Viral Kinetics of the Hepatitis C Virus

F.C. Bekkering

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Prof. dr. H.A.P. Pols

Prof. dr. H.A. Drexhage

Do you know what you are ?
You are what you is
You is what you am
(a cow don't make ham)
You ain't what you're not
So see what you got
You are what you is
And that's all it' is…

Frank Zappa

Voor Jasper

This thesis is based on the following articles:

1 **HCV: viral kinetics**

Bekkering FC, Brouwer JT, Leroux-Roels G, Elewaut A, Schalm SW. ***Hepatology*** 1997;26:1691-3.

2 **Ultrarapid hepatitis C virus clearance by daily high-dose interferon in non-responders to standard therapy**

Bekkering FC, Brouwer JT, Leroux-Roels G, Van Vlierberghe H, Elewaut A, Schalm SW.
Journal of Hepatology 1998;28(6):960-4.

3 **Clinical significance of qualitative and quantitative testing of viral load in hepatitis C**

Schalm SW, Bekkering FC, Brouwer, JT
Hepatitis C: A Series of Topical Reviews, 4D Communications, Oxford 1998:3-9

4 **Hepatitis C viral kinetics in difficult to treat patients receiving high dose Interferon and ribavirin**

Bekkering FC, Brouwer JT, Hansen BE, Schalm SW
Journal of Hepatology in press

5 **Estimation of early viral clearance with daily interferon and ribavirin using a mathematical model**

Bekkering FC, Stalgis C, McHutchison JG, Brouwer JT, Perlson AS. ***Hepatology*** 2001;33(2):419-23

6 **Can we routinely quantify Hepatitis C virus (HCV) RNA in clinical samples to monitor the effect of therapy in chronic hepatitis C?**

Niesters HGM, Bekkering FC, Hansen BE, Schalm SW, Osterhaus ADME submitted

7 **High sustained virological response in difficult to treat hepatitis C patients by combination of induction and prolonged maintenance interferon-ribavirin therapy**

Bekkering FC, Brouwer JT, Hansen BE, Niesters HGM, Schalm SW submitted

8 **Changes in anti-viral effectiveness of interferon after dose reduction in chronic hepatitis C patients: implications for treatment strategy**

Bekkering FC, Neumann AU, Brouwer JT, Levi-Drummer RS, Schalm SW
Hepatology (submitted)

9 **New treatment strategies in non-responder patients with chronic hepatitis C**

Schalm SW, Brouwer JT, Bekkering FC, Rossum TGJ van
Journal of Hepatology 1999;31:184-8

10 **Implications of viral kinetics studies for the treatment of hepatitis C, today and tomorrow**

Bekkering FC, Schalm SW

Contents

Chapter 1

General introduction

1.1 INTRODUCTION TO HEPATITIS C TREATMENT

1.1.1 Chronic hepatitis C infection

Hepatitis A virus and hepatitis B virus were identified as the cause of infectious hepatitis and serum hepatitis respectively in the beginning of the seventies [1-3]. After introduction of screening tests for hepatitis A and B [4] only 25% of the cases of post transfusion hepatitis were found to be caused by hepatitis B and none by hepatitis A. One or more viruses other than hepatitis A or B were suspected to be the cause of the remaining 75% of post-transfusion hepatitis. Initially, this hepatitis was named non-A, non-B hepatitis. After the discovery in 1989 of the hepatitis C virus (HCV), HCV was found to explain the large majority of post transfusion hepatitis [5,6]. HCV is an enveloped, single-stranded RNA virus, approximately 50 nm in diameter, that has been classified as a separate genus in the Flaviviridae family [7]. Occasionally acute viral hepatitis with jaundice occurs, but usually HCV presents as chronic hepatitis . In fact it appeared to be the most important causes of chronic viral hepatitis in Europe and the United States [8].

Chronic viral hepatitis is often asymptomatic or associated with non specific fatigue. The hepatitis (inflammation of the liver) is usually detected by an elevation of the enzyme alanine amino transferase which resides predominantly in hepatocytes. Over a period of 10 to 30 years chronic hepatitis C can induce fibrosis and cirrhosis followed by complications of cirrhosis or hepatocellular carcinoma [9].

Even before the virus was discovered, interferon treatment for 6 months was shown to normalize ALT in about 50% of patients, with persistent normalization of ALT after discontinuation of therapy in about 20-25% of patients [10,11]. With the availability of tests to detect the actual virus itself (polymerase chain reaction tests; PCR) , it became clear that the patients with a sustained biochemical response had cleared the virus, while in nonresponders the virus remained detectable. In a large multicenter Benelux study it was shown that early disappearance of HCV RNA from blood as well as early normalisation of ALT were predictive of a sustained response. In fact the predictive value of a HCV test at 4 weeks had a very strong predictive value; detectable HCV RNA virtually eliminates the change of a sustained response [12]. Current studies use loss of HCV RNA as endpoint of treatment, since prolonged

disappearance of HCV RNA (>6 months) from the blood seems to indicate a genuine clearance of HCV infection (sustained response) [13].

1.1.2 The problem in the treatment of chronic hepatitis C

The first standard treatment for chronic hepatitis C were bases on randomised controlled trials reported in 1989 by Davis and [10,11]. Since these reports, most treatment regimens in Western Europe and the United States uses a treatment schedule of 3 Mega Units (MU) IFN given subcutaneously thrice weekly (tiw). Initially the duration was 6 months, later 12 months became the standard therapy because of a lower relapse rate [14]. The second standard was combination of interferon alpha with ribavirin after documentation in randomised controlled trials that the addition of ribavirin doubled the virological sustained response [15-18]. Even with this improved treatment a sustained virological response only occurs in 40-45% of treated patients. The problem in the treatment of hepatitis C relates to the fact that more than 50% of patients are treatment failures. Certain baseline characteristics were found to be associated with nonresponse; sustained virological responses in patients with a genotype 1 infection were only about 20% [16-18].

1.1.3 The solution

Assuming that HCV RNA negativity at 4 weeks is almost a prerequisite for a sustained response our interest focussed on following the decline in HCV RNA between start of treatment and 4 weeks. The development of a new diagnostic tool in the form of a PCR to quantitate HCV RNA in blood could make such type of monitoring possible [19]. Furthermore, such an assay could allow us to assess within 4 weeks the effect of changes in dose (10MU instead of 3MU Interferon) as well as changes in the frequency of administration (daily instead of three times a week) in those patients who did not respond to standard treatment.

After having validated the assay on the Eurohep standard dilution panel [20] over a range of about 10^E6 to 10^E2 copies per ml, we could describe the actual decline in viral load between start of treatment and 4 weeks. The pattern of decline of HCV RNA during the first 4 weeks of treatment showed a 99% reduction in viral load in the first 2 days, followed by a steady, much slower decline over the following 26 days in most patients. The descriptive analysis of such a curve created the term viral kinetics

which provided insight in the half-life of the virus as well as the numbers of particles produced each day. The mathematical modelling of virological and cell-biological processes induced by treatment created the concept of viral dynamics. So the studies assessing the role of quantitative HCV RNA rapidly became studies on viral kinetics and viral dynamics in chronic hepatitis C infected patients.

1.2 AIM OF THE THESIS

To explore the value of quantitative HCV RNA measurements in the management of patients with chronic hepatitis C undergoing anti-viral therapy:

1) How does the hepatitis C virus decline after treatment initiation?
2) What is the influence of dosage and frequency of Interferon on the viral decline?
3) Can we mathematically model the decline in viral load?
4) Can we use viral kinetics analyses for early dose reduction in the treatment of chronic hepatitis C?
5) What are the possible biological mechanisms of interferon that explain the kinetics of the viral decline after treatment?

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Chapter 2

Clinical significance of qualitative and quantitative testing of viral load in hepatitis C

Solko W. Schalm, Frank C. Bekkering, Johannes T. Brouwer

Hepatitis C: A Series of Topical Reviews, 4D Communications, Oxford 1998:3-9

2.1 INTRODUCTION

Therapy with alpha-interferon for chronic non-A, non-B hepatitis, developed in the mid-eighties, was based on improvements in serum alanine aminotransferase (ALT) and liver histology. Since 1990 studies also aimed for clearance of hepatitis C virus (HCV) RNA from blood after the discovery of HCV and the development of a reverse transcriptase polymerase chain reaction (RT-PCR) in 1989 [1,2]. Since then, the endorsement of assessing HCV RNA by PCR before and during antiviral therapy has been clear and strong. However, the clinical advantages of testing HCV RNA by PCR compared to serum ALT are not entirely clear, but there are indications that RNA testing may be a more reliable parameter in determining the true response to interferon therapy.

The clinical relevance of HCV RNA testing has been difficult to determine due to introduction of quantitative tests as well as the increasing sensitivity of HCV RNA-PCR tests. In 1998 the clinical significance of HCV RNA testing can be viewed from three angles:

- generally accepted standard practice;
- new developments
- controversial issues.

2.2 STANDARD PRACTICE FOR PATIENTS RECEIVING ANTIVIRAL THERAPY

The two consensus reports published in 1997 [3,4] recommend testing HCV RNA by PCR at 4 specific time points:

a) before start of therapy
b) after 12 weeks
c) at the end of therapy
d) 6-12 months after stopping therapy.

In contrast, individual experts have concluded that the poorly standardized methodology and the high cost of testing HCV RNA restricted its clinical usefulness [5]. Therefore, we discuss here the key data that - in all likelihood - were the basis for the recommendations of the consensus panels.

2.2.1 HCV RNA before start of therapy

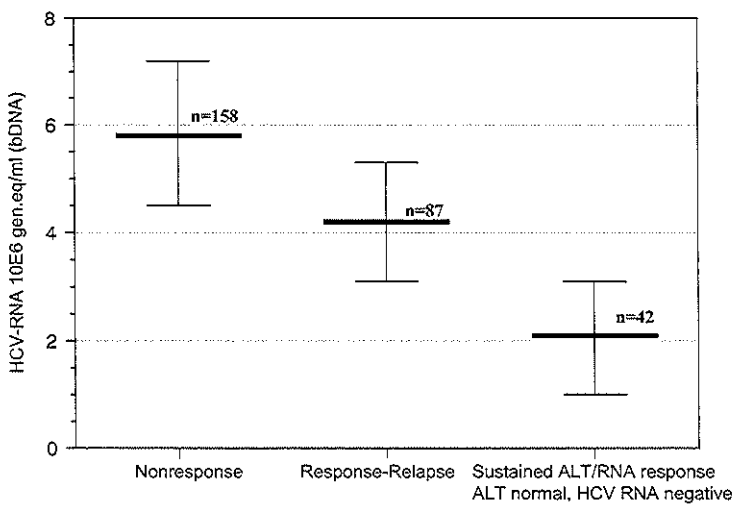
Patients found to be anti HCV-positive with elevated serum ALT are considered patients with liver disease, and should undergo HCV RNA testing by PCR as part of the process of diagnosis. If the result is positive, the diagnosis of ongoing hepatitis C virus infection is confirmed.

The need for antiviral therapy and the chances of sustained response should be established. In patients with active disease and signs of progressive fibrosis, chances of a sustained response to alpha-interferon are predominantly determined by three independent factors:

- stage of the liver disease (cirrhosis),
- virus genotype and
- viral load

In at least three studies [6-8] the level of viremia was on average predictive of the response to interferon. Non-responders had a threefold higher viral load than patients with a sustained response to interferon (Figure 1). Patients with high viremia had an 0-7% chance of sustained response versus 15% for patients with low viremia [7].

Figure 1



Pretreatment HCV RNA levels measured by bDNA version I (corrected for genotype 2 and 3 sensitivity), and response to 6 months alpha-interferon (3 MU tiw). Results (expressed as mean (95% C.I.)) are from the Benelux study in 336 patients show a significant difference between sustained responders and other patients [8].

The strength of two predictors - stage of liver disease and genotype - is so pronounced [8], that we have not used quantitative HCV RNA for deciding whether to institute standard interferon therapy (3 MU thrice weekly). However, Reichard et al. [9] have recently demonstrated that the combination of interferon and ribavirin is superior to interferon monotherapy in patients with viral load above 3 million genome-equivalents/ml. We therefore predict that a quantitative assessment of HCV RNA will become considered clinically useful for deciding the type and possibly duration of therapy, as reliable quantitative tests for HCV RNA become increasingly more available.

2.2.2 HCV RNA after 12 weeks

Patients on antiviral therapy for chronic hepatitis C have a 5-50% chance of obtaining a sustained response. In view of the cost and side-effects of antiviral therapy, non-responders should be identified early in the course of therapy.

Patients who do not have normal serum ALT after 12 weeks of standard treatment are very unlikely to respond to further treatment, according to expert opinion [5,10]. However, this criterion is not generally confirmed. Experience from a Californian group, that used a PCR with a high sensitivity level of (100 copies/ml), indicates that HCV RNA testing at 12 weeks actually provides more accurate information than ALT [11]. At week 12 of treatment of the sustained responders 7% (1 out of 15) were still HCV RNA positive whereas 33% (5 out of 15) had abnormal ALT. It is unclear whether this latter figure reflects a patient population with a high degree of steatosis or a different method for setting the upper limit of the normal value. However, the ALT level is quite sensitive to other factors including the toxicity of interferon if used in higher doses.

Therefore, to avoid making an important clinical decision solely on one ALT value, it is recommended that both ALT and HCV RNA by PCR be measured at week 12; those with an abnormal ALT and a positive HCV RNA test should stop therapy.

2.2.3 HCV RNA at the end of therapy

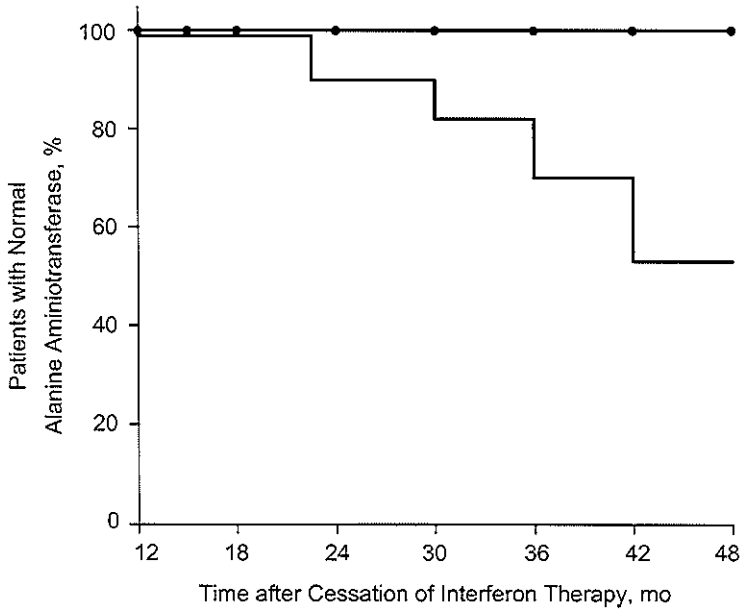
Patients at the end of therapy who have a normal ALT are considered biochemical responders, but many will relapse in the 6-12 months period after stopping therapy. Biochemical relapse is significantly more frequent in patients with a positive HCV RNA test by PCR at the end of treatment (95%) than in those with a negative HCV RNA test (57%) [8]. Another important justification for testing HCV RNA by PCR at the end of therapy in individual patients is that it provides an important guide for further therapy in case of relapse. If HCV RNA is negative, the original therapy might be reinstituted with appreciable chance of sustained response with longer therapy; if HCV RNA is positive at the end of therapy, retreatment with the same schedule is universally unsuccessful [12].

