently (15). Dividing our patients according to this limit, a borderline significant lower $CD8^+/CD3^+$ T-lymphocyte ratio was found in patients who did not have at least a 2-log drop of HCV RNA as compared to the other patients. Therefore, whether the CD8/CD3 ratio in pretreatment FNAB is predictive for long-term virological response to therapy needs to be investigated in a larger patient population.

It is not allowed to compare the present finding directly to our previous finding on the association between the numbers of $CD8^+$ T-cells in pre-treatment liver biopsies and response to anti-HCV therapy (7), since absolute numbers of cells cannot be measured in FNABS and the therapies in both studies are different. In the previous study the patients were treated with interferon-α and in the present study with Peg-interferon. The determining role of the immune system in obtaining a sustained response is probably much smaller in Peg-interferon therapy because of the long lasting and constant antiviral pressure and the relatively high dose when compared to 3 MU t.i.w of conventional interferon-α therapy.

In conclusion, we demonstrated that differences in numbers of intrahepatic $CD8^+$ T-cells during chronic HCV-infection are reflected in FNABs, and that there is an association between the composition of the T-cell population contained in pre-treatment FNABs and early response to subsequent anti-viral therapy. We therefore postulate that the FNAB-technique can be used as a tool for monitoring the cellular immune status in the liver during chronic HCV-infection on a frequent basis.

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Part III

(Peg) interferon related factors correlating with (non-) response

Chapter 8

A replicon-based bioassay for the measurement of interferons in patients with chronic hepatitis C

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8.1 ABSTRACT

Overall treatment results of chronic hepatitis C have improved markedly with the introduction of pegylated interferon-alpha (PEG-IFN-α) and ribavirin combination therapy. However, cure rates in the most common genotype 1 infection are still unsatisfactory. IFN-α dose-response studies on viral kinetics suggest that inadequate dosing might be a key factor but drug levels have hardly been tested, which is in part due to difficulties in measuring this cytokine in patient samples. We have shown recently that hepatitis C virus (HCV) replicons are highly sensitive to IFN-α. In this report we tested whether the replicon system could be used as a sensitive bioassay to determine the amount of biologically active IFN-α in serum or heparinized plasma of patients under therapy. To facilitate the measurements, a stably replicating subgenomic HCV RNA was developed that carries the gene encoding the firefly luciferase. Dose response studies with IFN-α demonstrate that the amount of expressed luciferase directly correlates with the level of HCV replication. By using this cell-based assay, serum samples of HCV patients treated with different types and doses of IFN-α were analyzed in parallel to IFN-α standards made by serial dilutions of the same type of IFN-α the patient was treated with. Based on nonlinear logistic models serum concentrations corresponding to 1.3 to 19 units per ml were determined in patients under standard or high dose IFN-α therapy, and from 3.8 and 4.1 ng/ml in patients treated with PEG IFN-α. In conclusion, the HCV-replicon based bioassay allows determining the levels of biologically active IFN-α in serum and heparinized plasma of patients under treatment.

8.2 INTRODUCTION

Since the first clinical trial of treating patients with non-A, non-B hepatitis in 1986 (1), successful treatment of this disease caused by infection with the HCV, has been improved steadily, particularly with the introduction of the combination therapy of IFN-α and ribavirin (2, 3). Recently, new forms of IFN-α became available. Results of large clinical trials indicate cure-rates of 76 to 82% with genotype 2 and 3, but only 42 to 46% with genotype 1 infections (4, 5). A possible explanation is a decreased sensitivity of genotype 1 infection for IFN-α, as suggested from studies on viral kinetics(6). However, high dose IFN-α treatment of these patients might still be effective in virus suppression (7, 8). Consequently, the study of correlations between suppression of genotype 1 virus replication and the levels of IFN-α in treated patients might provide more insight into this possibility.

Studying blood levels of biologically active molecules is preferably done by a bioassay, but replaced commonly by an immunoassay for reasons of ease and costs. However, the use of immunoassays for IFN-α measurements has two problems. First, all immunoassays make use of antibodies to immobilize the cytokine, but false-positive signals can be obtained if binding occurs to an inactive protein. Secondly, and most importantly, unmodified IFNs are standardized biologically and expressed in (international) units per ml whereas pegylated (PEG-) IFNs are specified on the basis of their molecular weights (ng/ml). Consequently, a direct comparison of the biological activities of the different IFNs has been difficult. The availability of a simple bioassay that allows the determination of the number of biologically active molecules present in a given sample would therefore be very helpful. In the case of IFN-α, many bioassays are based on the protection of cultured cells against the cytopathic effect of a challenge virus (9). However, in addition to working with infectious agents, another drawback is that these assays do not necessarily reflect the ability of IFN-α in suppressing HCV replication.

It has been shown recently that HCV replicons are highly sensitive to IFN-α (10-12). Therefore, the usefulness of the replicon system was assessed for measuring the amount of biologically active IFN-α in serum and heparinized plasma of patients under treatment. A rapid quantitative assay was developed which is based on measuring the activity of a luciferase reporter gene that was integrated into a subgenomic selectable HCV replicon. By using this system, the (bio) activities of

different forms of recombinant IFNs and PEG-IFNs in serum and heparinized plasma were compared and interferon levels in patients treated for chronic hepatitis C were determined.

8.3 MATERIALS AND METHODS

8.3.1 Patient sera

Serum samples obtained at various time points during different IFN-α treatment schedules were prepared within 2 hours of blood sampling and stored at -70^{o}C until assayed. All patients were infected chronically with HCV and enrolled in investigator initiated clinical trials after having given informed consent (Table 1). The trials were approved by the local ethical committee. Patient A, a virological non-responder to IFN-α monotherapy was enrolled in a study combining high-dose induction and prolonged IFN-α2b and ribavirin therapy (13). Blood samples used in this assay were drawn 24 hours after the third daily subcutaneous injection of 10 million units (MIU), 12 hours after 1 month of 3 MIU daily injections and 12 hours after the administration of 3 MIU three times a week. Patients B to E were included in a study designed to explore treatment strategies during PEG-IFN-α2a and ribavirin (14). In patient B and C, blood samples used in this assay were drawn 12 hours after the third weekly injection of PEG-IFN-α2a (180 μg) and in patient D and E after the seventh weekly injection of the same dose.

8.3.2 Generation of a selectable HCV replicon with a reporter gene.

The basic replicon construct pFK-I$_{389}$/NS3-3'/5.1 has been described recently (15). This construct carries three cell culture-adaptive mutations that enhance RNA replication cooperatively (E1202G, T1280I, and S2197P). The reporter gene luciferase from the firefly (*Photinus pyralis*) and the coding sequence for ubiquitin were inserted upstream of the neo gene by using standard recombinant DNA technologies (Fig. 1a). Details of the cloning procedure will be described elsewhere. Cell clones derived from Huh-7 cells that carry persistently replicating HCV replicons were generated as reported recently (16)

Table 1. Patient characteristic and time of blood sampling.

Patient	Medication	Duration (d)[1]	Sample time (h)[2]	Gender	Weight (kg)	Height (m)	BSA[3]	Histology[4]	Genotype
A	IFN-α2b 10 MIU/d + 1200mg Rbv/d	2	24	Male	106	1.78	2.23	0	1b
A	IFN-α2b 3 MIU/d + 1200mg Rbv/d	27	12	Male	106	1.78	2.23	0	1b
A	IFN-α2b 3 MIU tiw +1200mg Rbv/d	448	12	Male	106	1.78	2.23	0	1b
B	PEG-IFN-α2a 180 ug QW + 1000mg Rbv/d	22	12	Male	76	1.82	1.97	0	3a
C	PEG-IFN-α2a 180 ug QW + 1000mg Rbv/d	22	12	Female	71	1.68	1.81	0	2a/c
D	PEG-IFN-α2a 180 ug QW + 1000mg Rbv/d	50	12	Male	70	1.70	1.80	3	1b
E	PEG-IFN-α2a 180 ug QW + 1000mg Rbv/d	50	12	Male	72	1.65	1.79	1	1b

d: day; QW: once a week; Rbv: ribavirin; tiw: three times a week.

[1] time elapsed since start of therapy in days.

[2] time elapsed since last dose administration in hours.

[3] body surface area in m^2.

[4] scoring of fibrosis according to the METAVIR classification.

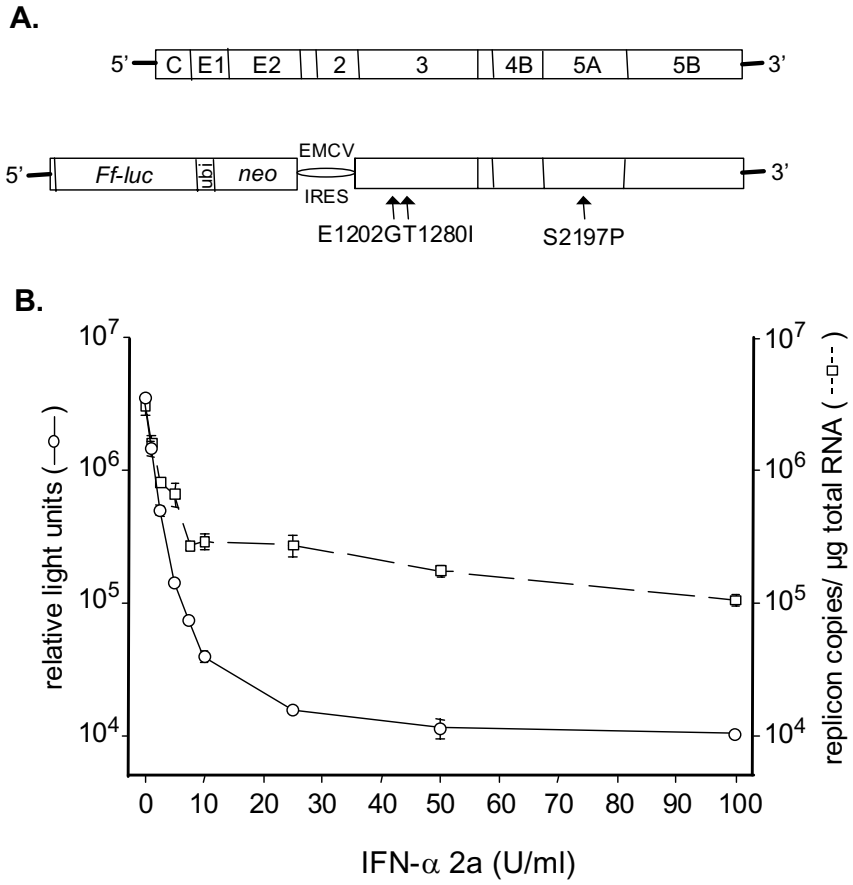

Figure1. Antiviral activity of IFN-α2a on a HCV replicon carrying a reporter gene. (A) A schematic presentation of the HCV genome organization, with the structural proteins core to E2, the nonstructural proteins NS2 to NS5B, and the nontranslated regions (thick lines) flanking the polyprotein is shown at the top. The structure of the HCV replicon used in this study is shown below. It contains the HCV 5' nontranslated region directing translation of a fusion protein that is composed of the first 16 amino acid residues of the HCV core protein, the firefly luciferase (Ff-luc), ubiquitin (ubi), and the selectable marker neomycin phosphotransferase (neo). The EMCV IRES mediates translation of NS3 to NS5B. A combination of three cell culture adaptive mutations (E1202G, T1280I, S2197P) derived from the highly adapted subgenomic replicon rep 5.1 was introduced to increase RNA replication (15). (B) Cells persistently carrying the replicon shown in panel A were treated with 0, 0.5, 1, 2.5, 5, 7.5, 10, 25, 50, and 100 U of IFN-α2a/ml for 72 hours. The inhibition of translation and replication was determined by measuring the amount of firefly luciferase activity (circles) and the amounts of replicon RNA (squares), respectively. Note that the copy numbers of replicon RNA as determined by quantitative RT-PCR were normalized to 1 µg of total RNA by using GAPDH that was co-amplified in the same reaction.

8.3.3 Cell culture and dose-response assays

Huh-7 human hepatoma cells carrying the HCV replicon were grown in Dulbecco's modified Eagle medium (DMEM; Life Technologies GmbH, Karlsruhe, Germany) supplemented with 2 mM L-glutamine, non-essential amino acids, 100 U of penicillin, 100 µg streptomycin, 10 % fetal calf serum (complete DMEM) and 250 µg/ml G418

(Life Technologies). Cells were passaged twice per week at a dilution of 1:3 – 1:5, depending on cell growth by using PBS containing 0.05% trypsin and 0.05% EDTA (Life Technologies). For the luciferase/ RT-PCR assay, cells were seeded in 9.6 cm^2 dishes at a density of 200,000 cells in complete DMEM supplemented with 250 µg/ml G418 (Life Technologies, Germany). After 24 hours, medium was replaced by complete DMEM without G418 and containing 100, 50, 25, 10, 7.5, 5, 2.5, 1 or 0 U/ml of IFN-α2a (Roche Applied Science, Mannheim, Germany) in triplicates. In all other cases, cells were seeded in 24-well plates (2 cm^2 per well) at a density of 40,000 cells per well. After 24 hours, medium was replaced by 1ml complete DMEM per well containing 10% human serum and one of the following concentrations of interferon: 1000, 500, 250, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20 or 0 U/ml of IFN-α2a (kindly provided by K. Weyer and E. K. Weibel, Hoffmann-La Roche AG, Basel) or IFN-α2b (Schering-Plough), or 180, 60, 30, 15, 7.5, 3.75, 1.88, 0.94, 0.47, 0.23, 0.12, 0.06, 0.02 or 0 ng/ml PEG-IFN-α2a (Roche Applied Science). All experiments were carried out at least twice each in sextuple and on several plates, such that the positions of individual samples relative to the controls were varied in order to reduce positional effects. In case of the cell proliferation assay, the medium in triplicate wells was replaced by 1 ml complete DMEM containing increasing concentrations of ribavirin (Schering-Plough). Proliferation was determined by measuring the optical density after staining the cells with the tetrazolium salt WST-1 (Roche Applied Sciences). A positive (1,000 U/ml IFN-α) and a negative control (no IFN-α) was included on each plate such that the position was different from plate to plate.

8.3.4 Incubation of cells with patient sera

Twenty four hours after seeding, cells in each well of a 24-well plate were incubated with 0.9 ml complete DMEM and 0.1 ml serum from patients specified in Table 1. All experiments were carried out in triplicate. On each plate a positive (1,000 U/ml IFN-α) and a negative control (no IFN-α) was included.

8.3.5 Cell harvesting and measurement of luciferase activity

Cells were harvested after 48 - 50 hours incubation (or after 72 hours in case of luciferase/ RT-PCR assays). After removal of the medium, cells were washed twice

with phosphate buffered saline (PBS) and incubated with 180 µl, or 350 µl in case of the experiment shown in Fig. 1, ice cold luciferase lysis buffer (1% Triton X100, 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM DTT added just before usage) for 10 min., after which the lysate was harvested. After 15 min. centrifugation at 13,000 g and 4 ^{o}C, 100 µl lysate was mixed with 360 µl assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 1 mM DTT, 2 mM ATP, 15 mM potassium phosphate buffer, pH 7.8) and 200 µl of a 200 mM luciferin stock solution (PJK Chemikalien, Kleinbitterdorf, Germany) in 25 mM glycylglycin was added. Luminescence was measured in a luminometer (Lumat LB9507 from Berthold, Freiburg, Germany) for 20 seconds and expressed as the number of relative light units (RLU) detected.