2.2.4 HCV RNA 6-12 months after stopping therapy

Patients who are followed 6-12 months after discontinuation of therapy and then have normal serum ALT's are termed sustained (biochemical) responders. Assessment of HCV RNA by PCR is then useful: absence of HCV RNA documents "cure" [10] but the presence of HCV RNA reflects a more than 50% chance of relapse in the following 5 years [11,13] (Figure 2). It is therefore recommended to measure both ALT and HCV RNA by PCR 6-12 months after stopping therapy to reliably predict the long-term outcome. In trials, the time of this assessment is usually 6 months after stopping therapy; in clinical practice there is a slight improvement in the predictive value of the test if the assessment is done 12 months after stopping [10].

Thus, the standard 1997 practice for assessing HCV RNA by PCR in patients undergoing antiviral therapy has clinical significance for each time point. These assessments are now considered essential for clinical trials; and - if reliable testing is available - should be introduced in clinical practice.

Figure 2



Probability of maintaining normal serum alanine amino-transferase levels at long-term follow-up (Kaplan-Meier curves) in patients groups with a sustained biochemical response, 12 months after discontinuation of a treatment with alpha-interferon. In HCV RNA positive patients (——), the probability of hepatitis relapse is 53%, 4 years after therapy; in HCV RNA negative patients (—•—), the probability is 0% (log-rank test; $P < 0.001$)
(adapted with permission of Chemello et al. [10])

2.3 NEW DEVELOPMENTS

The consensus reports recommended a decision to stop or continue treatment on the basis of the ALT and HCV RNA determination at week 12. This recommendation was based on the observation that nearly all patients who achieved a biochemical end-of-treatment response had normal ALT levels by treatment week 12 [14,15]; in addition testing HCV RNA by PCR at week 12 was said to be sensitive and specific for the outcome of treatment [16].

New insights in viral kinetics [17,18] have focussed attention on several reports mentioning week 4 after start of therapy as a clinically useful time point for predicting outcome of therapy. In addition, the changing sensitivity of HCV RNA-PCR makes it imperative to include quantitative measurement of HCV RNA into the discussion.

Therefore, the following sections will discuss new developments with regard to the questions:

a) Is a qualitative HCV RNA test earlier than week 12 advantageous for clinical decision-making about continuing or stopping interferon treatment?

b) Is quantitative HCV RNA testing advantageous?

2.3.1 Early HCV RNA testing

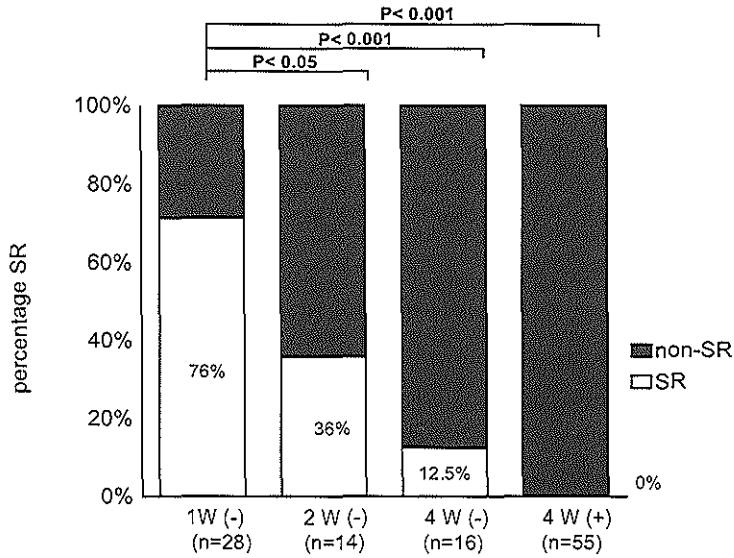
In the early phase of the Benelux study [2], it was observed that HCV RNA had disappeared from plasma by week 4 in some patients, and that HCV RNA measurement at that time might have a high predictive value. In the final study results [8], the predictive value of a positive test for non-response was 98% and the predictive value of a negative test for sustained response was 34%. Along the same line, Karino et al. [19] reported that in 114 patients that had received alpha-interferon therapy for six months, no sustained responders were found in more than 50 patients with HCV RNA positivity at week 4. The percentage of sustained responders was highest (76%) in those with undetectable serum HCV RNA at week 1, and decreased weekly till week 4 (Figure 3).

A Spanish study comparing the predictive value of HCV RNA at week 4 that of serum ALT confirmed the high clinical relevance of testing HCV RNA by PCR at week 4 [20]. The predictive value of a negative HCV RNA test or normal ALT for a sustained response and that of a positive HCV RNA test or raised ALT for patients without a sustained response is given in Table I. These findings have been confirmed in smaller studies [21,22].

Testing HCV RNA at week 4 therefore seems the best way to identify sustained responders and exclude almost all those patients that will not show a sustained response. By testing HCV RNA at week 4 of the treatment one could predict response or non-response early and avoid unnecessary treatment and cost [11] in many patients, that will become non-responders. Several studies [8,19] show that week 4 analysis of HCV RNA does not exclude a large percentage of potential sustained responders. While these studies suggest week 4 rather than week 12 should be the time point of measurement, there are arguments which would advise against this at the present time. The HCV RNA-PCR tests that were basic to the evidence had a detection limit of 1000-2000 copies per ml. With enhanced sensitivity of HCV RNA-PCR to 100 copies per ml [11] it is readily understandable that HCV RNA negativity will occur later in many

patients. Therefore the time to propose amendments to the consensus reports will be related to the general availability of quantitative HCV RNA.

Figure 3



Sustained response rate by time point of negativity of serum HCV RNA to IFN therapy in 114 patients with chronic hepatitis C receiving interferon therapy for 6 months. 1W(-), 2W(-) and 4W(-) indicate HCV RNA negative patients at the first, second and fourth week of therapy, respectively. 4W(+) indicates patients who showed serum HCV RNA positivity throughout the first four weeks of therapy. SR indicates sustained responders; non-SR other type of response (non-response or relapse). *(adapted with permission of Karino et al. [19])*

Table 1: Predictive values of HCV RNA and ALT at 4 and 12 weeks of treatment

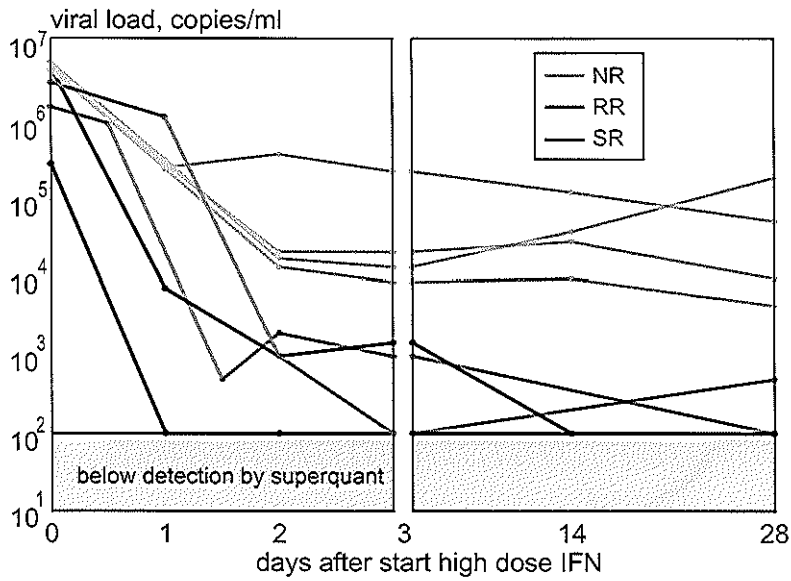
		Predictive value (%)	
		negative test for sustained remission	positive test for no sustained remission
HCV RNA	week 4	50	95
HCV RNA	week 12	44	93
ALT	week 4	41	78
ALT	week 12	34	78

From: Gavier B et al. [20] with permission.

2.3.2 Quantitative RNA testing

The predictive value of a HCV RNA-PCR test at a certain moment is dependent on both the velocity in the decrease of viral load during therapy and the sensitivity of the test used. When there is a gradual decrease in plasma HCV RNA concentration, a test with a higher sensitivity will remain positive for a longer period of time than a less sensitive test (Figure 4). Therefore, a better prediction of response or non-response might be achieved by determining the early kinetics of the hepatitis C virus after start of interferon therapy, with a highly sensitive test.

Figure 4

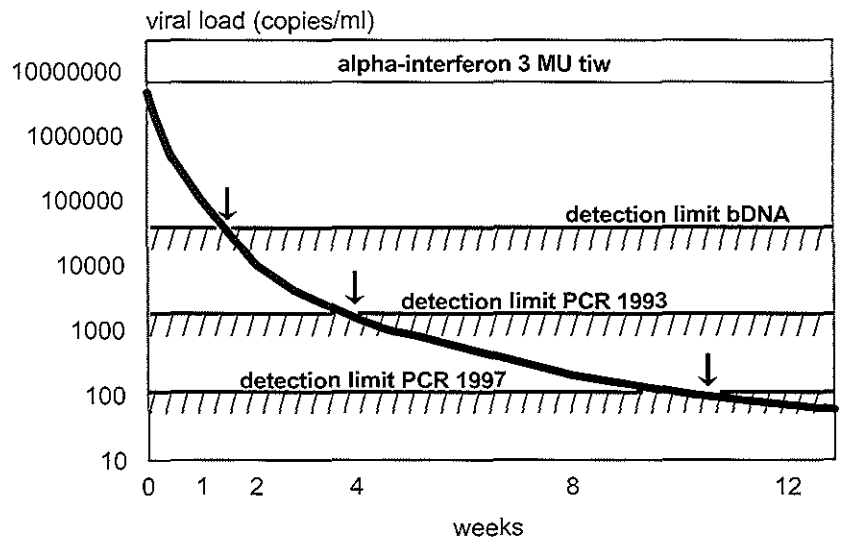


The effect of increased sensitivity of HCV RNA tests on the time-point of HCV RNA negativity. In a hypothetical patient the decline in viral load is depicted over time of interferon therapy. HCV RNA becomes negative at week 2 when using an insensitive bDNA test; increase in assay sensitivity will cause HCV RNA result to remain positive for a longer period of treatment.

Close examination of the kinetics of the hepatitis C virus reveals the crucial importance of an early response of the virus to interferon. Measurements from studies of viral kinetics reveal a very short viral half life of approximately 2-8 hours and a very high production rate of about 10^E11 viral copies per day [17,18]. Moreover, even after a few days there are significant differences in the drop in viral load between patients that will become responders and those that will remain HCV RNA positive after treatment [18] (Figure 5). These observations suggest that a reaction to medication can be expected early after the start of treatment and - vice versa - that non-response also can be determined early. Thus, quantitative HCV RNA measurement could prove a very welcome tool for decision making.

This discussion is becoming more clinically relevant through the emergence of a HCV RNA test, that is quantitative, has equal sensitivity for known genotypes, a detection limit of 100 copies/ml, and a cost comparable to that of a liver biopsy (Superquant™, National Genetics Institute, Los Angeles, USA). Other manufacturers of HCV RNA-PCR tests should follow rapidly with similarly qualified assays.

Figure 5



Results of viral kinetics study in 11 patients treated with an initial high daily dose of alpha-interferon. Differences in the drop in viral load between non-responders and responders become clear after day 2. No easy discrimination can be made between sustained responders and response relapsers in this study with early viral load measurement.

Patients who have insufficient suppression of HCV RNA (for instance, levels above 2000 copies/ml after 4 weeks of interferon monotherapy) should stop that regimen. This is particularly important because it has been confirmed that patients without clearance of HCV RNA from blood at 4 weeks of therapy frequently relapse after 12 months of interferon therapy.

Another way of assessing response to therapy is to calculate the drop in viral load at the end of 4 weeks of therapy. Several studies in a limited number of patients suggest that responders can be separated from non-responders by the percentage reduction of viral load from baseline [23] or by the reduction in viral load expressed in log units [24].

The development of early quantitative HCV RNA testing therefore has clinical relevance, since it may allow a decision on response or non-response about 8 weeks earlier in most patients; this is beneficial for the patient and reduces cost in case of non-response. It is clear that implementation of early quantitative HCV RNA testing should only be done when a validated HCV RNA test is available for the physician in care of the patient.

2.4 CONTROVERSIAL ISSUES

Identification of non-responders during therapy is quite reliable with current tests and therapies. However, identification of sustained responders - vs. potential relapsers - among patients without detectable serum HCV RNA after 6-12 months of therapy is unreliable.

The clinical significance of this test in relation to complexicity and costs is under debate. We will therefore discuss the following two topics:

a) can testing of HCV RNA in the liver or peripheral blood mononuclear cells (PBMC) be useful for clinical decision-making?

b) the complexicity and costs of HCV RNA testing.

2.4.1 Can testing of HCV RNA in the liver or PBMC be useful for clinical decision making?

Since hepatitis C is a viral infection affecting the liver, it is logical to be interested in the viral load in the liver. Again, the availability of an accurate quantitative test with high sensitivity makes the study of this question possible. Schlaak et al. [25] have described how the viral load per liver cell is significantly lower in those patients that will become sustained responders compared to non-responders.

However, the clinical relevance of testing the liver might be greater after treatment than before therapy. Conceivably, patients with undetectable serum HCV RNA by PCR at the end of therapy but still with HCV RNA positivity in the liver, will become relapsers - whereas those that have cleared HCV from the liver will be the sustained responders [26-28].

Partly because of easy accessibility, investigators have examined HCV RNA in PBMC in order to answer the same question of cellular viral persistence and correlation with relapse. Current data are inconclusive, in view of the low number of patients tested [28,29]. Further data should be accumulated with the use of validated methodology.

2.4.2 Complexicity & costs

Until recently, HCV RNA-PCR testing was complex and costly. Preparation of serum or plasma had to be undertaken within 2 hours of drawing blood, with subsequent storage at -20°C or below. Since both in-house PCR and commercial assays often had methodological problems [30,31] the clinician had to ask about negative controls (specificity), low positive controls (sensitivity) and - in case of quantitative assays - for results of dilution series and which standard was used. However, with the advent of plasma preparation tubes (Becton Dickinson MEYLAN Cedex-France), and validated assays [18] the complexicity of HCV RNA-PCR testing will disappear rapidly.

In relation to costs, our approach follows that of Tong et al. [11] When a treatment costing several thousand dollars is contemplated, it seems prudent to establish the need for therapy. Therefore the initial work-up should include a liver biopsy and an HCV RNA-PCR test, both costing $ 150 - $ 250.-

The use of HCV RNA-PCR testing early in treatment is extremely cost effective, leading to overall savings of 30% or more [11].

The cost-effectiveness of HCV RNA-PCR testing at the end-of therapy and during follow-up are debatable. However, at present they provide more prognostic information

than a liver biopsy, which is often included in evaluation of therapy and has similar cost. Furthermore, the information, provided by an HCV RNA-PCR test at the end of therapy may be of value for choosing a treatment schedule in case of relapse.

In conclusion, the evidence presented here suggests that HCV RNA-PCR testing before therapy and three times thereafter is good clinical practice and can save overall costs.

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Chapter 3

Can we routinely quantify Hepatitis C virus (HCV) RNA in clinical samples to monitor the effect of therapy in chronic hepatitis C?

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Submitted

3.1 ABSTRACT

Background. The quantitative measurement of HCV has become an important diagnostic and prognostic tool for the initiation and follow-up of treatment of patients chronically infected with hepatitis C virus (HCV). These techniques are changing constantly, and continuous adaptations have to be made.

Objectives and study design. The aim of this study was to validate the currently used PCR amplification techniques (COBAS Amplicor v2.0 and SuperQuant), and position them in relation to the Quantiplex bDNA 2.0 signal amplification assay.

Results. Regression analysis of the two PCR based amplification assays indicated a high correlation ($P<0.0001$) for both HCV genotype 1 as well as for HCV genotype non-1 specimens. Furthermore, Bland-and-Altman analysis clearly indicated that the differences between these two assays were independent of the viral load measured. We observed that validation of these assays using the Pelicheck HCV-RNA panel indicated that although both assays gave similar results, the SuperQuant assay, which is performed in an external reference laboratory, was more genotype dependent. Furthermore, the claimed detection level of this assay could not be confirmed using this reference panel, and the detection level of both PCR based assays were more identical than suggested previously.

Comparison of these two PCR based assay with the Quantiplex bDNA 2.0 assay showed a more clear correlation with the HCV non-1 genotype samples ($r=0.893$ for SuperQuant, and $r=0.932$ for Cobas Amplicor) than with the HCV genotype 1 samples ($r=0.734$ for SuperQuant, and $r=0.898$ for Cobas Amplicor). However, regression analysis identified in all cases a slope significantly different from 1.

Conclusion. Both PCR based assays can be used for the quantification of HCV. Improvements of commercially available PCR based assays warrant their use in a diagnostic setting. The use of the currently available Quantiplex v2.0 assay is limited due to its detection limit. Further effort and implementation of standardization is needed.

3.2 INTRODUCTION

It has been estimated that world-wide over 170 million individuals are chronically infected with the hepatitis C virus (HCV) [1,2]. Measurement of HCV RNA in serum or plasma has been used extensively as a prognostic marker for individuals undergoing treatment with interferon alone or in combination with ribavirin [3-8]. The ability to quantify HCV RNA accurately is important in chronic hepatitis C infections as the pretreatment level may predict the response to therapy. Furthermore, viral kinetics can predict an early response to therapy in chronic hepatitis C infections, identifying patients who will or will not benefit from therapy [4,9,10]. The quantitative measurement of HCV RNA can also be useful to identify and monitor patients with an incomplete response or relapse. The optimal time to measure HCV RNA to predict response in an individual patient undergoing therapy is not exactly known, but available data indicate that early measurement may be important [3,5].

Techniques to measure HCV RNA both qualitatively and quantitatively are changing constantly, but results from different studies cannot be compared easily due to lack of standardization [11]. Furthermore, commercial assays are subject to continuous adaptations to detect different genotypes equally, with increasing sensitivity [12-16].

In this study, we evaluated three available commercial assays for the quantitative detection of HCV RNA. Two of these assays are PCR based, the COBAS Amplicor 2.0 (Roche Diagnostics), and the SuperQuant assay (National Genetics Institute). The latter is performed in an external central laboratory. One signal amplification assay, the Quantiplex bDNA 2.0 (Bayer Diagnostics) is included. Their use in a routine diagnostic laboratory is discussed.

3.3 PATIENTS AND METHODS

3.3.1 Patients and samples

Plasma and serum samples from thirteen patients with a laboratory confirmed chronic hepatitis C infection undergoing alpha interferon therapy were used in this evaluation. Samples were drawn for the quantitative analysis of HCV RNA, and an average of 14 samples was analyzed from each patient. Samples were aliquoted and

frozen at -80°C within 2 hours after collection. This sample management provides the optimal condition for reliable results [17].

3.3.2 Genotyping of hepatitis C virus

EDTA plasma samples were selected for sequence analysis. The genotype of the hepatitis C virus was determined with primers directed against the conserved sequences of the 5' non-coding region [cDNA primer 5'-GTG. CAC. GGT. CTA. CGA. GAC. CT, primer HCV2 5'-TTG. GCG. GCC. GCA. CTC. CAC. CAT. GAA.]. Sequences reactions were performed on a Vistra Labstation (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) using dye terminator chemistry and analysed on an Applied 373 automated sequencer (Applied Biosystems, Nieuwerkerk, The Netherlands). Both strands were sequenced and analysed using Geneworks software (Oxford Molecular, Oxford, United Kingdom). The genotype was determined according recommendations of the International HCV Collaborative Study Group [18]. The thirteen patients were included proved to be infected with the HCV genotypes 1a (n=2), 1b (n=5), 2a (n=3), 3a (n=1), 3b (n=1), and 4a (n=1).

3.3.3 Quantiplex HCV RNA 2.0 Assay (bDNA)

The Quantiplex HCV RNA 2.0 assay (from Bayer Diagnostics, formerly Chiron Diagnostics) is a signal amplification nucleic acid probe assay for the quantification of HCV RNA in serum or plasma. The conserved 5'-noncoding region and part of the core region are used as targets for detection. HCV RNA is captured into wells coated with specific synthetic oligonucleotide target probes. A second set of target probes hybridises to the viral RNA. Branched DNA amplifiers and alkaline-phosphatase conjugated probes subsequently amplify the signal. The whole process is performed in a semi-automated Quantiplex bDNA System 340, which automatically performance the incubation, plate sealing, washing, reading, data processing, and reduces the hands-on labour requirements. Each plasma samples is tested in duplicate (50 µl per assay) and the concentration of HCV RNA is calculated as the mean of the two test results. The sensitivity of the bDNA version 2.0 assay is claimed to be 200,000 RNA copies per ml, with an upper limit of detection of 120 million RNA copies per ml.

3.3.4 COBAS Amplicor Monitor v2.0 Assay

The Cobas Amplicor Monitor v2.0 assay (called COBAS Monitor, Roche Molecular Systems) is a semi-automated target amplification assay for the quantification of HCV RNA in plasma or serum. The measurement of HCV RNA is carried out using an internal quantification standard (QS), which is a non-infectious RNA transcript that contains the identical binding sites as the HCV target and a unique primer binding site that distinguishes the QS RNA from the HCV amplicon. The QS is added during the sample preparation step, therefore enabling to correct for loss of HCV RNA during the whole procedure, and opens the possibility to monitor inhibition of the sample during the amplification step. The amplification and detection is performed automatically on the Cobas Amplicor. Samples with an optical density above 3.0 are recommended to be adequately diluted to ensure proper quantification. The detection range of this assay is according the manufacturer, between 1000 and 1 million HCV RNA copies per ml.

3.3.5 SuperQuant HCV quantification

A protocol for this assay (SuperQuant HCV) is performed and developed at the National Genetics Institute, Los Angeles, U.S.A. [19]. RNA is extracted from serum using a guanidinium thiocyanate phenol chloroform mixture followed by an ethanol ammonium acetate precipitation [20]. Extracted RNA for each sample is converted to cDNA in two separate reverse transcription reactions using random hexadeoxyribonucleotide primers and this cDNA is immediately used as a template for the PCR amplification using two different primer sets both from the 5'nontranslated region of HCV. The cDNA is split evenly and amplified in four separate PCR reactions terminated each at different cycle numbers. This enables both accurate quantification and sensitivity for each sample. Known numbers of diluted standards are included in the process to construct standard curves for quantitative measurement. The lower detection level is claimed to be less than 100 HCV RNA copies per ml [21]. An internal standard is used to monitor sample inhibition. The whole process of agarose gel electrophoresis of the PCR product, followed by Southern blotting, hybridisation and detection using a digoxigenin labelled DNA probe is performed automatically. The range of the SuperQuant assay is claimed to be between 100 and 5,000,000 HCV RNA copies per ml.

3.3.6 Pelicheck HCV-RNA panel

The Pelicheck HCV reference panel (Viral Quality Check –VQC- CLB, Amsterdam, The Netherlands) contains dilutions of the EUROHEP genotype 1 and 3 plasma standards. These standards have been extensively characterised in the collaborative EUROHEP study, and have been prepared from a plasma pool negative for HBsAg, anti-HBc, anti-HCV, anti-HIV-1/s, anti-HTLV-1, syphilis, HCV RNA, HBV DNA and HIV-1 RNA. The HCV RNA type 1 contains 36,000 genome equivalents per ml, while the HCV RNA type 3 contains 120,000 genome equivalents per ml [11]. The same lot of Pelicheck HCV RNA panel was used in our analysis of both the SuperQuant and the COBAS Amplicor assay.

3.3.7 Statistics

Logarithmic transformation of the readings of the different assays was carried out. XY scatter diagrams were drawn and the correlation coefficients (r^2) or Spearman correlation r as well as the linear regression analysis was done using the statistical functions of SPSS 9.0 software. Ninety-five percent confidence interval were calculated using the Fisher's Z transformation method.

In order to compare to what extent the data obtained with the COBAS Amplicor assay agree with the data obtained with the NGI assay, the data were furthermore analysed as described by Bland and Altman [22]. This analysis is based on comparing the differences between measurements for the same sample by plotting the differences against the average.

3.4 RESULTS

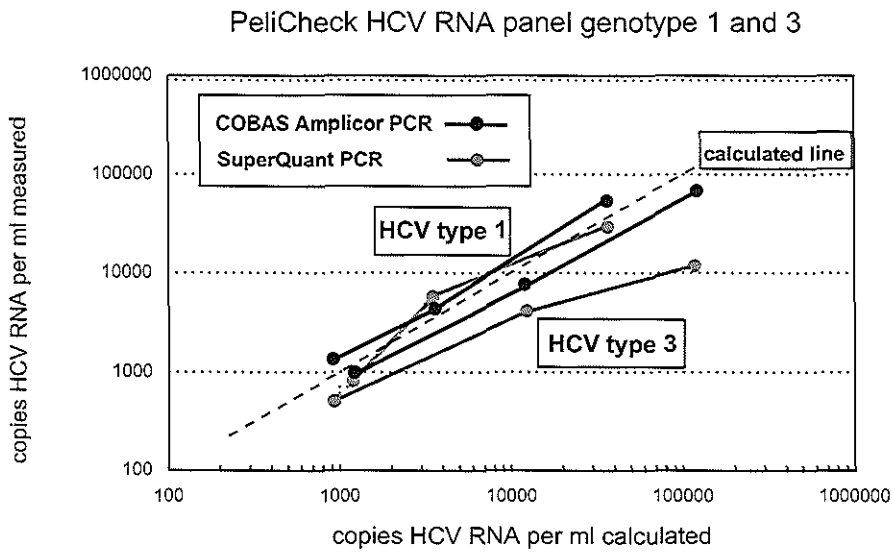
3.4.1 Pelicheck HCV-RNA panel

The HCV reference panel was used to evaluate the sensitivity and the linearity of both the NGI SuperQuant and the Roche COBAS Monitor assay. Furthermore, because two genotypes of HCV (genotype 1 and 3) are included in this panel, information on genotype dependency of the assays becomes available (Figure 1). The viral load of this reference panel is below the detection level of the bDNA assay, therefore no data for this assay are available.

Both assays were able to detect and quantify HCV genotype 1 equally well within two-fold of the assigned value. The COBAS Monitor assay proved to slightly overestimate the number of HCV RNA copies per ml (1.5 fold), in contrast to the SuperQuant assay for which values were around the actual assigned HCV RNA copy number. However, both assays were unable to detect the dilutions calculated 225 or less HCV RNA copies per ml.

For the HCV genotype 3, the COBAS Amplicor assay slightly underestimated the values assigned to the panel by not more than two-fold. The SuperQuant assay, however, had difficulties to detect the high HCV RNA copy number of this genotype (120,000 HCV RNA copies per ml) with a 10-fold differences. Both assays detected the lowest assigned value of 1200 HCV RNA copies per ml in this panel, but not the 300 HCV RNA copy number.

Figure 1



The linearity of the Roche COBAS Amplicor v2.0 assay HCV RNA assay (marked with black lines) and the NGI SuperQuant assay (marked with gray lines) was determined using the Pelicheck HCV RNA panel. This panel contains assigned HCV RNA dilutions of genotype 1 and 3. These assigned values were compared to the calculated line and expressed as HCV RNA copies per ml (calculated line).

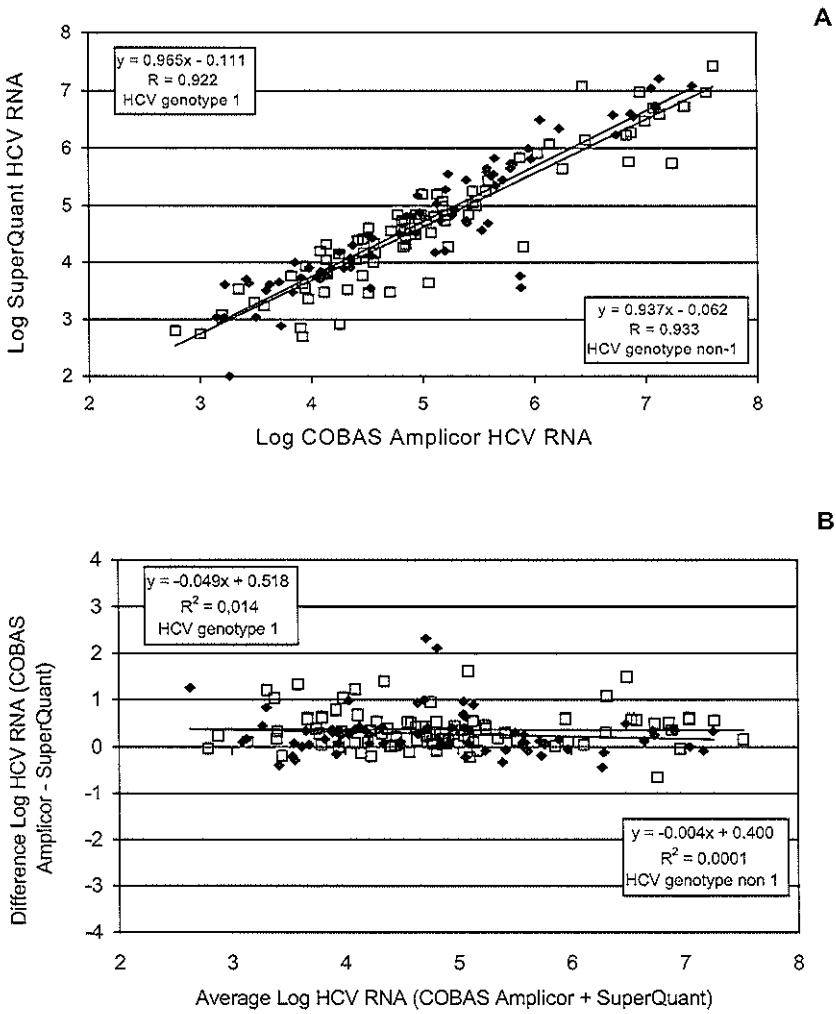
3.4.2 Quantification of HCV RNA by PCR based assays

The COBAS Amplicor and the SuperQuant assay were compared to determine their usefulness for a clinical laboratory, by comparing the HCV RNA values in patient samples during treatment for each type of assay. Furthermore, to establish to what extent these assays quantified HCV RNA of different genotypes, we divided the obtained HCV RNA values between patients infected with HCV genotype 1 (n=7), and patients infected with HCV genotype non-1 (n=6).

One-hundred-sixty-one samples were analysed in both the SuperQuant and COBAS Amplicor assays. The samples analysed with the COBAS Amplicor assay were diluted in negative plasma if the optical density was above 3.0. Furthermore, the samples were divided into HCV genotype 1 (n=77) and HCV genotype non-1 (n=84) samples. The log values were plotted (see Figure 2A) and regression analysis showed that both datasets were highly correlated for HCV genotype 1 ($r = 0.922$, $p < 0.0001$) and HCV genotype non-1 ($r = 0.933$, $p < 0.0001$). Furthermore, regression analysis showed that the slope for both HCV genotype 1 as well as HCV genotype non-1 approached 1 (HCV genotype 1: slope 0.965, 95% confidence interval 0.872 to 1.058; HCV genotype non-1: slope 0.937; 95% confidence interval 0.858 to 1.016), while the y intercept approached 0 (HCV genotype 1: y intercept -0.111, 95% confidence interval –0.591 to 0.369; HCV genotype non-1: y intercept –0.062; 95% confidence interval –0.472 to 0.348).