8.3.6 RNA preparation and quantitative RT-PCR

150 µl of cell lysate were mixed with 600 µl of GITC buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl, 0.1 M 2-mercaptoethanol), 1/10 volume of 2 M sodium acetate (pH 4.5), 1 volume of phenol, and 1/5 volume of chloroform. After a 15 min. incubation on ice, samples were centrifuged for 15 min. at 13,000 g and 4°C. Nucleic acids in the aqueous phase were transferred to a fresh tube, precipitated with 1 volume of isopropanol, washed once with 70% ethanol and resuspended in 50 µl of doubly distilled water. Three µl of the sample were used for quantitative RT-PCR analysis using an ABI PRISM^{TM} 7700 Sequence Detector System (Applied Biosystems, Foster City, USA). The HCV- and GAPDH-specific RT-PCRs were conducted in duplicates with the One step RT-PCR kit (Qiagen, Hilden, Germany) using the following probes and primers (TIB Molbiol, Berlin, Germany): HCV, 5´-6FAM (6-Carboxy-Fluorescine)- TCC TGG AGG CTG CAC GAC ACT CAT-TAMRA (Tetra-Chloro-6-Carboxy-Fluorescine)-3´; HCV-S66, 5´-ACG CAG AAA GCG TCT AGC CAT-3´; HCV-A165, 5´-TAC TCA CCG GTT CCG CAG A-3´; GAPDH, 5´-TET (6-carboxy-4, 7,2', 7'-tetra-chlorofluorescein) -CAA GCT TCC CGT TCT CAG CCT-TAMRA-3´; GAPDH-S, 5´-GAA GGT GAA GGT CGG AGT C-3´; GAPDH-A, 5´-GAA GAT GGT GAT GGG ATT TC-3´. Reactions were performed in three stages by using the following conditions: stage 1, 60 min at 50°C (reverse transcription); stage 2, 15 min at 95°C (heat inactivation of RT and activation of Taq-polymerase); stage 3, 15 sec 95°C, 1 min 60°C, 40 cycles (amplification). The total volume of the reaction mix was 15 µl and contained the following components: 2.66 µM 6-Carboxy-X-

rhodamine (Rox, passive reference), 4 mM $MgCl_2$, 0.66 mM dNTPs, 0.266 µM HCV probe, 0.3 µM GAPDH probe, 1 µM of each HCV and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer, 0.6 µl enzyme mix. The amount of HCV RNA was calculated by comparison to serially diluted in vitro transcripts and the amount of GAPDH RNA by comparison to serially diluted HuH-7 total RNA. Both RNA species were included in the RT-PCR amplifications.

8.3.7 Calculations

The IFN-α and PEG-IFN-α dose dependent inhibitory activity in terms of percentage was calculated by the following equation:

% Inhibition = ((log10 (PC) - log10 (S_n)) / ((log10 (PC) – log10 (NC)) * 100%

Where PC is the number of RLU after incubation with the positive control (1,000 U/ml IFN-α or 180 ng PEG-IFN-α2a), NC is the number of RLU after incubation with the negative control (no IFN-α2a/α2b or PEG-IFN-α2a), and S_n is the number of RLU after incubation with samples from the dilution range (as mentioned above). A nonlinear logistic model expressing the relationship between the inhibition and concentration of IFN-α and PEG-IFN-α, respectively, was estimated using the program SPSS for Windows (Version 10.1 SPSS Inc, Chicago, IL, USA).

8.3.8 Vesicular stomatitis virus cytopathic effect reduction assay

The vesicular stomatitis virus (VSV) bioassay used in this study is based on the reduction of cytopathogenicity by treatment of cells with IFN-α prior to VSV infection (9). In brief, 4 x 10e4 Hep-2 cells seeded in 96-well plates were incubated at 37°C with IFN-α2b or PEG-IFN-α2a diluted in 10 twofold steps in DMEM with or without 10% human serum or with serum samples from patients under treatment. On all plates, controls were added (no IFN-α2b or PEG-IFN-α2a). After 6 hours the supernatant was removed, VSV (Indiana strain) was added in a volume of 100 µl DMEM (m.o.i. of 1) and incubated overnight. To visualize vital cells, the overlay was removed and cells were stained and fixed with 1% crystal violet in 1% formaldehyde. The virus-induced cytopathic effect was quantified by measuring the optical density at 595 nanometer.

8.4 RESULTS

8.4.1 Development of a selectable HCV replicon carrying a reporter gene

The usefulness was explored of the HCV subgenomic replicon system that is based on the autonomous replication of genetically modified 'minigenomes' in the human hepatoma cell line Huh-7 for determining the amount of biologically active IFNs. Originally, these replicons that were derived from a cloned genotype 1b HCV full length genome carried the following elements: the HCV 5' non-translated region that directs the expression of a selectable marker (the neo gene), the internal ribosome entry site (IRES) from another virus (encephalomyocarditis virus, EMCV) directing translation of the HCV NS3 to 5B region and the authentic 3' non-translated region. These replicons were transfected into Huh-7 cells and after G418 selection cell clones were established that carry HCV RNAs replicating autonomously to very high levels (15). However, direct determinations of RNA replication e.g. by Northern hybridization or quantitative RT-PCR are time consuming and they require technical skills and certain equipment that are not readily available in every laboratory. Therefore, a novel replicon was devised which carried in addition to the neo gene a reporter gene that could be used as a marker for RNA replication. For this purpose, we chose the firefly luciferase for several reasons: (1) it can be easily measured, (2) it is very sensitive, and (3) it has a very short half life which is important when measuring rapid changes of RNA translation/replication in a cell. Ubiquitin was inserted between this reporter and the selectable marker to enable proteolytic separation of the luciferase from the neomycin phosphotransferase (Fig. 1a).

In the first set of experiments, the sensitivity of this replicon towards inhibition by IFN-α was determined. Since WHO international standards for interferons were not used but rather interferons specified by different commercial suppliers, all values given below are expressed in units per ml (U/ml) rather than international units. Cells were incubated with different concentrations of IFN-α, or were left untreated, and after 72 hours cells were lysed and luciferase activity was determined. In order to verify that the amount of luciferase activity correlates directly with the amount of replicon RNA, quantitative RT-PCR was carried out with total RNA isolated from the same cell lysate The data in Fig. 1b demonstrate that luciferase activity and replicon RNA decreased with increasing concentration of IFN-α albeit with different kinetics. At concentrations up to 7.5 U/ml, luciferase and RNA amounts were reduced in parallel.

Interestingly, at higher concentrations, luciferase activity was reduced further whereas the amount of RNA was affected to a much lesser extent. This may be due to a preferential inhibition of RNA translation by higher concentrations of IFN-α. However, within the concentration of up to 7.5 U/ml IFN-α, both RNA and luciferase were reduced with comparable efficiency. In fact, the IC_{50} we determined from this assay was 1 – 2.5 U/ml when measuring HCV RNA and about 1 U/ml when using luciferase assays. In summary, these data demonstrate that the luciferase activity is a simple and reliable indicator for HCV RNA replication in this model system.

Next the dose response curves of different IFN-α formulations in this HCV replicon system were determined. Cells were incubated with various concentrations of IFN-α2a, IFN-α2b, or PEG-IFN-α2a and replication was determined by measuring the luciferase activities (Fig. 2). To express the relation between inhibition and concentration, a nonlinear logistic growth model over a polynomial fit was chosen because the first is based on the interpretation of biological parameters producing the response and can thereby be considered as mechanistic. Moreover, a nonlinear model requires only half of the amount of parameters and provides more reliable predictions of the response variable outside the observed range (17). A disadvantage of nonlinear logistic growth models is that classical analysis criteria such as parallelism of dose-response lines can not be used due to the absence of linearity. The correlation between inhibition and concentration of (PEG-) IFN-α/ml was expressed as:

$$\% \text{ Inhibition} = 100/ (1 + \exp (- (\log10(\text{Concentration}) - \beta)/ \delta))$$

where β is the concentration at which 50% of inhibition is reached, thereby including the IC_{50} in the equation, and δ is the difference in log concentration between 50% and about 75% [$1/(1 + e^{-1})$] inhibition. The estimated concentration of cytokine required for a 50% reduction of RLU (IC_{50}) by IFN-α2a, IFN-α2b and PEG-IFN-α2a was 4.1 U/ml (asymptotic 95% confidence interval: 3.4 - 4.9 U/ml), 2.3 U/ml (asymptotic 95% confidence interval: 1.9 - 2.7 U/ml)) and 1.7 ng/ml (asymptotic 95% confidence interval: 1.5 - 1.9 ng/ml), respectively. The estimated δ for IFN-α2a, IFN-α2b and PEG-IFN -α2a were 3.3 U/ml (asymptotic 95% confidence interval: 1.6 - 3.9 U/ml), 3.3 U/ml (asymptotic 95% confidence interval: 2.8 - 4.0 U/ml) and 3.2 ng/ml (asymptotic 95% confidence interval: 2.9 - 3.6ng/ml), respectively. IFN-α2a and IFN-

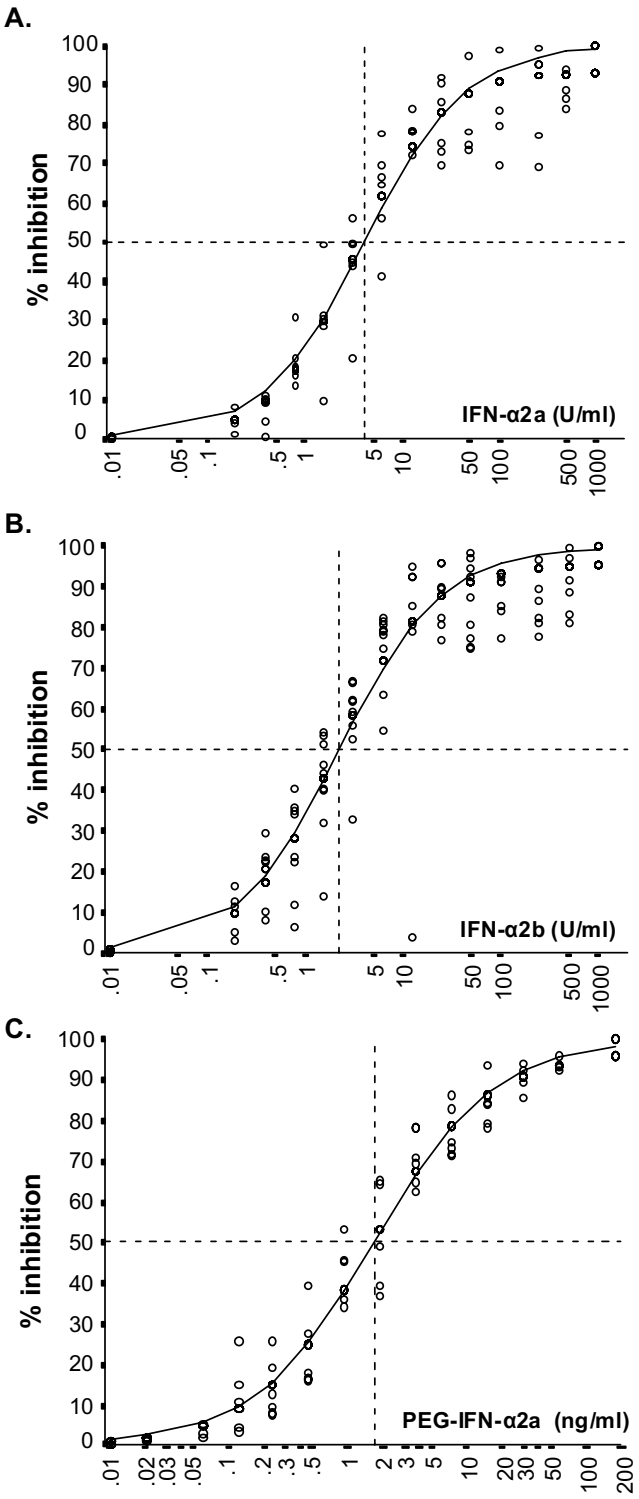

Figure 2. Dose response curves of recombinant IFN-α2a (a), IFN-α2b (b) and PEG-IFN-α2a (c). Cells carrying the HCV replicon were incubated with various concentrations of IFNs in a mixture of 0.9 ml complete DMEM and 0.1 ml human serum. The dose dependant inhibition by IFN-α2a, IFN-a2b and PEG-IFN-α2a, was calculated by the reduction of relative light units as determined by luciferase assays.

α2b appear similar in biological activity and based on the IC_{50} approximately 2 ng PEG-IFN-α2a can be compared to 2 - 4 U IFN-α2a or IFN-α2b.

8.4.2 Evaluation of the HCV replicon to measure biologically active IFN-α in patient samples

Having established a highly sensitive assay to measure the amount of biologically active IFN-α, it was determined whether this test could also be used to determine IFN-α in sera of patients under therapy. Therefore, sera from patients that were treated with different regimes of IFN-α (Table 1) were applied to cells with the HCV replicon as described in the Materials and Methods section. For comparison, on each plate a serial dilution of the same type of (PEG-) IFN-α the patients were treated with was analysed in parallel. The results are summarized in Table 2. Estimated serum concentrations in patients on treatment ranged from 1.3 - 19 U/ml (standard vs. high dose IFN-α) and from 3.8 - 4.1 ng/ml (standard PEG-IFN-α). The measured serum levels fell in the highest sensitivity range of the assay with inhibition of the replicon between 15 and 85%. Thus, the HCV replicon system can be used to determine the amount of biologically active IFN-α in sera of chronic hepatitis C patients that are under treatment.

Standard antiviral therapy consists currently of PEG-IFN-α in combination with ribavirin (4, 5). Pharmacokinetic studies have shown that ribavirin serum concentrations range from 1,000 to 2,750 ng/ml when measured after 4 weeks of daily administration of 1,200 mg (18). Since the patient sera which were used also contained ribavirin, tests were carried out to determine whether this drug would affect HCV replicons. However, it was found that concentrations up to 3,000 ng/ml did not inhibit HCV replicons nor did it affect Huh-7 cell proliferation as determined with a WST cell proliferation assay (data not shown). Thus, the inhibition we measured was only due to IFN-α present in patient samples.

Since in most cases plasma is prepared from blood samples of patients instead of serum, it was necessary to determine whether the replicon-based biotest could also be used to measure IFN-α and PEG-IFN-α in plasma samples. In the first set of experiments we performed IFN-α and PEG-IFN-α dose response assays in serum, EDTA plasma and heparinized plasma samples in parallel as described in the Materials and Methods section. Briefly, 24 hours after seeding, cells were incubated

with concentrations ranging from 0 to 1,000 U/ml IFN-α and from 0 to 180 ng/ml PEG-IFN-α added to cell culture medium that contained 1/10 volume of EDTA plasma or heparinized plasma or serum of naive patients. After 2 hours incubation, the cell culture medium containing 10% human EDTA plasma was gel, which was most likely due to coagulation that was induced by the fetal calf serum in the cell culture medium. Consequently, the medium could not be aspirated without affecting the cell monolayer making an analysis of these samples impossible. In contrast, cells incubated with serum or heparinized plasma could be easily harvested and replication was determined by measuring the luciferase activities after a 52 hours incubation. The results in Fig. 3 show that in spite of some variation cytokine activity in heparinized plasma and serum are comparable showing that the assay can be used for the measurement of IFN-α in both patient materials.

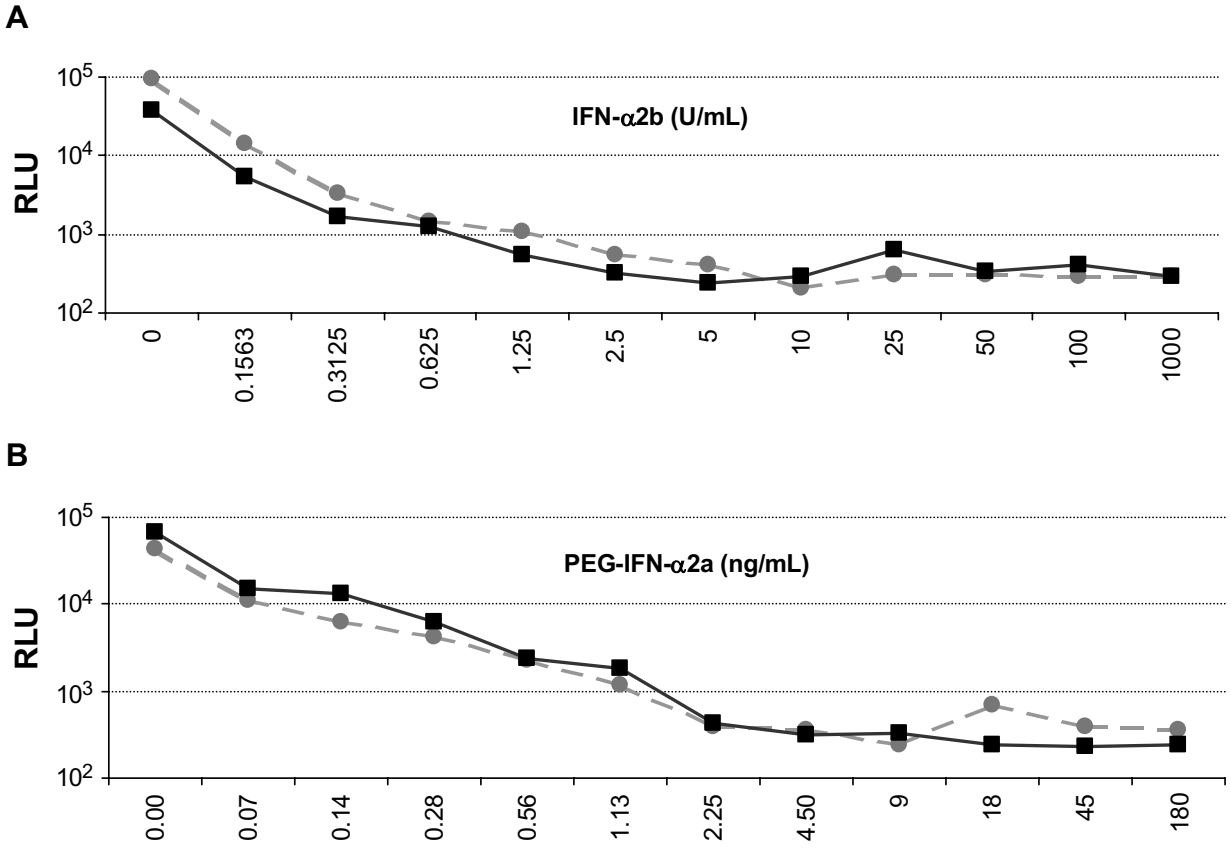

Figure 3. Dose response curves of recombinant IFN-α2b (a) and PEG-IFN-α2a (b) in serum and heparinized plasma. Cells carrying the HCV replicon were incubated for 52 hours with given concentrations of IFNs in a mixture of 0.9 ml complete DMEM and 0.1 ml human serum (grey dots) or heparinized plasma (black squares). The experiments were performed in triplicates and the medians are shown. RLU as determined by luciferase assays are displayed as a function of IFN-α concentration.

The biological activity of interferons is often determined by antiviral assays in which the reduction of virus yield or cytopathogenicity is used as an indicator for the activity of a given IFN-α preparation. Therefore, a comparison of the HCV replicon system was made with the VSV cytopathic effect reduction assay. This assay is based on the inhibition of cytolysis of infected cells by treatment with interferon and subsequent measurement of viable cells with a vital dye stain. In the first set of experiments, Hep-2 cells were treated with increasing concentrations of IFN-α2b or PEG-IFN-α2a in the presence or absence of human serum and subsequently infected with VSV. As shown in Fig. 4, in the absence of human serum, cells were gradually protected against VSV with increasing concentrations of IFN-α2b whereas only high concentrations of PEG-IFN-α2a confered antiviral resistance. However, in the presence of human serum, the cells were completely protected against VSV. The same was true when sera from patients under therapy (Table 2) were used. The assay could therefore not be used for the measurements of IFN-α2b or PEG-IFN-α2a levels in patient sera.

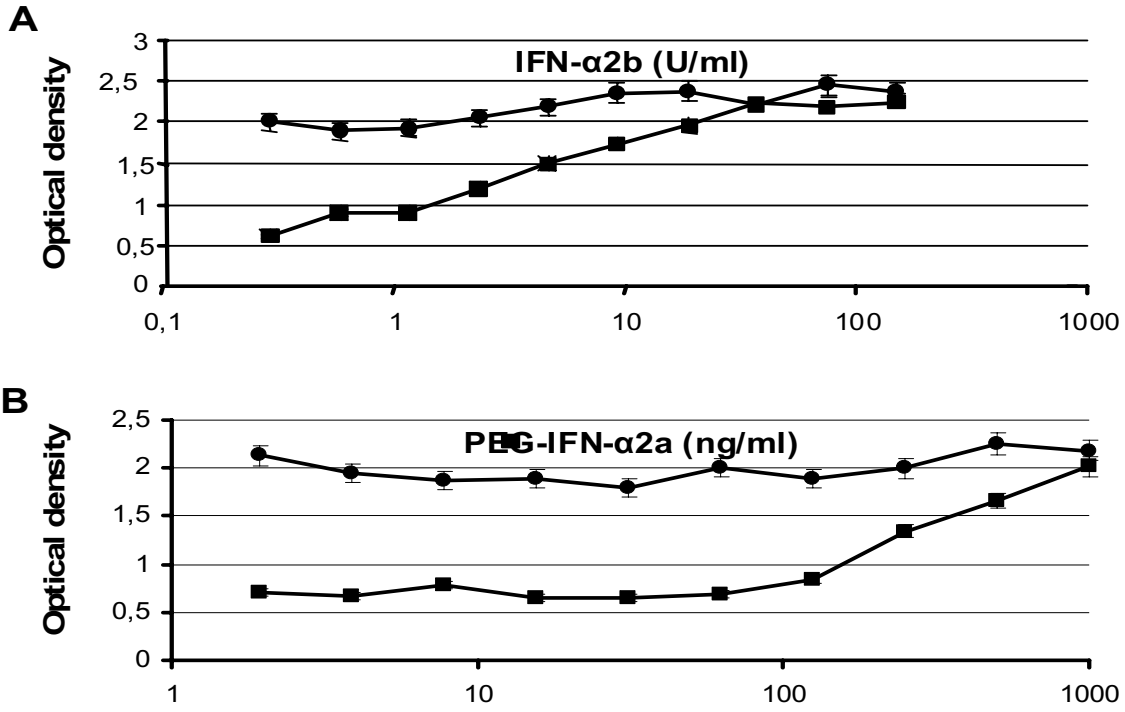

Figure 4. VSV cytopathic effect reduction assay. Hep-2 cells pretreated with given concentrations of IFN-α2b (A) or PEG-IFN-α2a (B) in the absence (black dots) or presence (black squares) of 10% human serum were infected with VSV (Indiana strain). After overnight incubation the virus-induced cytopathic effect was determined by optical density reading at 595 nanometer after staining of viable cells with the vital dye crystal violet. The experiments were performed in duplicates and the medians are shown.

Table 2. Estimated serum interferon concentrations

Patient	Medication	% inhibition[1]	Concentration (U or ng per ml)[2]
A	IFN-α2b 10 MIU/d + 1200mg Rbv/d	85	19 U/ml
A	IFN-α2b 3 MIU/d + 1200mg Rbv/d	69	6.3 U/ml
A	IFN-α2b 3 MIU tiw +1200mg Rbv/d	39	1.3 U/ml
B	PEG-IFN-α2a 180 ug QW + 1000mg Rbv/d	68	4.10 ng/ml
C	PEG-IFN-α2a 180 ug QW + 1000mg Rbv/d	67	3.85 ng/ml
D	PEG-IFN-α2a 180 ug QW + 1000mg Rbv/d	68	4.10 ng/ml
E	PEG-IFN-α2a 180 ug QW + 1000mg Rbv/d	67	3.85 ng/ml

[1]Reduction of luciferase activity relative to cells treated with naive human serum.

[2]Serum interferon concentrations as determined by comparison with cells that were treated with a serial dilution of the homologous interferon.

8.5 Discussion

In this study, a new bioassay for the measurement of IFN-α in serum and heparinized plasma of patients treated with this cytokine for chronic hepatitis C is described. The assay that is based on the inhibition of a subgenomic HCV replicon is sensitive and can be used for the measurement of IFN-α activity in clinical practice. The levels of IFN-α found in patient samples fell within the test range and could be determined by comparison with a dilution series of the homologous interferon analysed on the same plate. Compared to other bioassays (19), the HCV replicon system has the following advantages: First, it directly measures the inhibition of HCV RNA replication; second, it is very sensitive and allows measurements in patient samples, i.e. in the presence of human serum; third, it is easy to perform because HCV replication can be measured by simple bioluminescense assays; fourth, HCV replicons are non-infectious which is an advantage over the Sendai virus growth inhibition assay or the VSV cytopathic effect reduction assay that are often used to measure IFN-α levels. As an alternative to these antiviral assays, IFNs can be measured by immunoassays such as enzyme-linked immunosorbent assay (ELISA), immunoradiometric assay

(IRMA) and radioimmune assay (RIA). These assays are precise and accurate for measuring IFNs in vitro but loose sensitivity when measuring this cytokine in patient samples. This may be due to certain inhibitors such as heterophile antibodies, rheumatoid factors, or a soluble form of IFN-α/β receptors interfering with sensitivity (20, 21). Moreover, results obtained in those assays do not necessarily reflect the amount of the biologically active fraction of IFN-α in a given sample. Finally, immunoassays depend on the specific recognition of an antigen by the antibodies used. Therefore, apparent differences in amounts can be the result of differences in sensitivity and specificity with which the antibodies recognize a particular IFN.

Inspite of the benefits discussed above, potential drawbacks of using the HCV replicon system as a bioassy should also be mentioned. First, bioassays are often sensitive to culture conditions and therefore somewhat variable in outcome. However, with a strict standardization of the assay as described here, the variations can be kept at a minimum. Moreover, in spite of some interassay variability in the absolute amounts of luciferase activity detected in replicon cells, the linear range of this assay is very stable throughout continued passage of the cells. The assays described here were performed multiple times in the course of about one year and in two different laboratories with good reproducibility of linearity as long as samples were measured against a dilution series of an internal standard that was added on each plate. A gradual reduction in the absolute amounts of luciferase activity was observed which was most likely due to the emergence of mutations in the luciferase gene. These mutations probably accumulated during prolonged cell passage and led to a successive loss of reporter activity. However, this reduction took several months and in case that absolute values were too low, the cells in culture were replaced by a lower passage of the same cell clone that had been stored frozen in liquid nitrogen. It should be pointed out that cells carrying the HCV replicon described in this study could be frozen and thawed without loosing the replicon and luciferase activity. In a well growing culture of Huh-7 cells carrying this replicon, luciferase activity was about 5 x 10e5 RLU per 10e5 cells, which corresponds approximately to the total number of cells in a single well of a 24-well plate.

Second, it is possible that the inhibition we observed is not the result of an inhibition of HCV RNA replication but rather due to a block of the EMCV IRES. However, since monocistronic HCV replicons that lack this element and in which translation and

replication are exclusively controlled by HCV sequences are also blocked efficiently by IFN-α, this possibility is ruled out (A.K. and R.B., unpublished). Third, HCV RNA replication depends very much on host cell growth (22) and therefore, it is important to standardize the cell culture conditions and to avoid the use of over confluent cells. Moreover, substances inhibiting cell proliferation apparently block replicons. However, proliferation of Huh-7 cells is not substantially affected by IFN-α or naive patient samples, at least in the concentration ranges used here.

Huh-7 cells carrying stably replicating HCV replicons are robust and easy to handle. In fact, their growth properties and morphology are very similar to that of naive Huh-7 cells. When cells with the replicon are kept under continuous selective pressure (G418), the replicon is maintained for several years. An important parameter is passaging the cells at regular intervalls, because cell density very much affects HCV RNA replication. When cells are kept in a confluent state for prolonged times, replicon RNA levels are reduced drastically (22). Therefore, cells are passaged at regular intervalls at a dilution of 1:3 to 1:5, usually twice per week. However, even when replicon RNA levels (luciferase activities) were low, the linearity of this bioassay was not affected.

For high throughput analyses, one limitation of this bioassay in its current form is the preparation of cell lysates and the measurements of luciferase activities in lysates. In this study, we analysed and measured a total of 300 samples within a day which is at the limit a single person can do manually. However, the preparation of the lysate and the measurements can probably be automated which allows a much higher throughput of samples.

By using the replicon based bioassay, it was possible to measure and compare the inhibitory effects exerted by IFN-α2a, IFN-α2b, and PEG-IFN-α2a, in samples of patients treated with these cytokines. Because of its high sensitivity, this bioassay could be used for the establishment of international biological standards, and for comparing and standardizing relative activities of different IFN-α preparations, as approved by the WHO (19). In clinical healthcare, such standards can be important to correlate viral kinetics and drug levels, thereby contributing to the optimization of treatment in patients prone to non-response.

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Chapter 9

Population pharmacokinetics, early response and long-term outcome of pegylated interferon alfa-2a in the treatment chronic hepatitis C

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9.1 ABSTRACT

Background/Aims: Responses to combination therapy of peginterferon and ribavirin in chronic hepatitis C are variable. The objectives of this study were to describe the population pharmacokinetics of peginterferon-α2a and to correlate pharmacokinetic parameters with outcome like early virologic response (EVR), end of therapy (EoT) and end-of-follow-up (EFU) treatment results.

Methods: Two hundred seventy patients were treated with peginterferon-α2a (180 mcg QW) and ribavirin (1-1.2g qd). Based on the first month viral kinetics all patients were classified into one of three EVR categories (rapid (RVR), slow (SPR) or flat-null (FPR/NUR) responders) and thereupon randomized to either standard or tailored therapy. During the first month of therapy serial blood sampling was performed and serum interferon levels were quantified in 255 patients using a sandwich enzyme-linked immunosorbent assay (ELISA) at 7 time points. Population pharmacokinetics were assessed by nonlinear mixed effects modeling. Secondary parameters, a.o. AUC (area under the plasma concentration versus time curve), were derived.

Results: Clearance of peginterferon-α2a was significantly correlated with weight; clearance for a typical 74 kg patient was 1.36 L/day. Inter- and intra-patient variability of clearance was considerable with values of 35% and 25%, respectively. The AUC in week 4 was approximately 2-fold higher than in week 1, indicating accumulation of peginterferon-α2a. Combined over the first 4 weeks AUC was higher for patients with RVR than for SPR (borderline sign.:$p=0.07$) or FPR/NUR (sign.:$p<0.01$). Within the group treated with standard therapy (n=126), response categories were highly predictive for outcome ($p<0.01$). However, a relationship between the first 4 weeks AUC and EoT or EFU could not be found.

Conclusions: There is considerable inter- and intra-patient variability in the pharmacokinetics of peginterferon-α2a. Exposure during the first month of treatment can predict early virological response but appears not to be correlated with treatment outcome. Pharmacokinetic variability of peginterferon-α2a does not appear to explain variability in treatment response in patients with chronic hepatitis C.

9.2 INTRODUCTION

The most effective and current standard therapy for chronic hepatitis C virus (HCV) infection is the combination of peginterferon-α2a or -α2b and ribavirin with sustained viral response (SVR) achieved in more than half of treated patients (1, 2). The two peginterferon-α molecules used in the registration studies clearly differ in pharmacokinetic characteristics but the significance of this difference remains unclear. Detailed information on peginterferon-α2b pharmacokinetics has been published in the recent years (3-5). In contrast, the pharmacokinetics of peginterferon-α2a have only been investigated in a single published study in which a limited number of patients with renal cell carcinoma received the compound with escalating doses (6).

To test the hypothesis that pharmacokinetic variability may explain variations in treatment response, we performed a pharmacokinetic study ancillary to an investigator-initiated multicenter clinical trial (7, 8). In this randomized controlled study the efficacy of a dynamically individualized treatment schedule according to the early virologic response was evaluated against that of standard combination therapy with peginterferon-α2a plus ribavirin for 48 weeks. The objectives of this ancillary analysis were to describe the population pharmacokinetics of peginterferon-α2a and to correlate pharmacokinetics with outcome like early virologic response (EVR), end of therapy (EoT) and end-of-follow-up (EFU) treatment results.

9.3 MATERIALS AND METHODS

9.3.1 Patients

270 consecutive patients with chronic hepatitis C were recruited from nine university hospitals in France, Germany, Greece, Israel, Italy, Spain, Sweden, Switzerland and The Netherlands. Patients were eligible if they complied with the following inclusion criteria: age over 18 years, two serum alanine aminotransferase values above the upper limit of normal within 6 months of treatment initiation; a positive test for anti-HCV antibody; a HCV RNA level greater than 1000 IU/mL by PCR; liver-biopsy taken in the preceding 12 months compatible with the diagnosis of chronic hepatitis C, compensated liver disease and for women a negative urine or blood pregnancy test. Major exclusion criteria included interferon or ribavirin therapy at any previous time,

advanced cirrhosis or decompensated liver disease, co-infection with hepatitis B or human immunodeficiency virus, alcohol abuse or any other relevant comorbidity. All patients gave written informed consent before inclusion and the medical-ethical committees of participating hospitals approved the study.