Bland-and-Altman analysis furthermore indicated that the difference between both assays were independent from the average values measured, since both for HCV genotype 1 and HCV genotype non-1, the slope did not significant differ from 0 (HCV genotype 1: slope –0.049, 95% confidence interval –0.140 to 0.045; HCV genotype non-1: slope –0.004, 95% confidence interval –0.086 to 0.077). From these data it could furthermore be concluded that both for HCV genotype 1 and non-1, the assigned values by the COBAS Amplicor assay were higher than the assigned values by the SuperQuant assay. However, regression analysis showed that these differences indicated by y intercept of the Bland-and-Altman analysis were not significantly different from 0 (HCV genotype 1: y intercept 0.518, 95% confidence interval –0.054 to 0.982; HCV genotype non-1: y intercept 0.400, 95% confidence interval –0.007 to 0.806).

Figure 2



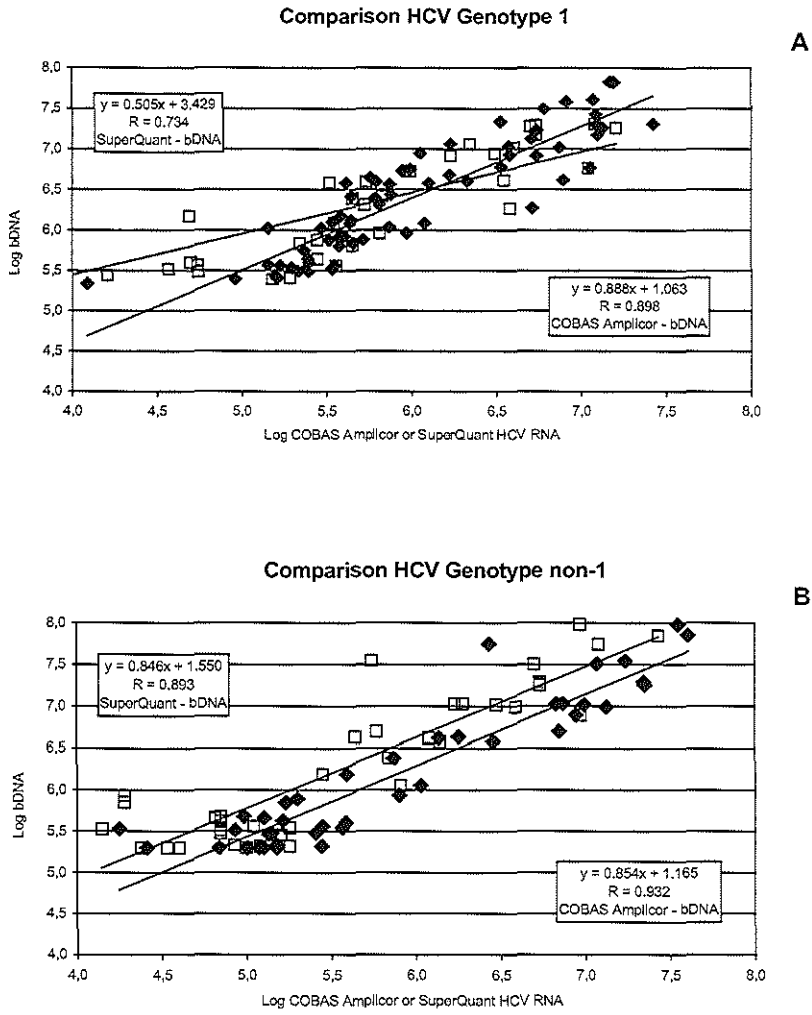
HCV viral loads in clinical samples determined with the COBAS Amplicor 2.0 and the SuperQuant assay. The correlation between both PCR based assays is divided into samples with HCV genotype 1 (marked with closed diamonds) and samples with HCV genotype non-1 (marked with open squares). The equation of the regression curve and the Spearman correlation coefficient *R* is given for each group of samples. (see panel A). Panel B shows the results of the Bland-and-Altman analysis performed for both groups of HCV genotypes. Values are given in copies HCV RNA per ml plasma.

3.4.3 Correlation of PCR based assays with the Quantiplex HCV RNA 2.0 assay

To demonstrate whether the two currently used PCR based assays can be used aside the Quantiplex bDNA 2.0 assay, samples were analysed by the three respective assays. Currently only samples above the detection limit of the Quantiplex bDNA 2.0 assay can be included (200.000 HCV RNA copies per ml). Samples were divided in HCV RNA genotype 1 (Figure 3a) and HCV RNA genotype non-1 (Figure 3b). The Quantiplex bDNA assay gave consistently higher values than both the COBAS Amplicor and the SuperQuant assay. For the HCV genotype 1 samples, the bDNA assay gave 0.197Log higher results than the COBAS Amplicor assay, and 0.539Log higher results than the SuperQuant assay. For the HCV non-1 genotypes, this was respectively 0.292Log and 0.626Log values.

Both the PCR based assays do highly correlate with the Quantiplex bDNA assay for HCV non-1 genotypes ($r = 0.893$ for SuperQuant, and $r = 0.932$ for the COBAS Amplicor assay). Regression analysis showed however that the slope of the curves did significantly differ from 1 (Quantiplex slope = 0.846, 95% confidence interval 0.699 to 0.992; COBAS Amplicor assay slope = 0.854, 95% confidence interval 0.747 to 0.960). Analysis of the HCV genotype 1 samples showed the best correlation between the COBAS Amplicor assay and the Quantiplex bDNA assay ($r = 0.898$, slope 0.888, 95% confidence interval 0.782 to 0.993). The correlation with the SuperQuant assay for HCV genotype 1 was less ($r = 0.734$), with a slope of 0.505 (95% confidence interval 0.342 to 0.668).

Figure 3

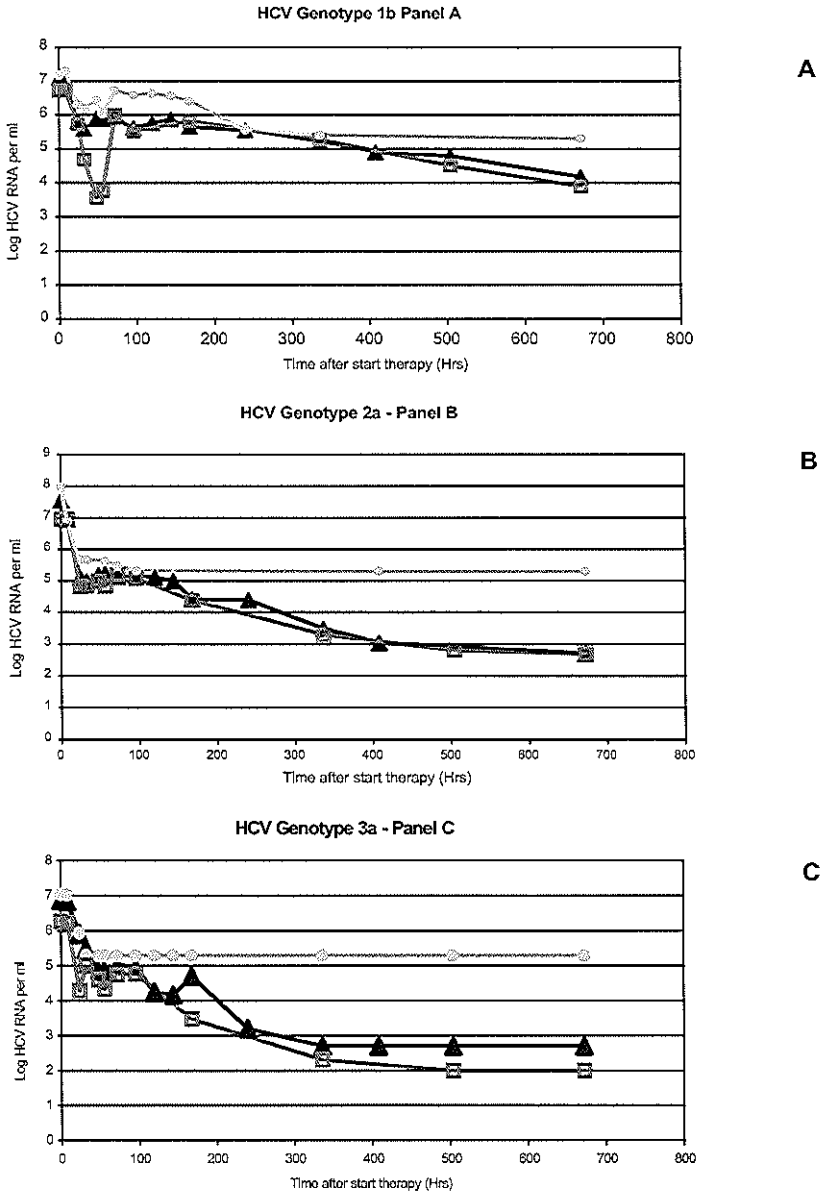


HCV viral loads in clinical samples determined with the Quantiplex bDNA 2.0 signal amplification assay and the relation with the COBAS Amplicor 2.0 (represented with closed diamonds) and the SuperQuant assay (represented with open squares). The correlation between both these assays are divided into samples with HCV genotype 1 (Panel A) and samples with HCV genotype non-1 (Panel B). The equation of the regression curve and the Spearman correlation coefficient *R* is given for each group of samples. Values are given in copies HCV RNA per ml plasma.

3.4.4 Clinical monitoring

To further demonstrate the clinical utility of the respective HCV RNA detection assays, chronically infected HCV patients treated with interferon were monitored with all three assays during the first period of this treatment. The appropriate dilutions were made in order to enable accurate determinations. The detection limit of the respective assays were used according the manufacturers information. In Figure 4, the calculated Log HCV RNA levels in copies per ml, measured with the respective assays were plotted for the three different assays. The data from three patients shown were infected with HCV genotypes 1b, 2a and 3b, respectively. Although all patients showed a similar rapid decline in HCV RNA levels, differences were observed due to differences in standardization and detection limits. Furthermore, in Figure 4A is can be clearly observed that the SuperQuant assay underestimates some individual samples from this patients, compared to the other two used assays.

Figure 4



Clinical course of three chronically HCV-infected patients during follow-up of interferon therapy. Three different HCV genotypes are represented in respectively panel A (HCV genotype 1B), panel B (HCV genotype 2A), and panel C (HCV genotype 3A). Values of HCV RNA are depicted in copies per ml and plotted against time after start therapy. The patients were monitored with the COBAS Amplicor 2.0 (closed light-gray dots), SuperQuant (open dark-gray squares) and Quantiplex 2.0 bDNA assay (closed black triangles), respectively.

3.5 DISCUSSION

The accurate measurement of HCV RNA is serum or plasma is becoming more and more important for individual patient management in a routine diagnostic setting. Data from large clinical trials have given convincing (clinical) evidence that the measurement of HCV RNA is an important factor in predicting the therapeutic response to interferon or combination therapy of interferon and ribavirin in patients chronically infected with HCV [7,23-25]. Furthermore, the HCV genotype has been implicated as a predictive factor, although the quantification of different genotypes has been a problem for several of the first generation of assays [6]. It should be clearly stated, however, that a large amount of these data in clinical trials have been generated using the PCR based, only externally available SuperQuant assay. No validation of this assay has been performed with the currently in use COBAS Amplicor assay, and no information is available whether this SuperQuant assay has been improved (or not) during the last years. Commercially available assays, which are currently available for routine in-house testing, have been changing constantly, and further improvements both in sensitivity and automation are to be expected in the next years [26-28].

In this study, we have evaluated the currently available assays for their use in a diagnostic setting, of which an essential part was the positioning of the SuperQuant PCR assay in relation towards the semi-automated COBAS Amplicor 2.0 assay and the Quantiplex bDNA 2.0 assay. This comparison is necessary because diagnostic laboratories have to assist in the implementation of the recommendations on the treatment of chronic HCV infections, like established at the International Consensus Conference on Hepatitis C [29].

From our data we could clearly conclude that there was a good correlation between the SuperQuant and the COBAS Amplicor 2.0 assay, both for HCV genotype 1 and HCV genotype non-1 samples. From regression analysis it could furthermore be concluded that both assays generated statistically significant related results, and that differences between these assays were independent from the average result. Although the COBAS Amplicor assay assigned higher values than the SuperQuant assay to the samples, these differences were not statistically different for the total set of data. Generally, data are generated for large studies, in which datasets from

individual patients will not influence the total outcome of the study. Looking at data from individual patients under treatment, like represented in Panel 4A, one could conclude however, that the SuperQuant assay underestimated HCV RNA values compared to both the Quantiplex or COBAS Amplicor assay. The large differences could most likely not been discovered due to the manner the assay is set up. In the COBAS Amplicor assay, an internal standard is used during the whole procedure and corrects for both loss of samples as well as inhibition of the amplification reaction.

From the analysis of the data from the Pelicheck HCV RNA panel, we could also conclude that the detection level for both PCR based assays were similar. Both assays could not detect less than 900 HCV RNA copies per ml. This indicates that the claimed detection level of 100 copies per ml for the SuperQuant assay is based on a different standard. Furthermore, the assay seems to be more genotype dependent than the COBAS Amplicor assay, definitely for the HCV genotype 3. No data are available for the other genotypes.
Comparing both PCR based assays with the Quantiplex bDNA 2.0 assay, indicated that although both correlate with this signal amplification assay, the slope of the curve did not reach 1. For the SuperQuant HCV genotype 1 comparison, the slope was even far from 1. These differences could be due to the intrinsic differences between signal and target amplification assays. The lack of sensitivity of this assay however, makes routine implementation not feasible.

Since the introduction of quantitative assays the detection of HCV RNA, many improvements have been made, both in detecting different genotypes of HCV and improvement of the detection level [27,30,31]. New generation of assays, similar to the new generation of Quantiplex bDNA assay for HIV-1, as well as developments in real-time amplification technology will be introduced in the near future. The final steps towards full proof introduction of quantitative detection techniques are automation and standardization. The introduction of an international standard for HCV has been initiated, although limited information is available on conversion towards currently used assay systems, stability in time. Furthermore, only HCV genotype 1 is included in this international standard.

In conclusion, our results indicate that there are no differences in the available PCR amplification assays. This enables the introduction of these assays in a routine diagnostic setting, enabling to support individual patient management.

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Chapter 4

Ultrarapid Hepatitis C Virus Clearance By Daily High Dose Interferon In Nonresponders To Standard Therapy

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4.1 ABSTRACT

Background. To analyse the kinetics of the hepatitis C virus (HCV) and the patterns of resistance to interferon alpha (IFN), we assessed the HCV RNA levels early during retreatment with high dose IFN in patients who did not respond to standard treatment.
Methods. Eleven nonresponders to previous therapy with 3-6 MU IFN three times a week were retreated with daily 10 MU. Plasma was sampled at day 0, 1, 2, 3, 14 and 28; all samples were prepared within 2 hours and stored at -70°C without thawing until analysis. The quantitative HCV RNA level was assessed by the Superquant™ assay, NGI, USA. The Eurohep reference panel, tested blindly, confirmed the linearity of the assay with a detection limit for genotype 1 and 3 between 10^2 and 10^3 copies/ml.
Results. All patients showed a fall in viral load between week 0 and week 2 (2.6 log, i.e. 99.7%, range 1.3—4.7 log), whereas no fall was detected after week 2. Closer examination in 9 patients revealed that all had a dramatic fall in the first 2 days (first day 1.8 log, 0.8—3.5; second day 0.8 log, -0.2—1.3), without any significant fall thereafter. The calculated half-life of viral decay in plasma was 5 (2—8.9) hours, corresponding to a clearance of 2.4 (0.2—13.7) $x10^{11}$ virions per day. Sustained responders showed a significant greater fall in viral load in the first day (3.2 log, 2.8—3.5) than those who did not respond (1.4 log, 0.8—2.1, p=0.001). All three sustained responders had undetectable plasma HCV RNA at day 14.
Conclusion. In patients without a response to standard IFN, the hepatitis C virus has a high daily turnover rate similar to that reported in naive patients. Our findings suggest that an early clearance of HCV-RNA from the circulation is the key to a sustained response, which might be induced in about 25% of these patients by treatment with high (10MU) daily doses of IFN. These findings have important implications for the concept of treatment of hepatitis C, which should shift its focus from long-term mild treatment towards aggressive therapy aiming at a fast viral disappearance within the first few days.