9.3.2 Treatment

All patients were initially treated with peginterferon-α2a (Pegasys, Roche, Switzerland) 180 µg once weekly (QW) subcutaneously injected (injections on day 0, 7, 14, 21, 28, etc.) in combination with twice daily orally taken ribavirin capsules (Copegus, Roche; 1-1.2g per day) for 6 weeks. A physician or a trained nurse instructed the patients how to perform the injection and administered the first injection of peginterferon-α2a. Initial viral kinetics were defined on samples taken on day 0, 1, 4, 7, 8, 15, 22 and 29.

After 6 weeks patients were classified into one of three early viral response categories as rapid viral responder (RVR), slow partial responder (SPR) flat partial or null responder (FPR/NUR) and randomized within each viral class to continue either with an individualized regimen or standard peginterferon-ribavirin combination therapy (48 weeks). Individualized treatment tailoring included discontinuation of ribavirin or shortening of treatment duration to 24 weeks in RVRs; the addition of histamine or prolongation of treatment to 72 weeks in SPRs; the addition of histamine in FPRs and retreatment with high-dose of PEG-IFN (360 µg QW) plus ribavirin in NURs (7, 8).

The primary end point was end of follow-up (EFU) treatment results, the secondary end point virologic end of therapy (EoT). Both end points were defined as undetectable serum HCV-RNA at the end of follow-up or the end of therapy, respectively (Cobas Amplicor^{TM} HCV, version 2.0, lower limit of detection 50 IU/mL).

9.3.3 Peginterferon-α2a measurements

Concentrations of peginterferon-α2a were measured in serum on day 0 (direct before the first injection) and days 1, 4, 8, 15, 22 and 29 using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) method (BMS216INST, Bender

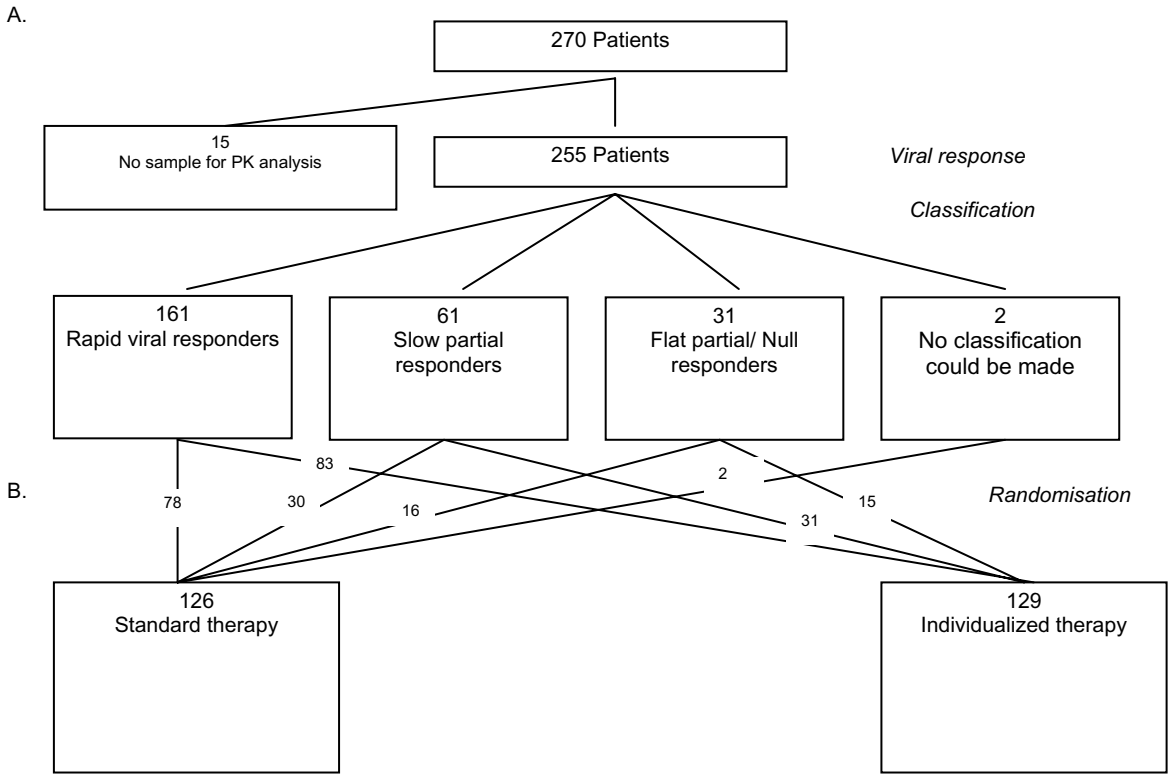


Figure 1. A) Flow diagram showing the classification and B) randomization outcome of all patients included in the study.

MedSystems Diagnostics GmbH, Vienna, Austria). The assay uses a murine antihuman interferon-α2 monoclonal antibody adsorbed onto microwells which captures interferon-α present in the samples or controls. A horse radish peroxidase-conjugated monoclonal anti-interferon-α antibody binds to captured interferon-α. After addition of substrate and activator a colored product is formed in proportion to the amount of interferon-α in the sample and determined photometrically as specified by the manufacturer. A calibration curve was prepared plotting optical density vs. the concentration of the standards. Standards containing 100.000pg–100pg/mL were prepared by diluting peginterferon-α2a in normal human serum (CLB, Amsterdam, the Netherlands). 5µl serum samples obtained from the patients on treatment were incubated on the same plates as the standards, all in duplicates. The detection limit of the assay is 3pg/mL and the assay is linear up to a concentration of 10.000pg/mL. In a subset of 55 patients sampling of blood and measurements of interferon-α levels was done more frequently; on days 0, 1, 2, 3, 4, 7, 8, 9, 11, 14, 15, 18, 21, 22, 25, 29.

9.3.4 Pharmacokinetic analysis

Pharmacokinetic models were fitted to data from all individuals simultaneously using nonlinear mixed-effect modelling (NONMEM). Measurements of the subset of 55 more frequently sampled patients were used for balancing the model. The NONMEM model accounted for inter-patient, intra-patient and residual pharmacokinetic variability (random effects) as well as pharmacokinetic differences predicted by patient factors (fixed effects). The typical population parameters, inter- and intra-patient and residual variances were estimated using the NONMEM software program (double precision; version V, level 1.1) (9). The first-order conditional estimation (FOCE) method was applied with the INTERACTION option. A one-compartment pharmacokinetic model with first-order absorption and elimination was used to describe the time profiles of peginterferon-α2a plasma concentration. The pharmacokinetics were parameterized in terms of absorption rate constant (Ka), volume of distribution (V/F) and clearance (CL/F). F refers to the unknown bioavailability. Inter- and intra-patient variability of the pharmacokinetic parameters was estimated using exponential error models. For instance, inter-patient and intra-patient individual CL/F was described using $CL/F=CL/Fpop\ e^{(\eta + \kappa)}$, in which CLpop is the typical population value and η and κ are the inter- and intra-patient random effects with mean 0 and variances ω2 and π2, respectively. The difference between the observed concentration (Cobs) and its respective prediction (Cpred) was modeled with a combined additive plus proportional error model: Cobs = Cpred * (1 + ε1) + ε2, where ε1 and ε2 are independent random variables.

On basis of the derived structural population model and the observed individual concentrations, individual pharmacokinetic parameter estimates were obtained by Bayesian (POSTHOC) analysis. The individual estimates were plotted against demographic and pathophysiologic factors (Table 1) for visual inspection according to the method described by Maitre et al. (10). Covariates that showed a correlation with a pharmacokinetic parameter were entered into the population pharmacokinetic model. In the NONMEM model the relationship between covariates (gender, weight, etc) and pharmacokinetics was statistically tested using the log-likelihood test (9). Both categorical and continuous variables were evaluated. For instance, a difference in the Cl between male and females was evaluated using the following equation: $CL= \theta1 \times \theta2^{FLAG}$, where the indicator variable FLAG has the value 0 (female) or 1 (male), θ1 is the typical Cl of females (FLAG = 0) and θ2 is the fractional change in θ1 with

males. The relationship between Cl/F and weight (WT) was described as follows: $CL/F = \theta3 \times (WT/74)^{\theta4}$,where Cl/F is a typical population value of clearance, θ3 is the Cl/F of a patient with WT of 74 kg and θ4 is an exponent.

Using the final population model (with significant co variables included) individual plasma concentration versus time profiles were generated by Bayesian analysis. On the basis of these individual profiles secondary parameters were generated as the area under the concentration-time curve (AUC), both over 1-week periods and cumulative, the maximal plasma concentration (Cmax), the time to maximal plasma concentration (Tmax).

9.3.5 Calculations and Statistics

Possible relationships between the pharmacokinetic parameters and early viral response (EVR) and end of therapy response (EoT) and end of follow-up (EFU) response were analyzed using the program SPSS for Windows (Version 10.1 SPSS Inc, Chicago, IL, USA). Groups were compared by parametric and non-parametric tests. Values of $p<0.05$ were considered to be statistically significant.

9.4 RESULTS

9.4.1 Patient characteristics

Of the 270 patients that entered the study, samples for virological and peginterferon-α2a measurements from the first treatment month were available in 255 patients. Demographic and baseline characteristics are shown in table 1. The mean (±1 SD) age of these 255 patients who are considered as the study group was 41.2±10.1 years. 172 patients were male (68%), 83 female (32%). 95% were Caucasians. The mean (±1 SD) weight, length and BMI were 74.3±13.5kg, 172±10cm and 25.1±3.6kg/cm^2. The average baseline ALT level was 2.6±1.8 times the upper limit of normal when corrected for center specific normal values, the log transformed serum viral load was 6.1±0.8 IU/mL. 167 patients were genotype 1 (66%), 22 genotype 2 (9%), 51 genotype 3 (20%), 14 genotype 4 (6%) and 1 genotype 5 (0%) infected.

Based on the first month viral kinetics, patients were classified into early viral response categories according the study protocol (figure 1A). 161 patients were classified as rapid viral responders (RVR; 63%), 61 as slow partial responders (SPR; 24%) and 31 as flat partial responders or null responders (FPR / NUR; 12%). After classification into response categories, patients were randomized to continuation of standard therapy to a total of 48 weeks (n=126) or individualized therapy (n=127). Two patients that could not be classified received standard therapy (figure 1B)(7, 8).

9.4.2 Pharmacokinetics

The time profiles of peginterferon-α2a plasma concentration for the first treatment month are shown in figure 2 (page 165). The population pharmacokinetic parameters of peginterferon-α2a are given in table 2 (page 162). In the structural model (no covariates included: model 1) considerable inter- and intra-patient variability in clearance was observed; respective values were 39% and 25% with a typical Cl/F value of 1.34 L/day. This indicates that within the studied population CL/F may vary from 0.29 to 2.39 L/day between patients (95% confidence interval: 1.34 ± 2 x 0.39 x 1.34). Within

Table 1. Baseline demographic and viral characteristics of the study group (n=255).

Population parameters		
	Mean (range)	SD
Weight (kg)	74.4 (946-126)	13.5
Height (cm)	172 (146-196)	10
BMI	25 (19-40)	3.6
age (years)	41 (18-66)	10
ALT (xULN)	2.6 (0.8-11.3)	1.77
Gender	n	(%)
male	172	68
female	83	33
Race	n	(%)
Caucasian	243	95
Asian	8	3
Black	4	2
Virological parameters		
Log Viral load (IU/mL)	6.8 (2.9-7.4)	0.8
Genotypes	n	(%)
genotype 1	167	66
genotype 2	22	9
genotype 3	52	20
genotype 4	14	6
genotype 5	1	0

a patient with average CL/F of 2 L/day this parameter may vary from 1 to 3 L/day (95% confidence interval: 2.00 ± 2 x 0.25 x 2.00).

Weight, body surface area and BMI correlated with clearance and volume of distribution when separately included in the NONMEM model ($p<0.01$). Due to the co linearity of these factors only weight was included in the final model as summarized in table 2 (model 2). For the other patient factors as listed in table 1 no significant relationships were detected. When weight is included in the model (unexplained) inter-patient variability in Cl/F decreases from 39% to 35%. The relationship between drug exposure and body weight is illustrated in Figure 3. Cumulative AUC over 1 month were lower in patients weighting between 80-100kg than in patients of 50-70kg (average: 382.000 vs. 514.000 pg/mL ; $p \leq 0.01$; figure 3b).
By application of Bayesian analysis individual time profiles of peginterferon-α2a were generated and secondary parameters as Tmax, Cmax and AUC were derived (Table 3). Cmax and AUC over 1 week increased approximately 2-fold from week 1 to week 4. Following subcutaneous administration maximal concentrations were obtained after 2-3 days.

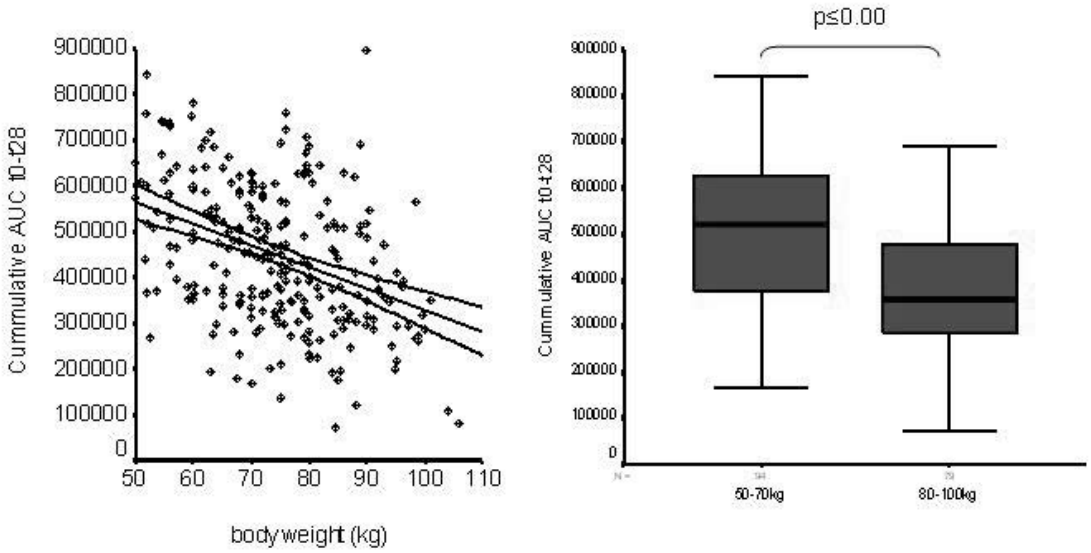


Figure 3: Cumulative AUC of Peginterferon-α2a from day 0 to day 28 in relation to body weight. A) Shown are the predicted regression line (straight line) and its 95% confidence interval (bent line). B) and box plots showing two weight categories significantly different in average cumulative AUC.

Table 4. Exposure to peginterferon-α2a. Compared are values from two early response categories.

	Rapid viral responders (n=161)	Flat partial / Null responders (n=31)	*p*-value*
$AUC_{day\ 0-7}$	78612	62816	0.004
$AUC_{day\ 7-14}$	114060	92924	0.003
$AUC_{day\ 14-21}$	133307	106880	0.003
$AUC_{day\ 21-28}$	143212	114565	0.006

AUC= area under the curve (pg/mL).