4.2 INTRODUCTION

Standard treatment of chronic hepatitis C with interferon alpha 2-b (IFN) 3 mega units (MU) thrice weekly (tiw) for 6 months leads to sustained virus clearance in about 15 percent of patients[1]. Longer treatment (12—18 months)[2] or combination with ribavirin[3] enhances the efficacy mainly by reducing the relapse rate after an initial response. However, HCV-RNA still detectable in plasma at 4 weeks of therapy virtually precludes sustained virus clearance[1,4], pointing to the crucial importance of an early response during therapy. In this study with daily high-dose IFN therapy in nonresponders to standard-dose IFN, we frequently measured quantitative plasma HCV-RNA levels in the first 4 weeks in order to assess the viral kinetics of the hepatitis C virus and to relate the patterns of viral decline to response to treatment.

4.3 MATERIALS AND METHODS

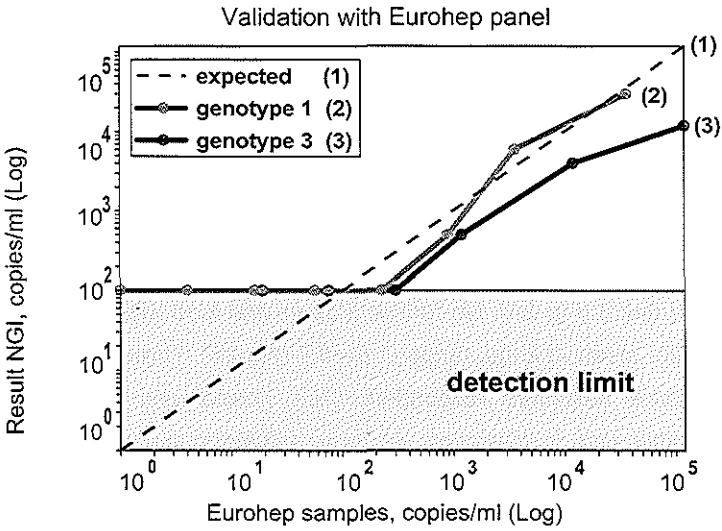
4.3.1 Study design

Nonresponders to standard IFN therapy 3-6 MU tiw were offered retreatment by daily high-dose IFN at the university hospitals of Rotterdam and Gent. Nonresponse was defined as lack of normalisation of serum alanine amino transferases (ALT) and persistent detectable viral RNA in plasma during IFN therapy. Sixteen patients received IFN 10 MU daily for 1 week, followed by 10 MU tiw until week 4 and 3 MU tiw until week 24. Treatment was discontinued because of nonresponse if plasma HCV RNA was still detectable at week 4 by a qualitative in-house PCR (detection limit 10^3 copies/ml). In 5 out of 16 patients no early high-quality plasma samples were available; these patients were comparable with those analysed with regard to their pre-treatment characteristics. Eleven patients had plasma samples suitable for quantitative HCV-RNA detection at week 0, 2 and 4 and were included in this analysis. Their mean age was 42 years (range 29—61); 8 of them had a genotype 1 and/or a liver cirrhosis, known characteristics for low responsiveness to IFN treatment[1]. Analysis of viral kinetics could be performed in 9 of 11 patients who had plasma samples available during the first 3 days of therapy. Blood was processed within 2 hours and stored without thawing at -70°C.

4.3.2 HCV RNA detection

HCV-RNA was quantified at National Genetics Institute, California, USA, by the Superquant™ assay, a HCV specific quantitative reverse transcriptase polymerase chain reaction, as previously described by Tong et al[5]. Briefly, RNA was purified and extracted from plasma samples using guanidinium-phenol-chloroform followed by isopropanolol precipitation. cDNA was made from this DNA using MMLV-RT and random primers. In four different reactions of 25, 30, 35 and 45 cycles, the 5'UTR region was amplified using two sets of specific primers. The PCR product was electrophoresed and hybridized in a Southern blot assay with a digoxigenin labelled specific PCR fragment. The bands were electronically scanned and the computerized images were automatically compared for size and optical density with the results of an internal series of standard RNA with a known number of virions; the result is presented as copies per ml plasma. If a samples reached the upper limit of the internal standard (5.10^6 copies/ml) quantification is achieved through 1:10 dilution. The range of the test is therefore between 10^2 —5.10^7 copies/ml. All samples were tested blindly; the Eurohep reference panel[6], which is a well characterized dilution series of genotype 1 and 3, was included as control. The detection limit as calculated from the Eurohep results was between 10^2 and 10^3 copies/ml for both genotypes, and dilution series yielded linear results which were close to expected for genotype 1 and about 0.6 log below expected for genotype 3.

Figure 1



The linearity of the NGI SuperQuant assay was determined using the Pelicheck HCV RNA panel. This panel contains assigned HCV RNA dilutions of genotype 1 (gray line) and 3 (black line). These assigned values were compared to the calculated line and expressed as HCV RNA copies per ml (calculated line).

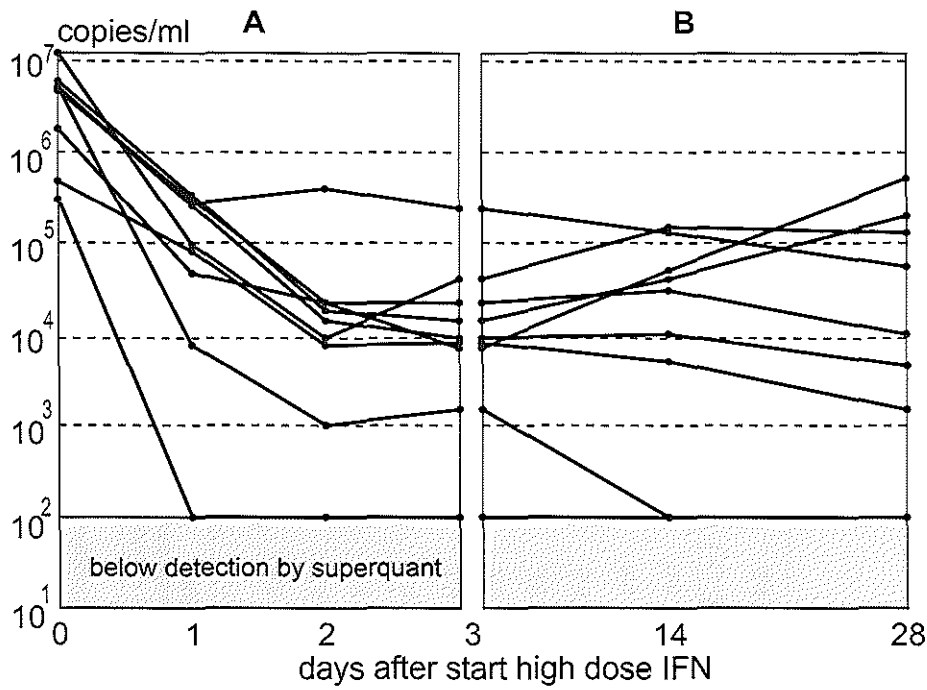
The rate of viral clearance (T½) was calculated according to standard methods[7,8], by which the half life ($T_{1/2}$) of viral decay in plasma is calculated for each patient from the initial slope (S) of decline between initial viral load and the viral load 24 hours after the start of treatment. As the initial decline is exponential by approximation, the half-life can be calculated according to $T_{1/2} = \ln(2)/S$, where $S = \ln(\text{viral load})_{T0} - \ln(\text{viral load})_{T24hours}$.

In this calculation the arbitrary value of 10^2 copies/ml was used for samples with HCV RNA below the detection limit of the Superquant^{tm} assay, so the half-life calculated in those who reached undetectable levels after 24 hours might even be shorter. Assuming that the virus is equally distributed in the extracellular water, the initial daily clearance rate can be estimated from the $T_{1/2}$, the concentration of viral particles in plasma and the extracellular fluid volume for each patient according to the equation: initial daily clearance = $V_{ec} \times C_0/T_{1/2}$ (V_{ec} = extracellular volume in litres, C_0 = pre-treatment viral concentration in copies/litre and $T_{1/2}$ = half-life in days).

4.4 RESULTS

The characteristics of the 11 patients with suitable plasma samples for HCV-RNA detection are listed in table 1. All patients showed a drop in the level of viraemia during the first 2 weeks. The mean decrease observed was 2.6 log (99.7%) with a range of 1.3—4.7 log. Between week 2 and 4 the fall in viral load was minimal (mean 0.2 log, range -0.7—1.4). Nine patients had day 1, 2 and 3 samples available; their mean reduction in viral load was 1.8 log (0.8—3.5) at day 1, 0.8 log (-0.2—1.3) at day 2 and 0 log(-0.7—0.5) at day 3 (Fig 2A). No further drop in viral load was observed between day 3 and day 28, mean -0.2 log, range -1.9—0.7 (Fig 2B). Three out of the 11 patients (27%) became sustained responders (ALT normal and HCV-RNA undetectable 6 months after end of treatment). All three sustained responders had undetectable HCV-RNA at day 14. In the group of 9 patients with samples available at day 1—3 the fall in viral load at day 1 in the two sustained responders was significantly greater (2.8 and 3.5 log respectively) compared to the seven others (mean 1.4 log, 0.8—2.1, p=0.001). The follow-up period for the sustained responders is more than 5 years. The calculated mean half-life of viral decay in plasma was 5 hours (range 2.0—8.9); the estimated half-life in the two responders who could be analysed was 2.6 and 2.0 hours when the calculation of undetectable samples was based on the arbitrary level of 10^2 copies/ml. Therefore the half-life is only a minimal estimate and may even be lower. The estimated initial daily clearance rate ranged between 0.2—13.7.10^{11} virions per (mean: 2.4.10^{11}).

Figure 2 A and B



Fall in viral load in patients with chronic hepatitis C following IFN 10 MU daily for at least 5 days and thereafter 10 MU thrice weekly. The mean drop in viral load in the first day was 1.8 log (0.8—3.5). Between day 1—2 there was a drop of 0.8 log (-0.2—1.3), whereas no drop in viral load was found between day 2—3 (0 log, range: -0.7—0.5). In total, the drop in viral load was 2.5 log between day 0—3 and between 0 log day 3—28.

Table 1 Baseline characteristics and viral dynamics, of patients treated with interferon 10 MU daily.

Patient	genotype	cirrhosis	age (years)	pre-treatment viral level (copies/ml)	decrease in viral load: day 0—2 (log)	estimated initial clearance rate (copies/day)	$T_{1/2}$ (hours)	outcome to treatment
1	1b	yes	61	$4.7.10^6$	2.3	3.10^{11}	6.0	NR
2	1b	no	29	$0.5.10^6$	1.8	$0.2.10^{11}$	8.9	NR
3	1b	yes	60	$5.0.10^6$	2.6	$3.4.10^{11}$	5.6	NR
4	1b	no	52	$5.2.10^6$	1.1	$3.6.10^{11}$	5.6	NR
5	4	no	35	$6.0.10^6$	2.5	$4.1.10^{11}$	5.6	NR
6	4	yes	43	$1.8.10^6$	1.9	$1.5.10^{11}$	4.5	NR
7	1b	no	50	$12.2.10^6$	2.8	$13.7.10^{11}$	3.5	NR
8	1a	no	30	$0.3.10^6$	3.5	$0.6.10^{11}$	2.0	SR
9	3	no	37	$5.2.10^6$	3.5	$7.7.10^{11}$	2.6	SR
10	1b	no	31	$2.9.10^6$	n.a.	n.a.	n.a.	NR
11	3	no	34	$2.8.10^6$	n.a.	n.a.	n.a.	SR
Range:			**29—61**	**$0.3—12.2.10^6$**	**1.1—3.5**	**$0.2—13.7.10^{11}$**	**2—8.9**	
Mean:			**42**	**$2.8.10^6$**	**2.4**	**$2.4.10^{11}$**	**5.0**	

4.5 DISCUSSION

Until recently, the hepatitis C virus was considered to have a low turnover rate and response to antiviral treatment was thought to occur gradually over several weeks. In a recent consensus at the National Institutes of Health of the USA, it was recommended to evaluate the initial response to therapy by testing HCV-RNA after 3 months[9]. Because of these concepts on the dynamics of hepatitis C virus infection, most of the large treatment studies have focussed on enhancing efficacy by prolonging therapy up to 18 months[2]. In this study we found an ultrarapid HCV-RNA clearance, with a mean half-life of 5 hours during the first day of 10 MU IFN therapy. In 1996 Zeuzem described an exponential decline of HCV-RNA with a mean half life of approximately 2 days; however, samples were not taken daily and patients received a lower dose of 3 MU IFN given three times per week. With a higher dose of 6 MU IFN given daily Yasui[10] observed a mean $T_{1/2}$ of 8 hours. Recently Lam et al[8]. described a dose-dependent effect of IFN in clearance of HCV genotype 1 patients, naive for IFN. With one injection of 10 MU IFN and by use of the bDNA assay (detection limit $3.5.10^5$ copies/ml), they found a mean half-life of 7.2 hours and an estimated daily clearance of $3.7.10^{11}$ particles per day. In our group of nonresponders to previous standard dose IFN we found similar results as Lam et al. with the validated sensitive Superquant^{tm} assay (detection limit 10^2 -10^3 copies/ml). A partial blockade in viral production induced by a sub-optimal dosing of IFN can cause an overestimate of the half-life because of the resulting sub-optimal decrease in viral load. The studies by Zeuzem, Yasui and Lam in naive patients together with our current study in nonresponders suggest that IFN 10 MU per day might block virion production more completely than IFN 3-6 MU. Interestingly, the two sustained responders showed the shortest half-life of viral decay in plasma (2.6 and 2 hours, table 1). After the initial very fast decline in viral load in the first two days in all patients, no further decline is seen thereafter. Lam et al. also found a rise in HCV-RNA about 48 hours after the IFN injection and proposed that daily dosing might help to continue the decline in HCV-RNA. However, we found this same rise in HCV-RNA after 48 hours while 10 MU IFN was continued daily. The reason for lack of a further fall in viraemia around 48 hours is unclear; it might be related to selection of IFN resistant virions or down regulation of IFN receptors and intracellular escape. Further studies on resistance need to focus on this early period.

Therapeutically, this study adds further evidence that nonresponders to a standard dose IFN can benefit from high dose treatment. This conclusion might however not be generalized for all nonresponders because in this pilot study none of the sustained responders had cirrhosis and only one had a genotype 1.

In conclusion this study reveals a very rapid replication of the hepatitis C virus with a frequent development of resistance within 48 hours after initialisation of treatment. The results of this study suggest that the key to a sustained response is an early clearance of HCV-RNA from the circulation.

4.6 ACKNOWLEDGEMENTS

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Chapter 5

Chronic hepatitis C: viral kinetics and duration of therapy

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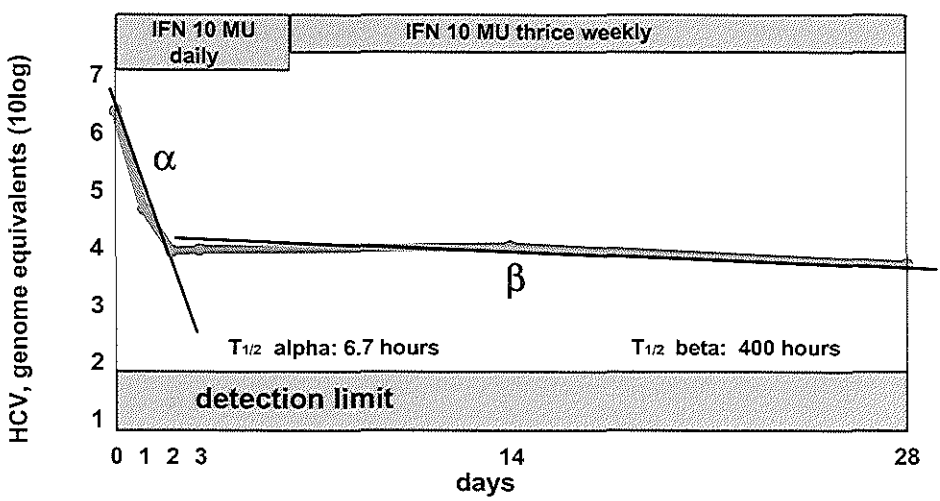
Hepatology 1997;26:1691-3

5.1 VIRAL KINETICS IN CHRONIC HEPATITIS C

Lam et al. reported an exponential decline in viral load after one injection of Interferon in hepatitis C, genotype 1 positive patients, naive for Interferon[1]. A dose dependent relation was found with the maximal drop in viral load after one injection of 10 MU Interferon. An exponential decline in viral load was described for the first 2 days after injection, followed by a decrease in the slope of viral decline after day 2. Based on these results prolonged therapy with 10 MU was suggested to continue this rapid viral decline. We conducted a study in which we re-treated nonresponders to previous standard dose interferon. Patients received 10 MU IFN daily for one week, followed by 10 MU thrice weekly thereafter till week 4. We collected the same early plasma samples as Lam et al. (pre-treatment and after 24, 48 and 72 hours) as well as later samples after 2 and 4 weeks of treatment. Samples were tested with a sensitive, validated quantitative PCR assay for HCV RNA (Superquant^{tm}, National Genetics institute, LA, detection limit between 10^2-10^3 copies/ml). We calculated the half-life and the production rate of the hepatitis C virus from the results of exponential decline, observed within the first days of treatment. To compare our data with those of Lam et al., we restricted our analysis to the six patients with genotype 1. During the first two days, a steep decline in viral load was observed similar to that reported by Lam et al. However, the loss of viral decline after 2 days which was attributed by Lam et al. to the weaning of the effect of the single dose of Interferon was also observed in five out of six patients despite continuation of treatment with daily high doses of interferon. The drop in viral load proved to have 2 phases, first an exponential rapid decline from day 1 till day 3 followed by a much slower second phase of viral decline from day 3 onward. The slope (a) of the viral decline can be used to calculate the half-life ($T_{1/2}$) according to $T_{1/2}$= a/log2. The mean half-life for these 6, genotype 1 patients was 6.7 hours for the initial phase (figure 1, $T_{1/2}$ alpha), with a range between 2—13 hours. This was followed by a much slower elimination in four out of 6 patients with a half-life of approximately 400 hours (figure 1, $T_{1/2}$ beta) and a range between 244—573 hours. In 2 patients no half-life of the second phase ($T_{1/2}$ beta) could be calculated; one patient had a very fast drop in viral load and became HCV RNA negative after 24 hours and remained a virological sustained responder, and one patient showed a very slight rise in viral load between day 2 and day 28. If we examine the observed drop in viral load, the decline in viral load can not be described by a simple exponential model but fits

into a 2 nd order kinetic model, designed to describe the decline in concentration for substances that are dissolved in 2 compartments with 2 different elimination rates. Based on the initial half-life of about 7.6 hours and the average pre-treatment viral load of about 3.10^7 copies/ml, it can be calculated that if a viral load of < 1 copy/ml is to be reached a high dose induction treatment would be needed for at least 7 days. This means that we need at least 20 x $T_{1/2}$ alpha before we reach HCV RNA negativity based on the initial fastest decline. However, prolongation of high dose interferon up to 1 to 2 weeks is probably not the simple solution for patients that fail to reach very low (below 100-1000 copies/ml) viral levels after the first 2 days of daily high dose treatment because of the appreciable change in elimination rate. If we consider the much longer half-life of the hepatitis C virus observed after day 2, much longer treatment is needed to reach HCV RNA negativity. Based on the $T_{1/2}$ beta of around 400 hours and an average viral load of 20000 copies/ml after the first 2 days of treatment, a prolonged therapy of about 250 days is needed, this equals 15 x $T_{1/2}$ beta. Long continued treatment in combination with a high dose induction treatment appears to be the best solution until we understand the biology of virus clearance and ways to modulate it.

Figure 1



Mean drop in hepatitis C viral load observed between day 0 and week 4 in 6 chronic hepatitis C patients, infected with genotype 1. Alpha: slope of viral decline observed between day 0 - 2, Beta: slope of viral decline observed between day 2 - 28.

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Chapter 6

Estimation Of Early Hepatitis C Viral Clearance In Patients Receiving Daily Interferon And Ribavirin Therapy Using A Mathematical Model

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Hepatology 2001;33(2):419-23

6.1 ABSTRACT

Background. Patients with HCV genotype 1 infection are resistant to standard interferon therapy. We used a mathematical model to estimate the duration of daily therapy necessary to maximize the number of patients achieving viral negativity before 12 weeks of therapy.

Design. Patients from a study to determine HCV RNA reduction over 4 weeks using 3MU, 5MU, or 10MU of alpha interferon daily plus ribavirin were compared with a group receiving alpha interferon 3MU tiw. All patients had HCV genotype 1 infection and baseline serum HCV RNA levels > 1 million copies/ml. HCV RNA was measured at days 0, 0.5, 1, 3, 5, 7, 10, 14, 17, 21, 24 and 28. Each measurement was log transformed and the means for each group computed. Using the mean kinetic data, linear regression and 95% confidence intervals were calculated and projected to the lower limit of detection of serum HCV RNA in order to estimate the treatment time needed for each treatment regimen. After day 28, patients continued therapy with interferon alpha 2b 3 million units tiw plus ribavirin.

Results. By extending the linear regression and prediction interval lines, the estimated time to negativity was greater than 12 weeks for the standard interferon group, 42- > 84 days for the 3MU interferon daily plus ribavirin, 39-60 days for 5MU interferon daily plus ribavirin and 25-45 days for the 10MU interferon daily and ribavirin group, respectively.

Conclusion. Thus, the use of a predictive model based on log transformation and linear regression of the early HCV RNA response suggests daily doses of 5 or 10 million units of interferon plus ribavirin will be theoretically necessary for longer than 4 weeks to maximize the number of patients who clear virus by 12 weeks of therapy. This model may be useful in predicting response in groups of patients receiving other therapies.

6.2 INTRODUCTION

Chronic hepatitis C infection is a slowly progressive disease that can lead to cirrhosis and/or hepatocellular cancer over a period of 10 to 20 years.[1] In the United States, it is estimated that nearly 4 million individuals are infected (1.8% of the population) and that there are approximately 30 thousand new cases of acute hepatitis C annually.[2] Estimates indicate that there are eight to ten thousand HCV-related deaths annually, and that this number is expected to triple in the next two decades. Currently, hepatitis C is one of the leading causes of chronic liver disease in the United States, and the most common indication for liver transplantation.[3]

The rationale for induction therapy in chronic hepatitis C infection include the suboptimal response rates observed with conventional alternate day interferon monotherapy, the large viral burden and high rates of viral turnover.[4-8] Also, an acute dose response has been reported with higher daily doses of interferon in chronic hepatitis C, and a biphasic exponential viral decline has been described in chronic hepatitis C virus infection during therapy.[5-7] In addition, most patients who achieve a sustained virologic response to antiviral therapy do so early during treatment, (in general before week 12), [9-13] and a greater than 3 log reduction in serum HCV RNA early during therapy has been reported to be a good predictor of eventual sustained response.[14] Early clearance of hepatitis C virus may also theoretically prevent the development of resistant quasispecies.[15-17] Taken together, these facts indicate that induction therapy with increased and/or daily interferon dosages may provide potential benefit in terms of long-term sustained response, although there is currently little prospective data to support this concept.

Induction therapy with interferon alone or in combination with ribavirin may thus provide a potential therapeutic strategy that may improve sustained response rates in individuals who are historically difficult to treat. These include patients infected with HCV genotype 1, and those with high viral load.

The aim of this study was to attempt to estimate the duration of daily combination therapy that would theoretically be necessary to achieve the maximum number of patients that have undetectable serum viral levels by 12 weeks of therapy.

6.3 MATERIALS AND METHODS

We collected data from a prospective 4-week trial of viral kinetics in 65 patients receiving initial treatment with interferon plus ribavirin. There were four groups of patients included in the study. The baseline demographics of the 4 patient groups are shown in table 1. The groups were well matched according to these baseline characteristics. There were 13 to 19 patients in each group. For the initial four weeks of this study the patients in these 4 groups received either interferon 3 million units (MU) subcutaneously tiw, daily doses of 3 MU of interferon plus ribavirin, 5 MU of interferon daily plus ribavirin, or 10 MU of interferon daily plus ribavirin, respectively. ribavirin was given orally in divided doses of 1,000 mg (weight < 75 kg) or 1,200 mg (weight >75 kg).

All patients had compensated liver disease and documented chronic HCV infection by hepatitis C antibody testing, histologic findings consistent with chronic hepatitis C and the presence of serum HCV RNA. Clinical, laboratory, and histologic evaluation excluded other causes of chronic liver disease. The Institutional Review Board of each participating center approved the protocol. The patients had not received prior treatment for their chronic hepatitis C infection, and all were infected with HCV genotype 1, and had high viral load prior to therapy defined as greater than 1 million copies/mL. None of the patients had histologic findings compatible with cirrhosis. Although these patients were not matched at enrolment, by virtue of the fact that they were chosen according to these characteristics, they were relatively homogenous.

6.3.1 Detection of Serum HCV RNA

Serum samples were collected frequently within the first 4 weeks of treatment (t=0, 0.5, 1, 3, 5, 7, 10, 14, 17, 21 and 28 days after treatment initiation). A quantitative multicycle RT-PCR assay was performed at the National Genetics Institute (NGI, Los Angeles, California), as previously reported.[18] The sensitivity of this assay is 100 copies/ml with an intra sample coefficient of variation of 26%. All samples were tested blinded and in duplicate.

6.3.2 HCV Genotype Determination

HCV genotyping was carried out on biotin-labelled PCR products by hybridization to oligonucleotides directed against the variable region of the 5' UTR, and then immobilized as a parallel line on membrane strips (Line Probe Assay, InnoLiPa HCV II, Innogenetics, Belgium) (19).

6.3.3 Statistical and Mathematical Modelling

The SPSS software package (SPSS Inc., Chicago) was used to perform linear regression of the log-transformed data from these patient groups. We log transformed the viral load data on each patient, calculated the geometric mean for each treatment group, and created linear regression lines. (Because viral loads fall many orders of magnitude when effective therapy is given, log transformation is used so that data at high and low viral loads have more equivalent weight in the linear regression analysis.) First, we included data from both the early first phase of viral decline, as previously reported[5-7] to observe the linear regression of phase one and two combined. Second, we included only data from the second phase (viral load data after 48 hours after treatment initiation) to test the hypothesis that the slope of the second phase alone can be used to predict treatment duration until viral negativity is reached, and to test whether the second phase of viral reduction follows an exponential decline as previously described.[5]

Using this software, 95% confidence intervals were also calculated for these linear regression lines for the four groups. Mathematically, the model is very simple: $y = at + b$, where t is treatment time, y = log transformed viral load data, a = the calculated slope of viral decline and b = log transformed viral load at t=0. The linear regression and prediction interval lines were extrapolated beyond the four weeks of the study to the lower limit of detection of the HCV RNA assay (less than 100 copies/mL).

We made two assumptions that we felt were theoretically reasonable and necessary. First, that the data would follow a biphasic exponential decline or equivalently that the log transformed data would follow two straight lines consistent with published data.[7] Second, that projecting the line corresponding to the second phase to the y intercept or level of viral detection will provide an estimate of the duration of daily therapy necessary to achieve viral negativity. Since induction therapy ended at 4 weeks, this assumption is equivalent to assuming that the rate of HCV decline will remain constant

when patients are switched to maintenance therapy. If the rate of decline slows, such as if there was a third or multiphasic response, then our estimates will be minimal estimates of the time needed to reach negativity.

6.4 RESULTS

The four patient groups were well matched prior to treatment for age, sex, ALT values, HCV RNA concentrations and severity of liver disease (table I). The individual regression plots with 95% prediction intervals indicated are shown in figure a-d for the 4 treatment regimens. The correlation coefficient (r) for the combined log transformed data is high for the daily 5 MU and daily 10 MU treated groups (.92), but much lower for the daily 3 MU (.72) and 3 MU tiw (.52) group. Combining the individual plots (table 2) shows that the estimated time to reach viral negativity is progressively shorter when the treatment dosage is increased. Only the treatment schedules using 5 MU and 10 MU interferon daily show a 95% prediction of viral negativity before week 12. The prediction interval for the 3 MU three times weekly interferon monotherapy group was too wide to make a reasonable prediction of the estimated time to reach viral negativity in this group, consistent with the poor clinical experience with this dosing in these patients.

Figure 1 A-D

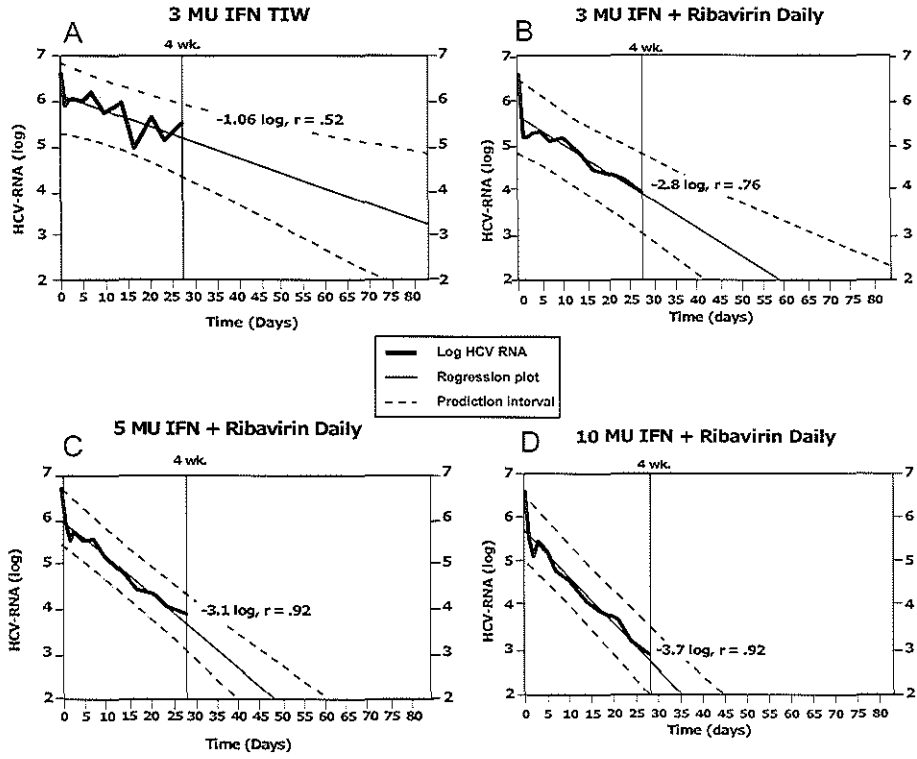

Figure 1 A-D: Represents the calculated linear regression lines and 95% confidence intervals for the patient groups that received interferon 3MU tiw (A), interferon 3MU daily plus ribavirin (B), interferon 5MU daily plus ribavirin (C) and interferon 10MU daily plus ribavirin (D) for the first 4 weeks of therapy respectively. The data were calculated using SPSS software of the log-transformed viral load data from each patient, the geometric mean for each treatment group at each time point, and then we created linear regression lines and confidence intervals. Data shown for each of the groups includes both phases of viral decline.