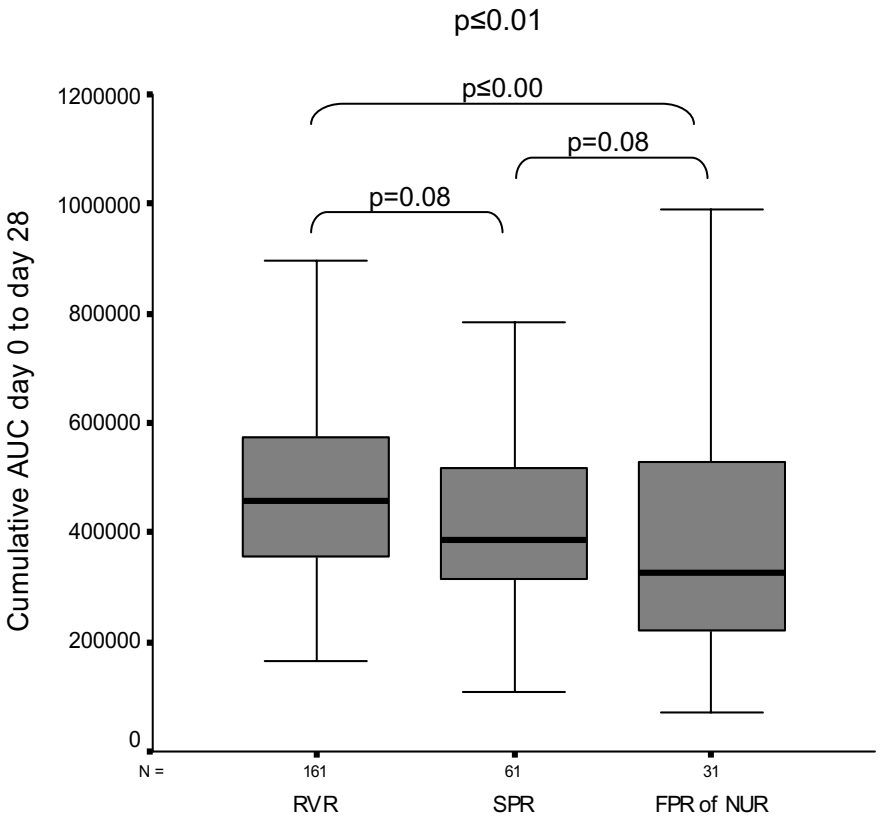
* Mann-Whitney test.

9.4.3 Pharmacodynamics

Significant differences between the cumulative first month AUC values were found between early viral response categories RVR, SPR, FPR / NUR (Kruskal-Wallis one-way analysis of variance (K-W test): p=0.006); figure 4a), indicating that higher serum peginterferon-α2a concentrations result in better early viral response. Detailed analysis for two response categories is displayed in table 4. Increasing weekly AUC values for FPR / NUR are 80% of RVR patients. Differences are highly significant (p<0.01).

Within the group treated with standard therapy (n=126), response categories were highly predictive for end of follow-up response outcome (Chi-square test: p<0.01). A relation between the cumulative first month AUC values value and the end of therapy or end of follow-up could however not be found ((K-W test): p=0.7; figure 4b). No significance for other pharmacokinetic parameters could be found; weight and BMI had no effect on treatment outcome.

A)


p≤0.01
p≤0.00
p=0.08
p=0.08
Cumulative AUC day 0 to day 28
1200000
1000000
800000
600000
400000
200000
0
N =
161
61
31
RVR
SPR
FPR of NUR

B)

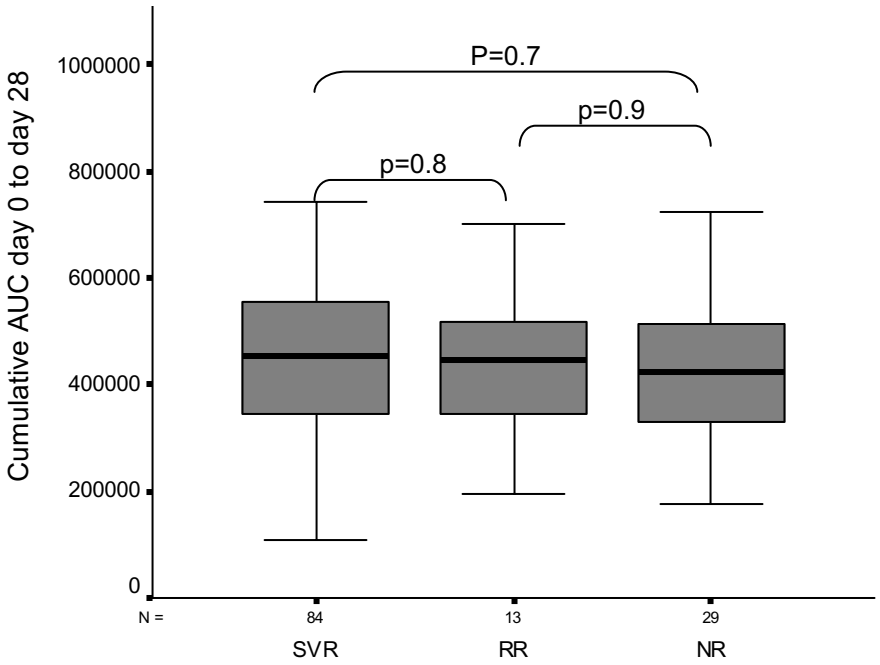

p=0.9
P=0.7
p=0.9
p=0.8
Cumulative AUC day 0 to day 28
1000000
800000
600000
400000
200000
0
N =
84
13
29
SVR
RR
NR

Figure 4:

A) Box plots of cumulative AUCs of Peginterferon-α2a measurements from day 0 to day 28 per response class based on first month viral kinetics. Testing for multiple independent samples (Kruskal-Wallis one-way analysis of variance (K-W test)) learned that the three response classes differ significantly in average cumulative AUC (p=0.006). The cumulative AUC is significantly higher in rapid viral responders (RVR) than in flat partial responder / non-responders (FPR/NUR) (Mann-Whitney test; p=0.004). RVR and slow partial responders (SPR) or SPR and FPR/NUR do not differ significantly. Displayed are data from 253 patients; data of 2 patients are missing.

B) Box plots of cumulative AUCs of Peginterferon-α2a values from day 0 to day 28 per treatment result (end of follow-up) for those treated for 48 weeks with standard therapy. No significance was observed (K-W test; p=0.9) between patients with a sustained virological response (SVR), response relapse (RR) or non-response (NR). Displayed are data from all 126 patients treated with standard dosed Peginterferon-α2a-ribavirin.

Explanation on boxes: The upper and lower limits of the boxes and the middle line across the boxes indicate the 75th and 25th percentiles and the median (the 50th percentile), respectively. The length of the box is thus the interquartile range; the box represents 50% of the data. The upper and lower horizontal bars indicate the 90th and 10th percentiles, respectively.

Table 2. Population pharmacokinetic parameter estimates for peginterferon-α2a.

	Model 1 Estimate (CV%)	**Model 2** Mean (CV%)
Population parameters		
Ka (1/day)	0.673 (6%)	0.673 (6%)
V/F (L)	10.1 (6%)	10.3 (6%)
Cl/F (L/day)	1.34 (3%)	1.36 (3%)
Fractional change in clearance (Cl/F)		
Exponent V-weight	-	0.697 (41%)
Exponent Cl-weight	-	0.868 (18%)
Between-patient variability		
Ka (%)	33 (53%)	35 (49%)
V/F (%)	71 (17%)	70 (17%)
Cl/F (%)	39 (17%)	35 (20%)
Between-dose variability		
V/F (%)	21 (41%)	21 (40%)
Cl/F (%)	25 (32%)	25 (32%)
Residual error		
Additive (pg/ml)	2090 (13%)	2080 (13%)
Proportional (%)	17.8 (21%)	17.6 (22%)
Minimal value objective function		
Value	35279.8	35237.4

Ka = absorption rate constant; V = volume of distribution; Cl = clearance; CV = coefficient of variation; F refers to the unknown bioavailability.

Model 1 and 2 were fitted to data from all individuals (n=255) simultaneously using nonlinear mixed-effect modelling. Model 1 is the structural population model with no covariates included and model 2 with the significant covariate "weight" entered in the model. Note that weight does not affect Ka, V, CL in a significant way since differences in model 1 and 2 are very similar

9.5 DISCUSSION

This study provides the first detailed description of peginterferon-α2a pharmacokinetics and pharmacodynamics in the treatment of a large cohort of chronic hepatitis C patients. Until now, the only fully published peginterferon-α2a pharmacokinetic data are from a small dose escalating study in renal cell carcinoma compromising only 6 patients treated with the dose standard used in HCV (6). Our study confirms the published data on mean level but also clearly shows the large variability between and in- patients. Subcutaneous administration of peginterferon-α2a led to rapid absorption from the injection side. After each peginterferon-α2a injection maximal concentrations for every week appeared between day 2 and 3 after each dose with declining intervals with chronic dosing, suggesting an increased absorption rate relative to treatment duration. Both C_{max} and AUC increased with chronic dosing to about 2 fold in 4 weeks due to accumulation. Variability in clearance between patients was 39% and only decreased to 35% when corrected for weight. The variability between doses within a patient was about 25%. The considerable intra-patient variability suggests other factors responsible for the great variance than included in the multivariate analyses. Both the intra- and inter-patient variability might be explained by the variability inherent to the process of subcutaneous administration of peginterferon-α2a. Local tissue blood flow, side and depth of injection as well as diffusion through the interstitial space and temperature of the farmacon might affect absorption rates.

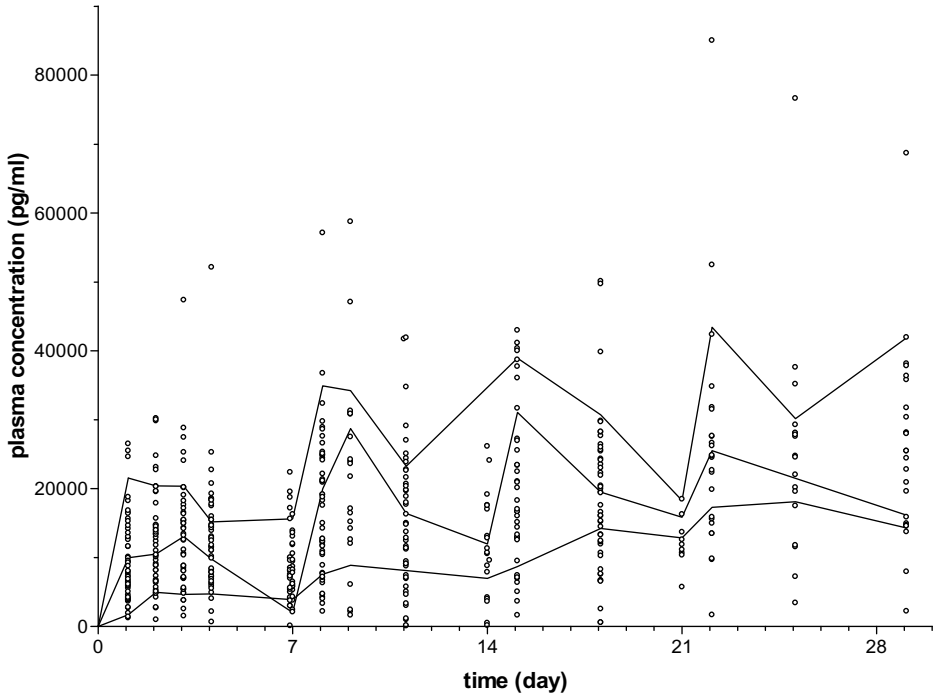
Within the study group individual weight varied widely. Variance in serum concentration could therefore be expected since all patients were treated with the same fixed dose. Indeed, a negative correlation between weight and C_{AUC} was found (figure 3). However, although lower serum concentrations of peginterferon-α2a were found in heavier patients, its significance on treatment outcome was lost. Therefore weight adjusted dosing are not expected to have beneficial effects in the average population. However, the results do not exclude that a small proportion of non-responder patients might be related to low drug levels. Only the heavier patient with more characteristics associated with unfavorable treatment response such as

Table 3. Peginterferon-α2a pharmacokinetic parameters in patients administered 180 mcg on days 0, 7, 14 and 21 (n=255).

day:	0-7		7-14		14-21		21-28	
	mean	SD	mean	SD	mean	SD	mean	SD
Ka (1/day)	0.676	0.166	0.676	0.166	0.676	0.166	0.676	0.166
V/F (L)	11.7	11.7	12.0	11.7	12.0	11.9	11.8	12.0
Cl/F (l/day)	1.37	.48	1.41	.55	1.39	.55	1.38	.52
$C_{max\ (pg/ml)}$	14200	7000	19700	8200	22100	8800	23800	9600
T_{max} (day) (rel.)	3.1	0.99	9.5 (2.5)	0.71	16.3 (2.3)	0.56	23.2 (2.2)	0.47
AUC week (pg/ml per period)	76100	33800	110000	45000	127000	50000	136000	56000
AUC- 4 weeks(pg/ml per period)	76100	33800	186000	73000	313000	117000	449000	164000

Ka = absorption rate constant; V = volume of distribution; Cl = clearance; CV = coefficient of variation; F refers to the unknown bioavailability; C_{max} = maximal PEG-IFN concentration per period; T_{max}= time of maximal concentration in days (relative to last injection); AUC= area under the curve

A)



B)

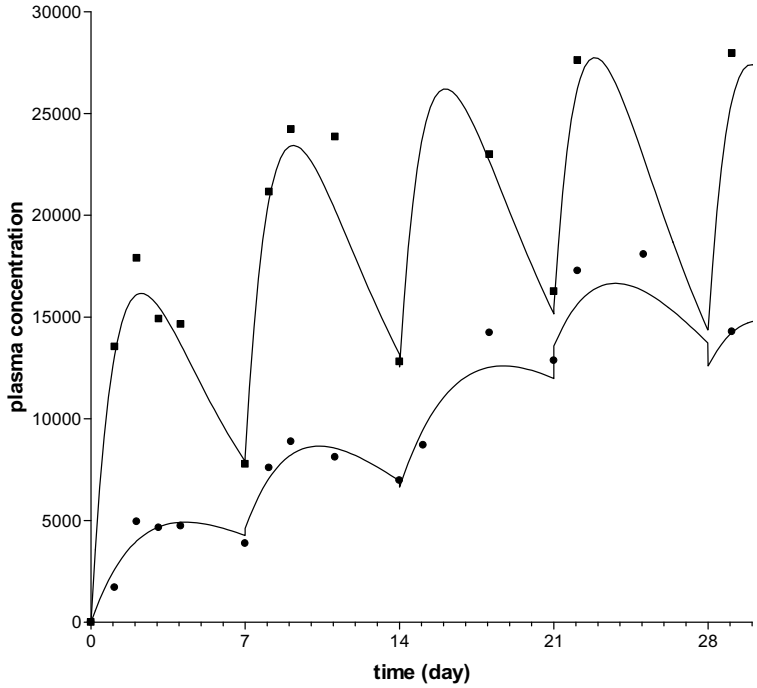


Figure 2. A) Time profile of Peginterferon-α2a plasma concentration of 255 patients. The patients received 180 mcg sc once weekly. The solid lines indicate the time profiles of 3 random patients showing the variation in intra- and inter-patient variability.B) Time profiles for two typical patients. The symbols are the determined concentrations. The solid lines represent the individual Bayesian pharmacokinetic fits.

genotype 1, male gender, high viral load, etc. might benefit from higher dosing or longer therapy duration.

A significant effect of C_{AUC} on early outcome but not on final outcome was found. The AUC in patients with favorable early responses (RVR) who more often became sustained virological responders were higher than in those without (FPR / NUR). Significance however declined with increasing treatment duration and was lost at the end of the treatment free follow up period. This could partly be explained due to power loss after halving the study group since the individualized tailored treatment patients could not be taken into the end of treatment and at the end of the treatment free follow up period analysis because the therapy they received was not comparable in length, intensity or dose. However, similar effects have been seen with non-pegylated interferon studies comparing high dosed daily interferon to standard dosed thrice a week interferon. In those reports, a significant increase of early virological response was found in the high dosed daily group although this effect did not result in an increased sustained virological response (11, 12). A possible explanation might be that the initial therapy results are (peg)-interferon serum level dependant and that the end of therapy result is determined by intracellular ribavirin levels.

In summary, the population pharmacokinetics of peginterferon-α2a have been characterized. Considerable inter-and intra-patient variability is present for clearance. Some of this variability can be explained by the relationship of clearance and weight. Variability in pharmacokinetic is also reflected in parameter describing drug exposure. There appears to be a correlation between exposure during the first month and early virological response. However no relationship has been demonstrated between the first months exposure and treatment outcome.

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Chapter 10

Discussion and summary

Jan Maarten Vrolijk, Solko W. Schalm

10.1 INTRODUCTION

In the treatment of chronic HCV, interferon based antiviral therapy has now been used for nearly two decades to slow down and prevent the progression to cirrhosis of the liver. In the past years, it's success rate has made remarkable progress from less than 10% up to 80% in selected patients (1). However, for patients that have unfavorable patient, disease or virus characteristics, sustained response rates are much lower. Major unfavorable characteristics are genotype 1 or 4 infection, liver cirrhosis or non-response to prior antiviral therapy. Among the minor but still important factors are high viral load, male gender and obesity. In patients with a single or combination of "difficult to treat" characteristics, the a priori chance to respond to standard therapy is considerably lower between 10 and 40%.

To overcome the effect of unfavorable characteristics and to identify factors associated with response we performed the studies described in this thesis. We imagined that response is the positive outcome after weighing the combination of "difficult to treat" characteristics

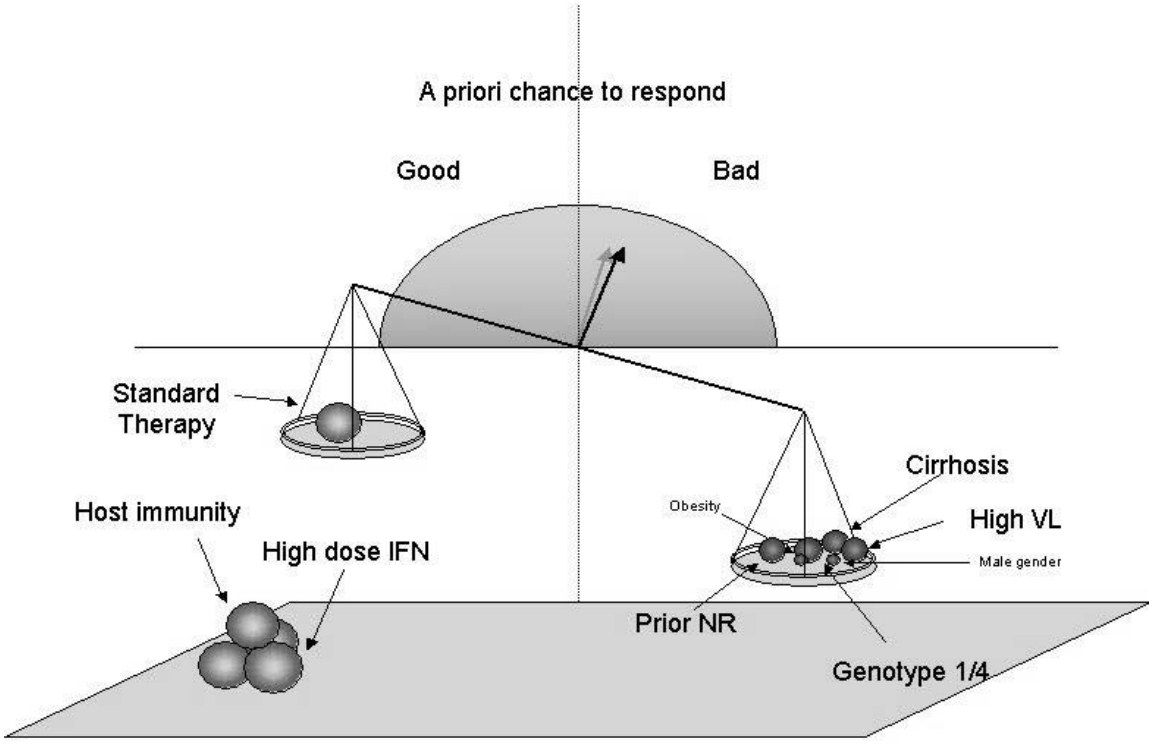

Figure 10.1. In patients with a single or combination of "difficult to treat" characteristics, the a priori chance to respond to standard therapy is limited.

on one side and the basic factors responsible for suppression of the virus and its replication on the other. Within these basic factors, we shared host immunity and in-vivo interferon level (figure 10.1).

Therefore, we a) treated prior non-responders and patients with unfavorable viral characteristics long-lasting and with high-dose (Peg-) interferon. We b) determined Peginterferon pharmacokinetics and dynamics and its relation to patients' characteristics and c) identified immunological factors in peripheral blood and in the liver associated with (non-) response. The thesis is therefore divided in three parts, each discussed here separately.

10.2 Treatment of non-responders and "difficult to treat" patients

When the first study described in this thesis (chapter 3) was designed, standard therapy for HCV was interferon-α 3 MU injected 3 times per week in combination with capsules of ribavirin taken twice daily. At that time the SVR for patients with "difficult to treat" criteria was about 28% (2, 3).

Failure to clear the virus during therapy was observed at 3 different phases: during the initial (4-12 weeks) treatment period (non-response, 40-50% of patients), during maintenance treatment after an initial response (breakthrough, about 10-20% of patients), and after treatment discontinuation (relapse, 25-50% of patients) (2, 3).

To reduce response failure at each phase an experimental treatment schedule was designed and investigated in a small exploratory non-controlled pilot study. Therapy started with high-dose daily induction to optimize the initial response (4, 5). After the initial phase, interferon administration was kept daily to obtain a more or less constant drug level that would optimize viral inhibition and minimize breakthrough. Moreover, all patients were advised to continue therapy for a total duration of 76 weeks. This schedule proved to be effective: the intention to treat sustained virological response was 67%.

To assess whether patients with unfavorable characteristics might benefit from such a modified treatment regimen in subsequent experience, we later analyzed the individual data of all patients (n=54) treated in our hospital with high-dose induction, daily and prolonged interferon-ribavirin combination therapy (chapter 4). The overall sustained virological response rate was somewhat lower than in the pilot study though still considerably improved over standard therapy: 57% (95%-CI: 43-71%).

Sustained virological response varied between 75-83% for patients with 1 unfavorable characteristic such as cirrhosis or non-response or genotype 1. However, in patients with combinations of such criteria the sustained virological response rates were considerably decreased: between 25-60% for patients with 2 unfavorable characteristics, and only 17% for those with 3 unfavorable factors.

At the moment of analysis (spring 2003) the superior treatment results of pegylated interferon (peginterferon-α) ribavirin therapy came available (6, 7). With high-dose, daily and prolonged interferon-ribavirin combination therapy we obtained similar

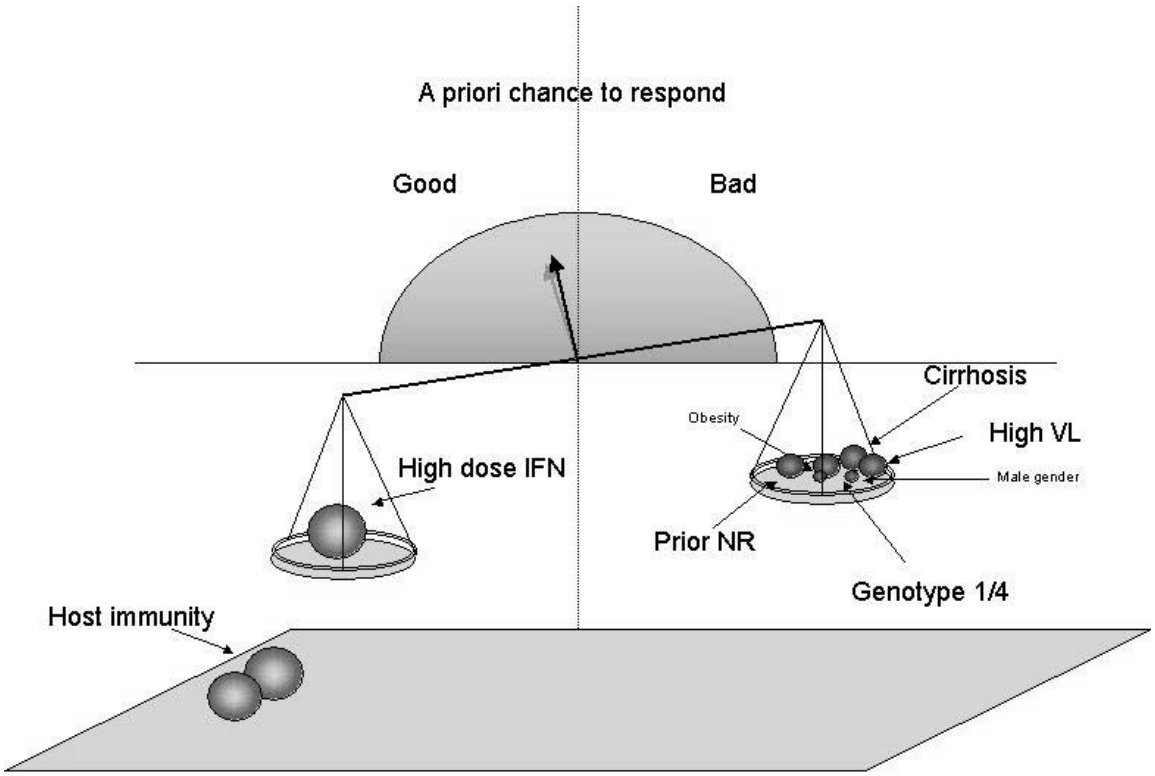

Figure 10.2. Patients with combination of "difficult to treat" characteristics might benefit from high-dose, daily and prolonged interferon-ribavirin combination therapy.

results as with modern schedules of peginterferon-α and ribavirin although baseline characteristics in our population were vastly more unfavourable. The intensive and long treatment schedules were expected to be associated with an increase of intolerance and side effects compared to standard treatment and the rate of non-compliance could only be kept low due to intensive monitoring. Given the augmented efficacy of peginterferon-α our results were no longer interesting for a wider audience. However, the results stimulated us to further research on initial high-dose peginterferon-α for the treatment of “difficult to treat” hepatitis C patients.

The early results of the first Dutch multicenter randomized trail considering the retreatment of hepatitis C patients with high or standard dosed peginterferon-α are described in chapter 5. Due to the improved treatment results with the introduction of peginterferon-α there was interest for re-treating patients who did not achieve SVR with previous therapy. However, although no conclusive results had been reported then, early Peginterferon-ribavirin re-treatment SVR estimates were only about 7-15%. We therefore assessed whether high-dosed Peginterferon-α2b (3.0 µg/kg once weekly; twice the standard dose) for 1 month had improved efficacy over standard Peginterferon-α2b therapy (1.5 µg/kg once weekly) on early virological response as earlier observed for high-dosed interferon. The safety and impact on “health related quality of life” of high dosing were also assessed.

Patients treated with high-dosed peginterferon showed a stronger response at all time points during the 29 days study period. Significance could however not be reached because of the limited sample size (n=40) and treatment success in patients infected with other genotypes then 1 or 4 of whom most were rapid responders independent of treatment arm.

High-dosed peginterferon was especially beneficial for genotype 1 or 4 infected patients. Most genotype 1/4 patients who were rapid responders were treated with high-dose peginterferon and within genotype 1/4 patients high-dose peginterferon induced a significant stronger suppression of HCV RNA replication at t=2, 7 (and borderline significant at t=29 days). Surprisingly, high-dosed Peginterferon-α2b therapy did not worsen the quality of life more than standard dosed Peginterferon-α2b.

The results of this study showed that in the majority of patients who previously failed to achieve a sustained response, an initial response could be obtained when treated with Peginterferon-ribavirin therapy. The decrease of HCV RNA was stronger in patients treated with high-dosed peginterferon than in those treated with the standard dose and especially genotype 1 and 4 infected patients might benefit from high-dose peginterferon. This finding should however be interpreted with caution, given the small number of patients included and the short study period. The end of treatment results are expected to become available in December 2005.

10.3 Immunological factors associated to (non-) response

Most patients in whom interferon therapy is unsuccessful are non-responders. (Some patients in whom interferon therapy is unsuccessful are responders who have a virological relapse after stopping interferon). In these non-responder patients, HCV RNA is detectable during the entire treatment period (2, 3) due to a lack of in-vivo interferon in combination with an inadequate host antiviral immune response (figure 10.3).

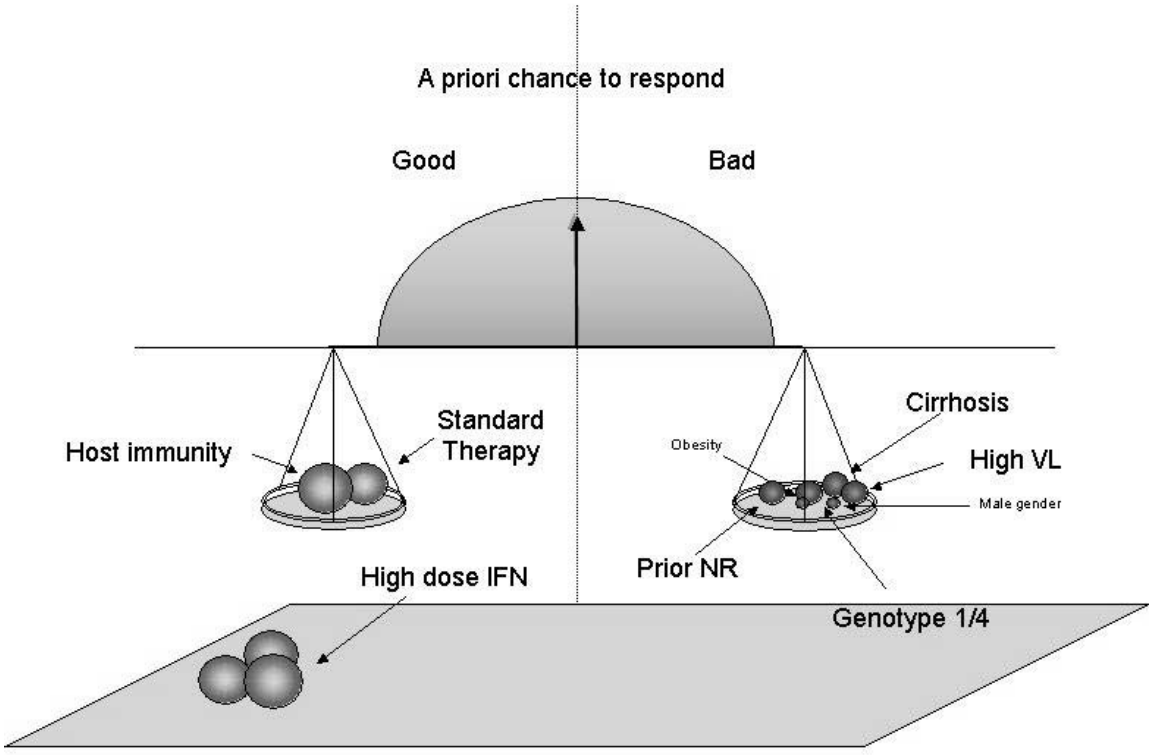

Figure 10.3. Patients with the combination of an adequate immune system and in-vivo interferon dose are thought to be more likely to have a beneficial virological response.

Although the immune-mediated mechanism responsible for the eradication of HCV is not well understood, there is consensus that intrahepatic localization of HCV specific T cells may be crucial to augment inhibition of viral replication and to clear infected hepatocytes during therapy (introduction to part II) (8, 9).

We therefore examined baseline localization of diverse subsets of intrahepatic immune cells by quantitative immunohistochemistry ($CD4^+$, $CD8^+$, and $CD68^+$ cells) in pre- and end of treatment liver biopsies and its causality to therapy response. For comparison also peripheral immune parameters (plasma interleukin (IL)-10, IL-12, IFN-γ levels) and circulating HCV-specific T cells were determined (chapter 7).

Numbers of intrahepatic $CD8^+$ cells located in the portal tracts of pre-treatment liver biopsies were found to be significantly higher in patients responding to therapy than in non-responders. The relation between portal $CD8^+$ cells and chance of response could be described by a logistic curve (univariate logistic regression analysis); its prognostic value was superior to that derived from genotype and other baseline factors (multivariate analysis). In contrast, neither peripheral cytokine levels nor HCV specific T cell reactivity in peripheral blood mononuclear cells did show a relationship to therapy response. These findings suggest that significant prognostic immune markers are to be found in the liver.

The liver biopsy procedure is however accompanied with considerable patient discomfort. We therefore investigated the feasibility of a less invasive and less traumatic fine-needle aspiration biopsy (FNAB) technique as a tool for monitoring the intrahepatic cellular immune status (chapter 8). Differences in numbers of intrahepatic $CD8^+$ T-cells during chronic HCV-infection are reflected in FNABs, and an association between the composition of the T-cell population contained in pre-treatment FNABs and early response to subsequent anti-viral therapy was found. We postulated that the FNAB-technique could be used as a tool for monitoring the cellular immune status in the liver during chronic HCV-infection on a frequent basis. Whether FNABs are predictive for long-term virological response to therapy needs to be investigated in a larger patient population.