Table 1: Baseline Characteristics

	3 MU IFN TIW	3 MU IFN/QD + Riba	5 MU IFN/QD + Riba	10 MU IFN QD + Riba
N	13	15	18	19
Age (years)	48	41	45	41
Sex (M/F)	8/5	11/4	11/7	14/5
ALT (IU/L)[a]	101	115	92	100
HCV RNA[b]	3.9×10^6	3.6×10^6	5.1×10^6	4.2×10^6

[a]ALT - normal range 0- 45 IU/L

[b]HCV RNA by RT-PCR, expressed as copies per ml serum. Detection limit < 100 copies/ml.

Table 2: Estimated time to reach viral negativity using combined data from the first and second phases

	3 MU IFN TIW	3 MU IFN/QD + Riba	5 MU IFN/QD + Riba	10 MU IFN QD + Riba
Log Reduction at week 4 (observed)	-1.06	-2.8	-3.1	-3.7
Negative by 4-wk	0/13 (0%)	3/15 (20%)	5/18 (28%)*	7/19 (37%)*†
2-log reduction at 4-wk (99%)	2/13‡ (15%)	9/15‡ (60%)	12/18‡ (66%)	15/19‡ (79%)
Linear regression (r)	.52	.76	.92	.92
Estimated time to negativity (days)	>84	65	52	36
95% CI (days)	78->84	42->84	39-60	25-45

*: $p<0.05$ (3 MU TIW vs 5 MU QD and 10 MU QD)

†: $p<0.05$ (3 MU QD vs 10 MU QD)

‡: $p<0.05$ (3 MU TIW vs 3 MU QD, 5 MU QD and 10 MU QD)

Results at week four of treatment also showed a faster decline in viral load with increased interferon dosage (Table 2). A significant difference in viral negativity at week 4 was observed between the 2 high dose (5 and 10 MU) daily dosing groups and the standard 3 MU IFN tiw group, and between 3 MU IFN plus ribavirin daily and 10 MU IFN plus ribavirin daily (Table 2).

The data for the second phase only (regression and confidence interval) did show that viral reduction followed an exponential decline and a dose -dependent response only for the daily interferon groups. Again a significant difference was observed in the drop in viral load within the observed 4 weeks for the daily 3 MU interferon plus ribavirin group compared with the daily 10 MU interferon plus ribavirin group (P = 0.025) (Table 3).

Table 3: Estimated time to reach viral negativity using only data from the second phase of viral decline (day 2-day 28)

	3 MU IFN/QD + Riba	5 MU IFN/QD + Riba	10 MU IFN QD + Riba
Log reduction at wk 4	-1.46*	-1.63	-2.25*
Linear regression (r)	.96	.95	.95
Estimated time to negativity (days)	57	52	37
Days (CI) 95% CI (days)	(52-64)	(46-58)	(33-42)

* p<0.05 3 MU IFN/QD + Riba vs 10 MU IFN QD + Riba

All other comparisons not significant

6.5 DISCUSSION

With the introduction of combination therapy with interferon alpha-2b plus ribavirin, the chance of achieving a sustained virologic response has dramatically increased for chronic hepatitis C patients. However, patients with genotype 1 infection and a high viral load (defined as higher than 1-2 x 10^6 copies/ml) still have a limited chance of achieving a sustained response.[11,20,21] Even though not all patients that become sustained responders show a rapid disappearance of HCV RNA,[11] one of the strongest predictors of a sustained viral response in the majority of patients is still the early (within 12 weeks) clearance of serum HCV RNA. This rapid HCV RNA clearance will not be reached in the majority of HCV genotype 1 patients with a high viral load. Thus, improved treatment options for these patients are necessary.

To assess the efficacy of higher dose interferon strategies on the initial response we used a linear regression model that described the overall change in viral load for groups of patients. Various models have previously been used to describe the decline in viral load during treatment.[7,8,22] To compare the efficacy of different treatment regimens within a group of standardized patients we calculated the geometric mean value of the viral load in each group at each time point. Linear regression of the geometric means for each time-point of HCV RNA measurement provided a line that described the average slope of viral decline in each group of patients. Since we only included genotype 1 infected patients with high viral load (although the patients were not matched for other variables), the differences in the calculated slopes of viral decline are most probably attributable to the differences in treatment that these groups received.

We propose that both the rapid and the slower phase of viral decline[5,7] are important for reaching viral negativity: a patient with an extremely large first phase viral decline will reach undetectable levels of HCV RNA much earlier than someone with a minimal first phase viral load decline, assuming both have the same second phase of viral decline. Since it is rare for a patient to reach viral negativity during the first phase, the slope of the second phase will ultimately determine if someone reaches negativity. Since the second phase generally includes all data from the second day of treatment onward, use of only second phase data has been shown to provide a good prediction

for viral negativity of individual patients at 12 weeks[7] and thus should be sufficient to determine the expected duration of daily therapy to reach viral negativity.[5]

The linear regression model that we have proposed can be used with the combined data of both phases or with data of the second phase alone. In our study, two measurements of HCV RNA before 2 days of treatment (0.5 and 1 day) and 8 points of the slower second phase were included in the overall model, leading to a prediction interval that did not differ significantly from an evaluation including only the second phase. Both analyses showed a more rapid viral decline with higher doses of interferon. Whilst combining both phases in the predictive model produced a more conservative estimate of the interval required to clear HCV RNA, data obtained using the second phase only may be more clinically relevant, and more readily available to practicing clinicians.

The first phase of viral decline has previously been shown to be exponential and dose dependent.[6] This study is the first to show a clear dose-dependent and exponential decline in viral load also during the second phase of viral decline. A significantly faster mean second phase slope was observed, even in this small study, in the group treated with 10 MU interferon plus ribavirin daily than in the group treated with 3 MU interferon plus ribavirin daily. This model – when applied to our data – strongly suggests a dose dependent efficacy of interferon in genotype 1 patients and clearly shows the inefficacy of interferon monotherapy when given as 3 million units IFN thrice weekly for this group of patients. Since this study was designed to show differences between different treatment schedules, it is not clear that this method for determining the necessary daily treatment period can be used for individual patients. This would require further prospective evaluation.

A large number of patients reaching early viral negativity theoretically allow for a high rate of end of treatment response, provided that breakthrough does not occur when interferon dosage is decreased. Unfortunately, this study used data from a 4-week treatment schedule and therefore the effect of a faster viral decline on sustained response could not be examined. Within the observed 4 weeks of the study the previously reported biphasic decay in viral load[7] was confirmed in our study. Whilst the possibility exists that a further slower phase of viral decline occurs after these initial two phases observed in many studies, to date there is no published data to support the possibility of a multiphasic decline beyond this period. Because our study was limited

to the first 4 weeks of therapy we cannot rule out that the rate of viral decline slows or is altered at later time points. If this latter scenario applies, our estimates would be minimal estimates of the time needed to reach negativity, and thus may not predict long term outcome after therapy.

The failure of the standard tiw regimen to achieve negativity before week 12 is consistent with the poor response of this regimen in the genotype 1 patient population. However, the 95% prediction interval of the 3 MU daily IFN also fell outside the 12 week "window" suggesting that this regimen, even though it appears much better than the standard tiw regimen, will be insufficient to maximize the number of patients achieving viral negativity early in therapy. These daily dose regimens may be superseded by the introduction of newer, longer acting forms of interferon and thus will likely not be used extensively clinically. However, this model could be used to determine the initial doses of new therapies to maximize the chances of success.

What are the implications of this study for the clinician? The most important factors identified to influence response to treatment with interferon plus ribavirin in two large multicenter studies were HCV genotype and viral load.[11,21,23] Other factors including the degree of histologic fibrosis, gender, body weight and age were less predictive of response, but also identified by stepwise logistic regression analysis.[11,21,23] In a clinical setting with an individual patient, information regarding HCV genotype is important, since it is generally recommended that genotype non-1 infected patients require only 24 weeks of combination therapy, and those with genotype 1 receive 48 weeks. Because of the fluctuations in viral burden seen in untreated patients and the numerous HCV RNA assays, which are currently not standardized and have variable sensitivities, viral load has a more limited clinical value. It is thus difficult to make recommendations regarding outcomes according to viral load reductions during therapy and baseline values. Because the majority of chronic hepatitis C patients have an unfavourable profile and are in need of more effective therapy, we have attempted to show in this study that for these patients, the dose of interferon when combined with ribavirin and the dosing interval are also factors which can influence early clearance of serum HCV RNA.

In conclusion, after log-transformation of viral load data, a simple linear regression analysis can be used to compare the kinetics of viral decline between treatment strategies. Interferon monotherapy at .a dose of 3 MU tiw is predicted to be insufficient

to lower the viral load below the limit of detection within 12 weeks in genotype 1 patients with high pretreatment viral load. Based on extrapolation of data obtained during the first four weeks of treatment, daily dosages of 5 or 10 MU IFN daily plus ribavirin will be necessary to reach viral negativity in the majority of these difficult to treat patients. None of the treatment schedules caused such a rapid viral decline that daily treatment could be stopped before 4 weeks.

New studies should focus on higher doses of daily interferon or longer acting interferon preparations and/or the combination of interferon and ribavirin in order to improve the low response rate in genotype 1 patients. Prospective studies to assess the efficacy of initial daily interferon therapy plus ribavirin for 24 weeks are currently in progress. The simple model used in this study might be helpful in comparing future treatments in order to evaluate the differences in efficacy for this difficult to treat patient population.

6.6 ACKNOWLEDGEMENTS

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Chapter 7

Hepatitis C Viral Kinetics In Difficult To Treat Patients Receiving High Dose Interferon And Ribavirin

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7.1 ABSTRACT

Background. Hepatitis C viral kinetics show an increased antiviral effect of higher than standard interferon dosages and of daily treatment schedules. Since interferon has a short half-life, twice-daily administration of interferon may be even more effective.

Methods. We evaluated HCV kinetics in daily versus twice-daily high dose interferon (IFN) therapy in combination with ribavirin in 24 difficult to treat patients. Patients were randomised to 10 MU IFN daily or 5 MU twice-daily for 4 weeks.

Results. Interferon efficacy (ε) was similar and very high for both groups (range 99.83-99.97%). Clearance of infected cells (beta phase) tended to be slightly faster for patients on 5 MU bd (T1/2 70 vs 90 hrs, ns). Clearance of infected cells was strongly related to initial viral load (T1/2 103 vs 53 hrs, p=0.002, for above versus below $2x10^6$ copies/ml). An additional phase with a temporary rise in viral load was observed between the alpha and the beta phase.

Conclusion. daily high induction dose is associated with nearly complete inhibition of viral replication even in difficult to treat patients. A twice-daily schedule did not lead to further improvement. Clearance rate of Infected cells was significantly correlated with initial viral load.

7.2 INTRODUCTION

Viral kinetic studies have demonstrated the increased antiviral effect of higher than standard dosages of interferon and of daily versus thrice weekly treatment schedules. The anti-viral effect can be described by a fast initial decline, reflecting the efficiency of inhibition of viral replication, and a slower second phase presumably related to the clearance of infected cells. Since interferon has a short half-life of only about 8 hours, twice-daily administration of interferon may be more effective.
With standard doses of interferon, even in combination with ribavirin, about 50% of patients do not achieve a sustained remission [1-3]. These difficult to treat patients are mainly identified by the pre-treatment characteristics genotype 1 virus infection, high viral load and presence of cirrhosis. In this group of patients we analysed whether twice-daily treatment could increase the inhibition of viral replication compared to daily dosing. In addition, the effect on the other parameters of the viral dynamics were studied, especially the effects on the clearance of infected cells, using both individual as well as group-based methods.

7.3 MATERIALS AND METHODS

7.3.1 Patients

Twenty-four difficult to treat chronic hepatitis C (detectable HCVRNA for longer than 6 months) patients were included in this trial; 21 with a previous non (-sustained*) response and 3 without previous treatment but having a liver cirrhosis. Standard exclusion criteria were applied with regard to contraindications to interferon and/or ribavirin, immune suppression, concomitant other causes of liver disease, other severe diseases, or clinical signs of decompensated liver disease (ascites, variceal bleed, encephalopathy) [4]. However, the threshold for bilirubin level and platelets count was adjusted to twice the upper limit of normal and 50.000 /mm^3, respectively; this adjustment was made to allow inclusion of patients with more advanced liver disease, the group which needs treatment the most.

* Non (-sustained) response: nonresponse (no HCV RNA negativity during treatment) or relapse (HCV RNA negativity during treatment followed by a relapse of HCV RNA after treatment)

7.3.2 Randomization

After informed consent was signed, consecutive patients were assigned a treatment schedule based on a computer-generated list by an independent person of the research co-ordinating centre.

7.3.3 Treatment schedule

After a first induction dose of 10 MU, group one continued with daily 10 MU IFN for 4 weeks while group 2 continued with 5 MU every 12 hours for 4 weeks **. Ribavirin was given twice daily orally in divided doses of 1,000 mg (weight < 75 kg) or 1,200 mg (weight ≤ 75 kg).

7.3.4 Measurement of serum HCV RNA

Blood samples were taken frequently during the first 4 weeks (T= 0, 8, 24, 32, 48, 56, 72, 96 hours and at week 1,2,3 and 4) of treatment. Blood was collected in (PPT) tubes (Bacton-Dickerson) which were centrifuged directly after collection in order to minimise RNA breakdown [5].

After centrifugation, the tubes were transported within 72 hours to the central virology department where plasma was aliquotted in separate tubes that were stored at -80°C. Viral load was quantified by NGI (Culver City, Los Angeles, CA, USA), using a quantitative multi-cycle RT-PCR assay [6]. The sensitivity and linearity of the assay has been validated for genotype 1 and 3 with the use of the Eurohep standard series [7]. Qualitative HCV RNA determination was performed in-house, using the Roche Cobas Amplicor assay. The lower detection limit for both assays was similar and less than 10^3 copies/ml.

7.3.5 Mathematical modelling and biostatistics

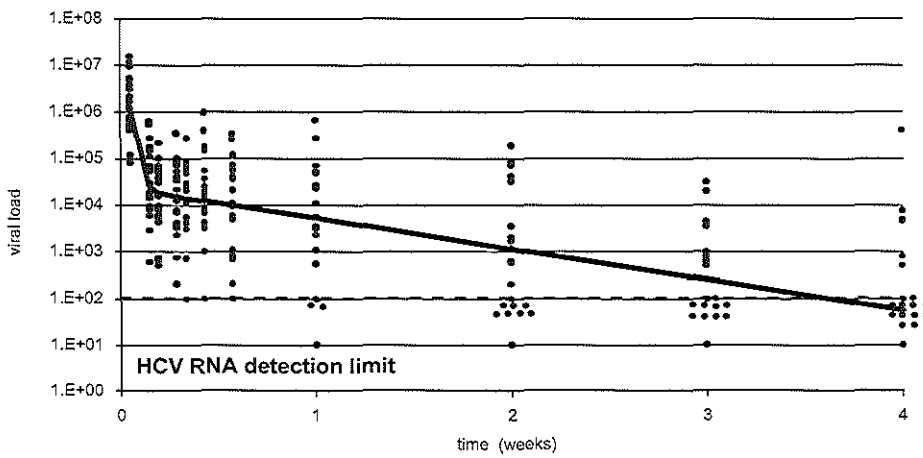
We analysed the data according to the bi-phasic mathematical model of Neumann et al. [8], using a biostatistical mixed model with random effects (Macro NLINMIX in SAS 6.12 software). The benefit of this modelling technique is that the entire data set can be analysed and that an overall population curve can be generated (Figure 1). Since this technique can be applied to an entire group, it is possible to analyse the effect of patient characteristics or treatment modulations on the viral kinetics parameters (Figure 2).