10.4 IFN pharmacokinetics and its relation to (non-)response

The third part of this thesis compromises the studies on plasma (peg-) interferon pharmacokinetics and its relation to (non-)response. Why HCV RNA falls rapidly in one and slowly or even not at all in comparable patients treated with the same (peg-) interferon dose remains unclear. It appeared that, on group level, the rate of decline was dependent on the administered dose of Peginterferon-α (12). We therefore tested the hypothesis that pharmacokinetic variability may explain variations in treatment response and affect its chance to achieve SVR.

To measure levels of biologically active IFN-α in serum we first developed a sensitive assay (chapter 8) not disturbed by the limitations of an ELISA. Therefore, we tested whether hepatitis C virus replicons, which are highly sensitive to IFN-α, could be used as a sensitive bioassay to determine the amount of biologically active IFN-α.

The developed HCV-replicon based bioassay allowed determining the levels of biologically active IFN-α in serum and heparinized plasma of patients under treatment. It was however replaced by an ELISA for reasons of ease and costs esteemed the enormous workload.

In more than 5000 samples from 255 patients sampled at 7 time points serum interferon levels were quantified during the first month of Peginterferon-ribavirin therapy. Population pharmacokinetics and secondary parameters were assessed and correlated to response characteristics.

Pharmacokinetics could predict early virological response at week 4. Significance however declined with increasing treatment duration and was lost at the end of the treatment free follow up period; pharmacokinetics had no effect on treatment outcome.
Loss of significance could partly be explained by the loss of statistical power after halving the study group. Our study was ancillary to a randomized controlled trial in

which half of the patients were treated with a dynamically individualized treatment schedule according to their virologic response and the other half with standard dose. The variation in the individualized treatment schedules was so large that the patients treated accordingly (n=126) could not be taken into the pharmacodynamic study. Another reason of the loss of significance during the study might be that the end of therapy result are determined more by intracellular ribavirin levels than by peginterferon levels alone. Whereas the initial therapy results are more (peg)-interferon serum level dependant.

Our study confirmed the earlier published data of Peginterferon-α kinetics on mean level. We were however surprised by the inter-and intra-patient variability present for clearance. Some of this variability could be explained by the relationship of clearance and weight.

10.5 Finally

In the last five years we conducted several studies to improve and understand response and non-response in the treatment of HCV. Most studies were performed with emphasis for those who are in the greatest need for therapy; patients with cirrhosis, genotype 1/4 and others with major and minor difficult-to-treat characteristics.

Success in the treatment of difficult-to-treat patients can only take place under the right circumstances with the right combination of factors like sufficient serum interferon levels and an adequate host antiviral immune response.

We believe future strategies in HCV patients should include pretreatment screening and identification of difficult-to-treat patients. When as such identified, difficult-to-treat patients should (when indicated) be treated in an experimental setting with an intensified regimen under close monitoring of viral kinetics and pharmacodynamics. At this moment, we postulate for combining one month of daily interferon-α induction on top of Peginterferon-ribavirin standard therapy.

Hopefully, in the future in all patients a sustained virological response will possible (figure 10.4. Currently, completely different forms of medications are being investigated. It is however unlikely that such new therapy will be available in the next 5 years.

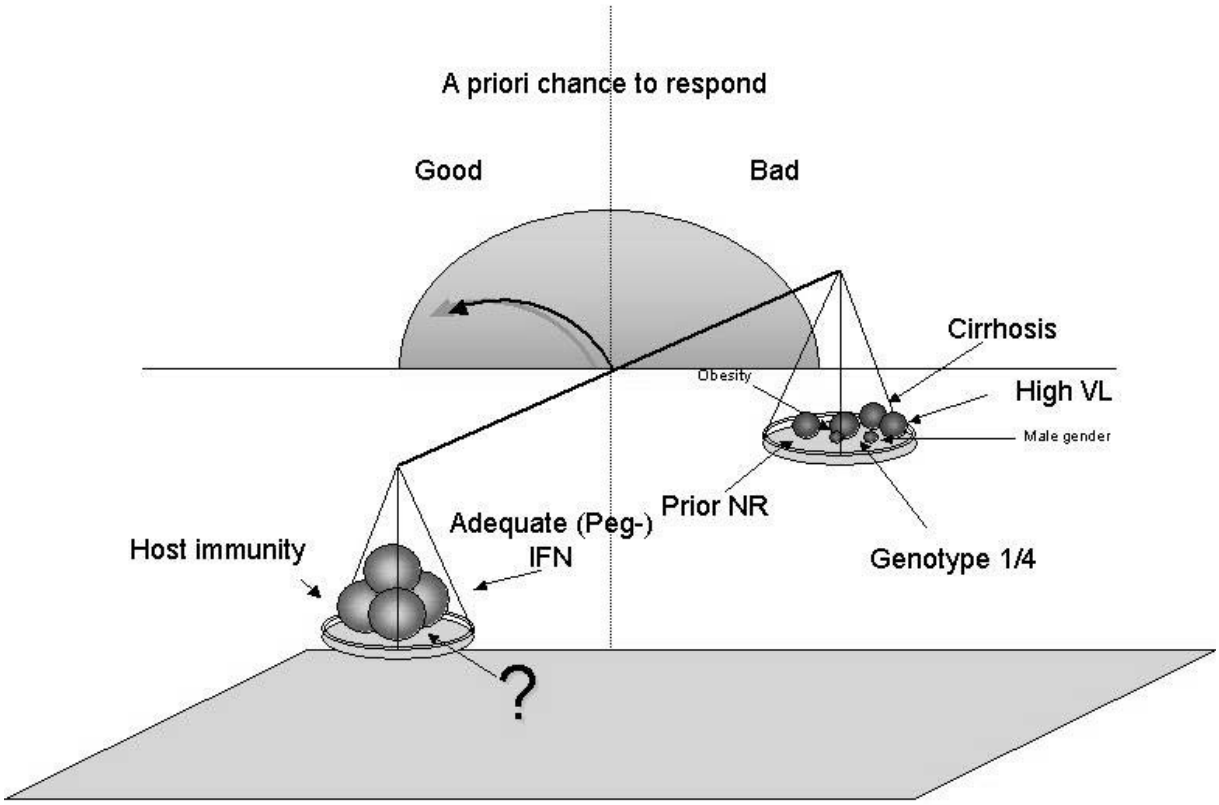

Figure 10.4. Some day, with the help of new therapeutic agents even in all patients who are now considered as difficult-to-treat a sustained response will be available.

Nederlandse samenvatting en discussie

10.6 Inleiding

Sinds bijna twintig jaar wordt ter behandeling van chronische hepatitis C virus (HCV) infectie gebruik gemaakt van op interferon gestoelde therapie om progressie tot lever cirrose tegen te gaan. De kans op succesvolle behandeling is de afgelopen jaren gestegen van minder dan 10% tot bijna 80% in geselecteerde patiënten (1). Echter, bij mensen met ongunstige kenmerken liggen de kansen op blijvende virale response (SVR; van het Engelse “sustained virological response”) aanzienlijk lager. Belangrijke ongunstige kenmerken zijn infectie met HCV genotype 1 of 4, het al hebben van cirrose van de lever of in het verleden niet gereageerd hebben op antivirale behandeling (zogenaamde non-responders). Minder belangrijk maar toch nog wezenlijke factoren zijn onder andere drager zijn van een groot aantal virale deeltjes in het bloed, man zijn en het hebben overgewicht. In dergelijke patiënten met één of een combinatie van dergelijke “moeilijk te behandelen” criteria ligt de *a-priori* kans te reageren op de standaard behandeling aanzienlijk lager namelijk tussen 10 en 40%.

Om het nadelige effect van de ongunstige kenmerken te niet te doen, en om factoren die samenhangen met een goede response te identificeren werden de studies zoals beschreven in dit proefschrift uitgevoerd. We stelden ons voor dat virale respons een positieve uitslag is na afweging van het totaal aan nadelige kenmerken tegen het totaal aan basale factoren verantwoordelijk voor onderdrukking van het virus en zijn vermenigvuldiging. Onder de basale factoren schaarden wij de immunologische afweer van de gastheer en de *in-vivo* (Latijn voor “in de patiënt”), aanwezige hoeveelheid interferon (figuur 10.1; pagina 172).

Zodoende werden a) non-responders en patiënten met ongunstige virale kenmerken langdurig en met hoge doses (peg-) interferon behandeld. Bepaalden wij b) de farmacokinetiek en dynamiek van Peginterferon en haar relatie met patiënt kenmerken en c) werden immunologische factoren samenhangend met (non-) respons in perifeer bloed en de lever geïdentificeerd. Dit proefschrift is daarom verdeeld in drie delen, hier elk afzonderlijk samengevat en bediscussieert.

10.7 De behandeling van non-responders en andere “moeilijk te behandelen” patiënten.

Op het moment dat de eerste studie zoals beschreven in dit proefschrift (hoofdstuk 3) werd ontworpen bestond de standaard behandeling van chronische HCV uit drie maal per week 3 miljoen units (MU) geïnjecteerde interferon-α gecombineerd met twee maal daags te slikken capsules ribavirine. De kans op een blijvende virale response bedroeg toen voor “moeilijk te behandelen” patiënten om en nabij de 28% (2,3).

Onvermogen het virus te klaren werd tijdens drie verschillende fasen van de behandeling waargenomen: wanneer tijdens de initiële (4-12 weken) behandel periode geen of onvoldoende daling van het aantal virus deeltjes plaats vond (non-response, 40-50% van de patiënten), wanneer na een initiële response de hoeveelheid virus deeltjes weer toenam tijdens de behandeling (breakthrough, ongeveer 10-20% van de patiënten), en wanneer nadat de behandeling werd gestaakt het aantal virus deeltjes weer meetbaar werd na een aanvankelijke response (relapse, 25-50% van de patiënten (2,3).

Met als doel de kans op falende therapie in elk van de drie fasen te reduceren werd een experimenteel behandel schema ontworpen en getest in een kleine exploratieve niet-gecontroleerde pilot studie. Om de initiële therapie kans te optimaliseren werd begonnen met een inductie fase bestaande uit dagelijks toegediende interferon in hoge dosering (4,5). Na de initiële fase werd de dagelijkse dosering gecontinueerd (in plaats van drie maal per week zoals standaard gebeurde) opdat een meer constantere hoeveelheid geneesmiddel zou worden verkregen ter bevordering van de onderdrukking van het virus en om breakthrough te voorkomen. Bovendien werd alle patiënten geadviseerd om de behandeling gedurende in totaal 76 weken voort te zetten. Dit schema bewees effectief: de “intention to treat” * SVR bedroeg 67%.

*“intention to treat” wil zeggen dat uitvallers, om wat voor reden dan ook, in de analyse zijn meegenomen als een niet succesvolle behandel uitkomst.

Om te bepalen of “moeilijk te behandelen” patiënten nogmaals baat zouden hebben bij een aangepast behandel schema werd later de individuele data van alle patiënten (n=54) in ons ziekenhuis behandeld met hooggedoseerde inductie, dagelijks en langdurige interferon-ribavirine combinatie therapie geanalyseerd. (hoofdstuk 4).
De SVR van de totale groep was iets lager dan die van de pilot studie maar toch nog aanzienlijk beter dan die van de standaard behandeling: 57% (95%-betrouwbaarheids interval 43-71%).
Blijvende virale response (SVR) varieerde tussen 75-83% bij patiënten met 1 ongunstig kenmerk zoals lever cirrose, voorafgaande non-response op eerdere therapie of genotype 1 infectie. De SVR was echter aanzienlijk lager bij patiënten met combinaties van ongunstige kenmerken: tussen 25-60% in patiënten met 2 ongunstige factoren en slechts 17% bij hen met 3 ongunstige kenmerken.

Op het moment dat we bovenstaande data analyseerden (voorjaar 2003) werden de eerste resultaten van de destijds juist geïntroduceerde peginterferon-α ribavirin therapie gepubliceerd (6,7). Onze resultaten met hooggedoseerde inductie, dagelijks en langdurige interferon-ribavirine combinatie therapie, waren vergelijkbaar terwijl de de *a-priori* kans op response in de door ons geselecteerde patiënten veel lager was.

Van de intensieve en langdurige behandel schema’s werd verwacht dat ze gepaard zouden gaan met een toename van bijwerkingen en intolerantie ten opzichte van de standaard behandeling en de mate van non-compliantie kon alleen maar laag gehouden worden door intensieve begeleiding en sturing.

Door de vergrote effectiviteit van peginterferon-α waren onze resultaten niet langer interessant voor een groot publiek. De resultaten hebben ons echter gestimuleerd tot verder onderzoek naar initiële hoog gedoseerde peginterferon-α ter behandeling van “moeilijk te behandelen” hepatitis C patiënten.

De vroege resultaten van de eerste Nederlandse gerandomiseerde multicenter studie betreffende de herbehandeling van hepatitis C patiënten met hoog- of standaard gedoseerde peginterferon-α zijn beschreven in hoofdstuk 5.

De interesse voor de herbehandeling met peginterferon-α van patiënten die geen SVR behaalden bij voorafgaande interferon-α therapie ontstond door de sterk verbeterde resultaten bij patiënten die nog nooit eerder waren behandeld. Echter, hoewel er op dat moment geen definitieve resultaten voorhanden waren, bedroegen vroege schattingen van de te verwachten SVR voor herbehandelden slechts ongeveer 7-15%.

Geïnspireerd door het eerder geobserveerde toegenomen effect van hooggedoseerde interferon wilden wij het effect van hooggedoseerde peginterferon bestuderen. Hiertoe werd gedurende een periode van 1 maand de afname van de hoeveelheid virus deeltjes bij patiënten behandeld met hooggedoseerde Peginterferon-α2b (3.0 µg/kg lichaamsgewicht 1x per week; 2x de standaard dosering) vergeleken met die bij standaard gedoseerde Peginterferon-α2b (1.5 µg/kg 1x per week). Bovendien werd het effect van hoog doseren op de kwaliteit van leven bestudeerd.

Patiënten behandeld met de hooggedoseerde Peginterferon-α2b toonden een sterkere response blijkend uit een grotere afname van het HCV RNA op alle tijdstippen tijdens de 29 dagen durende studie periode. Significantie kon echter niet bereikt worden doordat de studie groep klein (n=40) was en door het grootte succes behaald in patiënten met genotypen anders dan 1 of 4 onafhankelijk van het behandel schema.

Hooggedoseerde Peginterferon-α2b bleek vooral gunstig voor patiënten geïnfecteerd met HCV genotype 1 of 4. De meeste genotype 1/4 geïnfecteerde patiënten met een snelle response werden behandeld met hooggedoseerde peginterferon en binnen alle genotype 1/4 geïnfecteerde patiënten had hooggedoseerde peginterferon een significant sterkere daling van de HCV RNA replicatie tot gevolg op t=2, 7 (en borderline significant op t=29) dagen. Hooggedoseerde Peginterferon-α2b behandeling had echter geen verslechterend effect op de kwaliteit van leven ten opzichte van standaard gedoseerde Peginterferon-α2b.

De resultaten van deze studie laten zien dat in de meerderheid van de patiënten die eerder geen SVR behaalden een initiële response kan worden verkregen door

behandeling met Peginterferon-ribavirine combinatie therapie. De afname van HCV RNA was sterker in patiënten behandeld met hooggedoseerde peginterferon dan in vergelijkbare patiënten behandeld met de standaard dosering. Vooral patiënten besmet met genotype 1 en 4 kunnen baat hebben bij hooggedoseerde peginterferon. Echter, gezien het kleine aantal bestudeerde patiënten en de korte studie periode moeten deze bevindingen met voorzichtigheid worden benaderd. De definitieve resultaten zullen naar verwachting in december 2005 ter beschikking komen.

10.8 Immunologische factoren samenhangend met (non-) response

In het grootste gedeelte van de patiënten waarbij interferon therapie faalt, is dat ten gevolge van virologische non-response. (Bij de overige patiënten waarbij therapie faalt, daalt de hoeveelheid virus tot onmeetbaar kleine getallen waarna het weer terug lijkt te komen: virologische relapse en rebound.) Bij deze non-responder patiënten is HCV RNA gedurende de gehele behandel periode meetbaar (2,3) ten gevolge van een gebrek aan *in-vivo* interferon in combinatie met gebrek aan een adequate gastheer antivirale immuun response (figure 10.3).zie pagina 176).

Het mechanisme verantwoordelijk voor de immuun gemedieerde eradicatie van HCV wordt (nog) niet goed begrepen. De heersende opvatting is dat HCV specifieke T-cellen gelokaliseerd in de lever een rol spelen in de onderdrukking van de virale replicatie. Bovendien zouden zij het opruimen van geïnfecteerde levercellen tijdens de behandeling bevorderen (Introduction to part II) (8,9).

Wij onderzochten de lokalisatie van diverse immuun cellen in de lever door middel van kwantitatieve immunohistochemie ($CD4^+$, $CD8^+$, en $CD68^+$ cellen) op lever biopten genomen voorafgaand aan en direct na een behandeling en een eventueel verband met behandel uitkomst. Ter vergelijking werden bovendien niveau's van perifere immunologische parameters (plasma interleukine (IL)-10, IL-12, IFN-γ) en circulerende HCV-specifieke T-cellen bepaald (hoofdstuk 7).

Het aantal $CD8^+$ cellen in de portale velden van de lever bleek significant hoger te zijn bij patiënten die vervolgens respondeerden op de behandeling dan bij non-responders.

De relatie tussen het aantal portale $CD8^+$ cellen en de kans op response kon beschreven worden met een S-vormige logistische curve (univariate logistische regressie analyse); zijn voorspellende waarde bleek groter dan die van genotype of andere factoren (multivariate analyse). Dit in tegenstelling tot perifere cytokine niveau's of HCV specifieke T-cel reactiviteit van perifere mononucleaire cellen waarbij geen relatie met behandel uitkomst kon worden gevonden. Deze resultaten suggereren dat significant prognostische immunologische factoren zich in de lever bevinden.

Het nemen van lever biopten is voor patiënten echter een onprettige procedure gepaard gaande met pijn, stress en het risico op complicaties. Wij hebben daarom de bruikbaarheid getest van een minder invasieve en traumatische dunne naald aspiratie biopsie (FNAB van het Engelse "fine-needle aspiration biopsy") techniek als instrument voor het meten van de cellulaire immuun status in de lever van patiënten met chronische HCV infectie (hoofdstuk 8).

Het relatieve aantal $CD8^+$ T-cellen in de FNAB bleek niet samen te hangen met dat in perifeer bloed maar wel significant gecorreleerd te zijn met de aantallen $CD8^+$ T-cellen in lever biopten. Bovendien was het relatieve aantal $CD8^+$ T-cellen in de FNAB significant hoger bij patiënten die vervolgens bij de ingestelde Peginterferon-ribavirine therapie een snelle initiële response lieten zien dan bij langzame responders. Wij denken dat dit bewijst dat de FNAB techniek gebruikt kan worden als een instrument om met regelmaat de cellulaire immune status in de lever te bestuderen tijdens chronische HCV infectie. Of met de FNAB techniek een voorspelling gedaan kan worden van de lange termijn resultaten van een behandeling moet nog worden bezien. Dit dient bij voorkeur te worden onderzocht in een grotere populatie.

10.9 Peginterferon farmacokinetiek en -dynamiek en (non-) response

Het derde deel van dit proefschrift beslaat de studies naar plasma (peg-) interferon farmacokinetiek en haar relatie tot (non-)response.

Waarom HCV RNA in vergelijkbare patiënten behandeld met gelijke doses (peg-) interferon bij de één snel en bij de ander langzaam of zelfs niet daalt blijft onduidelijk. Het bleek dat op groeps niveau, de snelheid van afname samenhing met de toegediende dosis Peginterferon-α (12). Wij onderzochten daarom de hypothese dat variatie in farmacokinetiek, variatie in behandel uitkomst tot gevolg kan hebben en dat dit effect heeft op de kans tot het bereiken van een blijvende response.

Om concentraties van biologisch actief interferon-α in serum te meten ontwikkelden wij allereerst een gevoelige test methode (hoofdstuk 8) die niet verstoord zou worden door de beperkingen kenmerkend voor een ELISA. We testten of hepatitis C virus replicons, die zeer gevoelig zijn voor interferon-α, gebruikt konden worden als een gevoelige bioassay voor de bepaling van de hoeveelheid biologisch actieve interferon-α IFN-α in een monster.

Met de op het HCV-replicon systeem gebaseerde test bleek het mogelijk te zijn om concentraties van biologisch actief interferon-α te meten in serum en geheparinieerd plasma afgenomen tijdens de antivirale behandeling. Voor de daarop volgende studie (hoofdstuk 9) werd de test echter alsnog vervangen door een ELISA in verband met de kosten en de te verwachten hoge werkbelasting gezien het grootte aantal monsters.

In meer dan 5000 bloedmonsters afgenomen bij 255 patiënten op 7 verschillende tijdstippen tijdens de eerste maand van een Peginterferon-ribavirine behandeling werd de serum interferon-α concentratie gemeten. Vervolgens werden de populatie farmacokinetiek en secundaire parameters bepaald en gecorreleerd met response karakteristieken.

Met de farmacokinetiek resultaten kon de vroege virologische response (na 4 weken behandeling) voorspeld worden. De significantie nam echter af bij toename van de behandel duur en was verdwenen op het einde van de behandeling en had derhalve geen voorspellende waarde op het behandel resultaat.
Deels kon dit verklaard worden door verlies aan statische kracht nadat de studie populatie gehalveerd werd. Onze studie maakte gebruik van monsters afgenomen tijdens een gerandomiseerde studie (DITTO-HCV) waarbij bij de helft van de

populatie (n=126) de behandeling werd aangepast aan de hand van de eerste virologische resultaten. De variatie in aangepaste behandel schema's was echter zo groot dat deze patiënten niet konden worden meegenomen in onze analyse naar de rol van vroege farmacokinetiek in relatie tot het behandel resultaat.

Een andere reden voor het verlies aan significantie zou kunnen zijn dat het uiteindelijke resultaat van een behandeling niet zozeer wordt bepaald door uitsluitend peginterferon concentraties maar dat intracellulaire ribavirine concentraties ook een belangrijke rol spelen.

Onze studie bevestigde de Peginterferon-α kinetiek resultaten van een eerder gepubliceerde kleine studie. We waren echter verbaasd over de enorme variatie in klaring tussen en binnen patiënten. Slechts een gedeelte van deze variabiliteit kon verklaard worden door het verband tussen klaring en gewicht.

10.10 Tot slot

In de afgelopen vijf jaar hebben wij diverse studies opgezet en uitgewerkt ter verbetering en lering van response en non-response tijdens de behandeling van chronische hepatitis C infectie. Het merendeel van de studies werd uitgevoerd met genegenheid voor hen die het meeste baat hebben bij een succesvolle behandeling; patiënten met een lever cirrose, genotype 1/4 infectie en anderen met meer en minder belangrijke "moeilijk te behandelen" kenmerken.

Een succesvolle behandeling van patiënten met "moeilijk te behandelen" kenmerken vindt uitsluitend plaats onder de juiste omstandigheden bij een combinatie van factoren zoals afdoende hoge serum interferon spiegels en een adequate gastheerantivirale immuun response.

Wij denken dat toekomstige behandelstrategieën van HCV onder andere het screenen op en identificeren van "moeilijk te behandelen" patiënten zou moeten bevatten. Dergelijke "moeilijk te behandelen" patiënten zouden in onderzoeksverband met een intensief schema en onder voortdurende toetsing van virale kinetiek en farmacodynamiek behandeld moeten worden. Op dit moment zouden wij willen

pleiten voor het toetsen van gedurende de eerste behandel maand dagelijks geïnjecteerde interferon-α inductie gecombineerd met standaard gedoseerde Peginterferon-ribavirine ten opzichte van huidige standaard Peginterferon-ribavirine behandeling.

Hopelijk zal er in de toekomst een behandeling bestaan waarmee alle HCV geïnfecteerde patiënten genezen kunnen worden (figuur 10.4; pagina 180). Op dit moment worden daartoe totaal nieuwe medicijnen ontwikkeld. Het is echter onwaarschijnlijk dat een dergelijke “wonder pil” binnen 5 jaar ter beschikking komt.

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List of often used abbreviations

ALT	serum alanine aminotransferase
APC	Antigen-Presenting Cells
AUC	aria under the curve
BMI	body mass index
CD8+ CTL	cytotoxic T cell
CL/F	clearance
Cmax	maximal plasma concentration
cpm	counts per minute
CV	coefficient of variation
EFU	end-of-follow
ELISA	enzyme-linked immunosorbent assay
EoT	end of therapy
EVR	early virologic response
FNAB	fine needle aspiration biopsy
FPR/NUR	flat partial or null responders
HCV	hepatitis C virus *or* hepatitis C virus infection
HRQoL	health related quality of life
IFN-α	interferon-α
IFN-γ	interferon-gamma
IL	interleukin
ITT	intention to treat
Ka	absorption rate constant
MU	million units
NONMEM	nonlinear mixed-effect modelling
NR	non-response
PBMC	peripheral blood mononuclear cells
PEG	polyethylene glycol
PEG-IFN-α	pegylated interferon-α
PP	per protocol
QW	once weekly
RR	response relapse
RVR	rapid virological responders
SF-36	Short Form 36
SPR	slow partial responders
SVR	sustained virological response

TCR	T cell receptor
Th1	helper T cells with a type 1 cytokine profile
Th2	helper T cells with a type 2 cytokine profile
Tiw	thrice in week
Tmax	time to maximal plasma concentration
TNF-α	tumor necrosis factor-alpha
V/F	volume of distribution
WHO	World health organization

Nawoord

…..en toen ineens was het af. Na bijna vijf jaar lezen, discussiëren, dromen, plannen maken, documenteren, analyseren, het schrijven van tientallen protocollen en honderden conceptmanuscripten kwam het einde toch nog als een verassing. En het voelde ook meteen onwennig. Het altijd knagende gevoel dat er nog veel te doen is en dat er niet verzaakt mag worden sloeg ineens nergens meer op. “Het boek is dicht” of eigenlijk: het manuscript ligt bij de drukker. Er kan niets meer veranderd worden. Hoe was het ook al weer begonnen?

In mei 2000 verrichtte ik voor het verkrijgen van beenmerg een punctie van het borst been bij een fors gebouwde maar angstig kijkende Kaap Verdiaan. Na een moeizame maar geslaagde introductie van de naald leek het borstbeen, dat eerst zoveel weerstand had geboden, de naald niet meer los te willen laten. Met de linkerhand tegendruk biedend op de borst van de patiënt trok ik met steeds grotere kracht aan de naald. Het resultaat was dat deze ineens omhoog schoot met de hand die hem omklemde en daarna in een reflex terugschoot. Op een haar na ontkwam de arme man aan een tweede punctie; de naald stond nu direct naast de nagel in mijn linker duim.

Uit de medische status bleek een mogelijke hepatitis C infectie. In ieder geval waren de antistoffen positief. Ongeveer een week later bleek (tot ons beider geluk) de infectie doorgemaakt en dus niet besmettelijk te zijn. In de periode tussen het prikken en de uitslag heb ik mij verdiept in hepatitis C infectie en de behandeling van de ziekte die zij veroorzaakt. Daardoor werd mij belangstelling gewekt. Vierhonderd miljoen mensen bleken geïnfecteerd te zijn met een virus waarvan ik eerder dacht dat het zelden voorkwam.

Enkele maanden later bezocht ik de “Rotterdamse Leverdag” alwaar ik in contact kwam met Prof. dr. Solko Schalm. Hij adviseerde mij een sollicitatie brief te sturen en gaf me vervolgens de kans “onderzoek te verrichten dat bij voorkeur resulteert tot een promotie”. Het product hiervan ligt voor u.

Promoveren bij Solko betekent niet dat er een pasklaar plan op je te wachten ligt. Aan de ene kant is er de “rijdende trein” met lopende studies die ook weer verlaten dient te worden terwijl de rit in volle vaart door gaat. Gezien de looptijd van de studies is dit haast onvermijdelijk bij patiënt gebonden onderzoek. Voor de promovendus heeft deze werkwijze het voordeel dat zij bijna altijd wel “iets” oplevert.

Aan de andere kant is er de wens en mogelijkheid dat de promovendus zijn eigen vragen vormt en uitwerkt. Ik ben Solko hier zeer erkentelijk voor. Het gaf mij de kans projecten te starten die a-priori een kleine kans van slagen hadden, zoals de “depressie studie”. Zoals hij al voorspelde is daarvan in deze thesis niets terug te vinden. Andere voorbeelden die beter lukten zijn het laboratorium werk en de multicenter studie waarvoor dankzij zijn uitstekende connecties, banden met andere afdelingen en ziekenhuizen werden aangeknoopt. Zijn gedrevenheid en neiging tot perfectie brachten me soms tot wanhoop maar altijd tot een beter eindproduct.

Een kant-en-klaar proefschrift is, zoals velen die mij voorgingen weten, een product van velen. Ik wil dan ook graag alle co-auteurs bedanken voor hun bijdrage. Dr. Frank Bekkering ben ik erkentelijk voor de studies die hij als promovendus opstartte en mij liet uitwerken, Bart Veldt voor het verder uitwerken van de studies die in mijn tijd begonnen. Dr. Hans Brouwer bewonder ik om zijn gave altijd in korte tijd de zwakke punten van een manuscript, protocol of zomaar een droom te identificeren. Zijn wijze van omgang met en behandeling van patiënten strekken mij tot voorbeeld. Dr. Rob de Knegt nam later het stokje van hem over. Ik ben hem dankbaar voor zijn inzet en voor het opschudden van de kussens waarop ingeslapen studies lagen te sluimerden. Ik hoop nog veel van hem te kunnen leren tijdens de klinische stages en hoop in de komende maanden de nog lopende onderzoeken met hem verder uit te werken.

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De behandeling van HCV is “teamwork”. Zonder de begeleiding van Heleen van Santen, Cokkie van der Ent en eerder Marjan van de Vrie hadden vele patiënten de eindstreep (en dus een kans op genezing) nooit gehaald. Graag wil ik hen ook bedanken voor de tomeloze inzet tot het uitwerken en uitvoeren van de studieprotocollen en de daarbij behorende duizenden bloedmonsters.

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En tot slot de paranimfen: Gert-Jan van Dalen en Gerben te Velthuis. Daar staan we straks schouder aan schouder in een outfit die we onder geheel andere condities al vaker droegen. Vrienden van een goed leven buiten het ziekenhuis. Sta me bij.

Rotterdam, 6 maart 2005.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 17 februari 1972 te Bilthoven. Na het behalen van het V.W.O. eindexamen aan het “Het Rotterdams Lyceum” werd in 1991 gestart met de studie geneeskunde aan de Erasmus Universiteit Rotterdam. Hij behaalde het doctoraal examen op 21 november 1996 en het artsexamen op 21 mei 1999. Van 1 juni 1999 tot 1 juni 2000 werkte hij als arts-assistent interne geneeskunde in het IKAZIA ziekenhuis te Rotterdam (opleider dr. R.J.Th. Ouwendijk) alwaar hij na een prik accident geïnteresseerd raakte in virale hepatitiden. Van 1 juni 2000 tot 31 december 2003 was hij werkzaam als arts-onderzoeker op de afdeling Maag-, Darm- en Leverziekten (MDL) van het Erasmus MC Rotterdam (hoofd prof. dr. E.J. Kuipers). Tijdens deze periode werd onder begeleiding van prof. dr. S.W. Schalm het onderzoek verricht zoals beschreven in dit proefschrift. De klinische studies vonden plaats op de polikliniek MDL onder supervisie van dr. J.T. Brouwer en dr. R.J. de Knegt. De laboratorium studies werden verricht op de laboratoria van de afdeling MDL (hoofd dr. J.G. Kusters) onder begeleiding van dr. J. Kwekkeboom, de afdeling Moleculaire Virologie Ruprecht-Karls-Universitat Heidelberg onder begeleiding van prof. dr. R. Bartenschlager, en de afdeling Virologie Erasmus MC (hoofd prof. dr. A.D. Osterhaus) onder begeleiding van dr. B.L. Haagmans. Sinds januari 2004 is hij in opleiding tot Maag-, Darm- en Leverarts. Momenteel is hij werkzaam in het IKAZIA ziekenhuis te Rotterdam in het kader van de vooropleiding interne geneeskunde (opleider dr. A. Dees). Hij is sinds gehuwd met Ellen Zwijnenburg; zij hebben een zoon Olivier.