**All patients completing 4 weeks of treatment were rolled over in an associated study to determine the durability of an early virological response during maintenance therapy and after stopping treatment.

In addition, the viral dynamic data were calculated for each individual as described previously by Neumann using Proc NLIN in SAS 6.12.

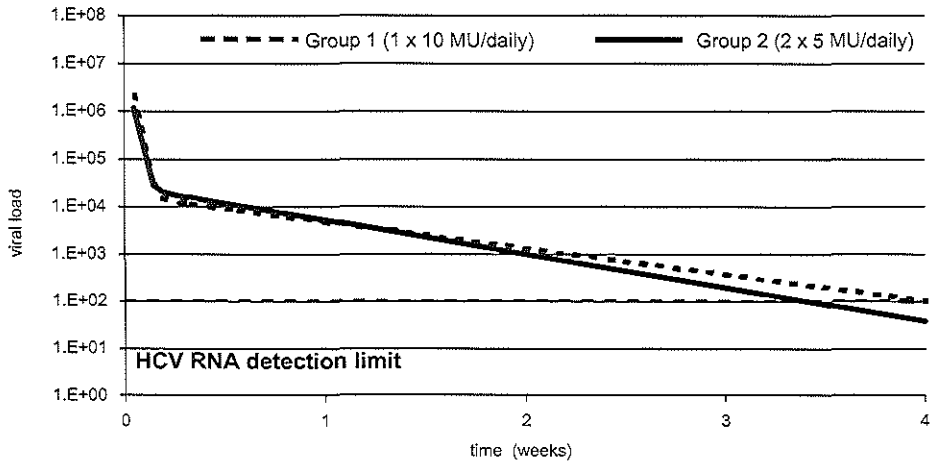
In accordance with guidelines recently proposed by a European collaborative group on viral dynamics (Ditto-HCV workshop, Paris 1999), patients were only included for viral dynamics analysis if a minimum of quantitative HCV-RNA data were available (T=0, at least one value within the first 2 days of treatment and at least 3 quantitative measurements between day 2 and day 28). Differences between pre-treatment characteristics (Kruskal-Wallis test) and HCV RNA negativity at week 4 (Fisher-exact test) were calculated with SPSS version 9.0 software.

Figure 1 Estimated viral decline using the viral load data of all patients



The closed line is the mathematical estimation of the overall viral decline, based on the mathematical model of Neumann et al. (1998) [8] and a new statistical analysis method (macro NLINmix in SAS software). All actual measured viral load data are showed as dots. Viral load data below the detection limit of the quantitative assay are displayed as dots at the level of the detection limit (10^2 copies/ml).

Figure 2



Estimated viral decline can be calculated for sub groups separately by using a statistical mixed model analysis. In this figure the separate lines for group 1 (receiving 10 MU IFN/daily; striped line) is plotted together with the estimated decline for group 2 (receiving twice daily 5 MU IFN; solid line). No significant difference in viral decline is present between these separate groups (see also table 3)

7.4 RESULTS

The pre-treatment patient characteristics are shown in table 1. As shown, both groups were balanced according to baseline patient characteristics. Most (21/24) patients were non-sustained responders to previous interferon treatment. Six out of 24 patients (25%) fitted our criteria of more advanced liver disease, having elevated bilirubin levels and/or platelet counts below 100.000 /mm^3.

Table 1: Pre-treatment patient characteristics.

Group		All patients	10 MU/d	5 MU/b.i.d
Number		24	12	12
Male		17	8	9
Age	mean median	46.5 47.5	43 44	50 50.5
Previous NR/relapse/naïve		14/8/2	7/4/1	7/4/1
ALT baseline (x ULN)	mean median	2.7 2.6	2.6 2.7	2.9 1.9
Pre-treatment HCV RNA* (copies/ml)	mean median	1.5×10^6 2.6×10^6	8.3×10^5 3.0×10^6	2.6×10^6 2.2×10^6
Genotype 1		11	7	4
Cirrhosis		11	4	7

- Mean and median viral load is calculated on the log-transformed viral load data

Out of the 24 patients, two stopped early after treatment initiation (<10 days): one because of non-compliance and one because of cardiac complains. Out of the 22 patients that completed the first 4 weeks 17 (77%) became HCV RNA negative by qualitative PCR (Table 2). The number of patients that reached PCR negativity within 4 weeks did not differ between the 2 groups of therapy. Genotype 1 and cirrhosis were patient characteristics that appeared to correlate negatively with HCV RNA response at week 4, but the only pre-treatment factor that correlated significantly with viral response was a low viral load (less than 2×10^6 copies/ml).

Viral dynamics parameters could be calculated in 20 patients as two patients reached HCV RNA negativity within 24-48 hours after treatment initiation, providing insufficient viral load measurements for viral dynamics analysis.

All 20 evaluable patients showed a fast drop in viral load between 8 and 32 hours after treatment initiation followed by a second slower phase in viral decline. Analysis of the viral dynamics showed a high interferon efficacy in all patients (range: 99.83 – 99.96%). Patients with unfavourable baseline characteristics (cirrhosis, genotype 1 infection, high viral load or previous nonresponse) had a similar rapid first phase viral decline as the others. However, patients with cirrhosis, a high viral load and genotype 1 tended to have a slower second phase viral decline (Table 3).Only for the subgroup of patients with a low viral load (cut-off < 2×10^6 copies/ml) the clearance of infected cells was significantly more rapid (p=0.002).

Analysis of the individual viral dynamics showed similar results, with a median blocking efficacy of 99.88% and a half-life of infected cells of 67 hours (Table 4).

Hand plotting of the viral load data suggested that the actual data did not completely follow a bi-phasic decline. To validate this observation we analysed the data without trying to make it fit into a bi-phasic model. A significant positive deviation from the calculated bi-phasic curve was observed between 32 till 120 hours after treatment initiation (Figure 3). In all 20 evaluable patients, a short rise in viral load followed the initial decline, creating a hump in the viral decline. This short rise in viral load was observed when sera were analysed by the NGI assay as well as by using the Cobas Amplicor assay (95% of the data comparable).

Table 2: HCV RNA negativity at week 4 of treatment.

Variable		PCR negativity at week 4 (Intention To Treat analysis)	PCR negativity at week 4 (Per Protocol analysis)
ALL	70 MU IFN/week	71% (17/24)	77% (17/22)
Schedule	1 x 10 MU daily	67% (8/12)	73% (8/11)
	2 x 5 MU daily	75% (9/12)	82% (9/11)
Previous treatment	Nonresponse	64% (9/14)	69% (9/13)
	Relapse /no treatment	80% (8/10)	89% (8/9)
Genotype	1	45% (5/11) *	56% (5/9) *
	non-1	92% (12/13) *	92% (12/13) *
viral load	$> 2 \times 10^6$	50% (7/14) †	69% (7/12) †
	$< 2 \times 10^6$	100% (10/10) †	100% (10/10) †
Cirrhosis	Yes	56% (6/11)	60% (6/10)
	No	85% (11/13)	92% (11/12)

* ITT analysis p=0.02, PP analysis p=0.12

† ITT analysis p=0.02, PP analysis p=0.04

Tabel 3: Estimated viral dynamics parameters by statistical analysis of all patients combined and by subgroup analysis.

	Initial load (Copies/ml) **V0 †**	Virion clearance (1/day) **c**	Virus half-life (hours) $T_{1/2}$	Efficacy (percent) **ε**	Infected cell death (1/day) **δ**	Infected cell half-life (hours) $T_{1/2}$
All	6.17	7.9	2.1	99.89	0.22	77
Group 1	6.31	7.4	2.2	99.93	0.18	90
Group 2	6.06	7.4	2.2	99.81	0.24	70
Previous NR	6.19	7.1	2.4	99.83	0.22	75
Others(RR/no Rx)	6.12	10.3	1.6	99.96	0.21	79
Genotype 1	6.44	7.9	2.1	99.89	0.18	91
Genotype non-1	5.95	7.9	2.1	99.88	0.24	70
High viral load*	6.41	7.8	2.1	99.90	0.16 ‡	103‡
Low viral load	5.82	8.4	2.0	99.89	0.31 ‡	53‡
Cirrhosis (+)	6.13	9.5	1.7	99.88	0.17	99
Cirrhosis (-)	6.19	7.8	2.1	99.92	0.25	68

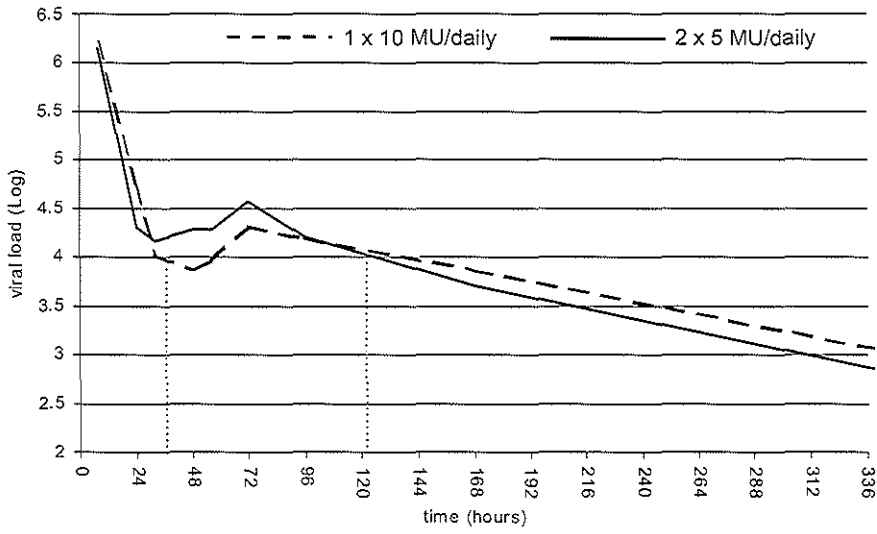
†: Initial viral load has been calculated with the assumption of an average 8 hours lag phase in viral decline
‡: p=0.0017

Tabel 4: Calculated individual viral dynamics parameters using the model of Neumann et al.

	Initial load (Copies/ml) **V0 †**	Virion clearance (1/day) **c**	Virus half-life (hours) $T_{1/2}$	Efficacy (percent) ε	Infected cell death (1/day) δ	Infected cell half-life (hours) $T_{1/2}$
All (median)	7.19	7.38	2.25	99.88	0.23	67
1	6.98	5.19	3.21	97.82	0.39	43
2	6.66	5.92	2.81	99.64	0.35	47
3	7.75	5.04	3.30	99.79	0.25	66
4	6.77	4.31	3.86	99.02	0.06	264
5	6.11	5.99	2.78	99.81	0.35	47
6	7.25	6.36	2.62	99.09	0.44	38
7	7.41	5.23	3.18	96.43	0.15	108
8	7.19	8.01	2.08	99.93	0.14	121
9	6.27	3.43	4.85	98.13	0.42	40
10	6.67	4.01	4.15	98.44	0.18	92
11	9.12	7.95	2.09	99.86	0.26	64
12	7.25	6.90	2.41	99.69	0.17	97
13	8.63	5.82	2.86	99.40	0.23	73
14	7.54	2.05	8.10	88.13	0.35	48
15	7.42	4.12	4.04	99.11	0.05	344
16	No fit	-	-	-	-	-
17	7.72	3.72	4.47	99.69	0.15	114
19	6.42	13.86	1.20	97.31	0.02	673
21	6.96	7.22	2.30	99.89	0.19	86
22	6.28	6.14	2.71	99.94	0.18	90

Patients No. 18 and 20 discontinued study medication due to non-compliance and side-effects.
Patients No. 23 and 24 could not be included due to lack of sufficient samples.
Patient No. 16 showed a rise in viral load; no correct individual fit could be obtained.

Figure 3 nonlinear curve fitting



In this figure, both group one and group 2 data is plotted without using a model that dictates a bi-phasic decline.
The linear rapid first phase and the linear slower second phase of viral decline are confirmed in this figure. However between these two phases a third phase can be observed that can not be explained in a bi-phasic model. Both for each patient separately (data not shown) as for the combined data shown in this figure a hump like curve can be plotted using the data between 32 hours and 120 hours after treatment initiation.

7.5 DISCUSSION

With standard interferon-ribavirin therapy, about 50 percent of patients do not achieve a sustained response [2]. Viral characteristics are the key factor in nonresponse (high viral load and genotype 1) [2,9], and explanations are sought by suggesting resistant quasispecies strains or mutations in the NS5A region [10]. In this study we treated a group of difficult to treat patients by a high initial interferon dose (10 MU). Our results show that high rates of viral suppression (>99% IFN efficacy) can be achieved in the large majority of patients, therefore the concept of interferon resistance is rather dose related than an intrinsic characteristic of the virus.
This efficacy is comparable to that in a previously reported study in treatment naïve patients who also received 10 MU interferon [8]. In that study, the efficacy was calculated for each individual separately. Our data are based on a group analysis that includes all data. When we analysed our data by using the individual method [8], the calculated median efficacy of blocking viral production was equally high (99.9%). No individual difficult to treat patient had an efficacy of lower than 88% which challenges the concept of intrinsically resistant HCV to interferon and ribavirin.
Our statistical method of group analysis is particularly suitable for group comparison and an additional tool for analyses of viral dynamics [11], in particular for analyses of (sub)-groups.
In this small randomised controlled trial the interferon efficacy was 99.9 versus 99.8% for the group with once daily treatment (group 1) versus twice daily (group 2). In addition, PCR negativity at week 4 did not show a significant difference (67% versus 75% for group 1 versus group 2 respectively). Therefore, the hypothesis that twice-daily dosing would be more effective could not be supported. In view of the unexpected high efficacy found for interferon in this difficult to treat patient group and the high rate of PCR negativity at 4 weeks with once daily treatment, the lack of an additional effect of twice daily treatment comes less as a surprise.

Biostatistical analysis showed that a high viral load (>$2x10^6$ copies/ml) was the only pre-treatment characteristic that had a significant correlation to a slower second phase viral decline ($T_{1/2}$ of 103 versus 53 hours for high versus low viral load respectively, p=0.002). This negative correlation, previously reported by Neumann et al., might reflect the pre-treatment immune reactivity of the host. Patients with a low

pre-treatment immune response against HCV tend to have a higher HCV RNA load, presumable due to a low spontaneous clearance rate of HCV infected hepatocytes. The observation that the immune status is reflected in both a high viral load and a slow second phase viral decline can explain the reported low sustained response rate in patients with a high viral load. The predictive value of early kinetics for sustained response has not been proven. However, results of this study make us believe that early viral kinetics yields more information for the prediction of sustained response than viral load alone.

The main difference between the 2 methods of viral dynamics analysis was found in the estimation of the interferon efficacy. The mixed model analysis estimated a very narrow range for the interferon efficacy. An overall interferon efficacy of more than 99% was estimated. If we compare this with the individual calculations, we find that for most cases, this very high efficacy can be confirmed, but in a few patients the interferon efficacy was found to be much lower (as low as 83%). The strength of the individual method is the accurate mathematical description of the viral decline where the strength of the mixed model analysis is in the comparison of groups.

Analysis of the individual curves of viral decline revealed that a true bi-phasic decline does not fully describe the fall in viral load. An additional phase between the currently identified phases was observed in all evaluable patients. The pattern of this additional phase could be described as a hump in the viral load curve. In retrospect this additional phase could also be observed in data of others [12]. Several possible mechanisms may be accountable for this observation. First, the efficacy of interferon may change after the first day due to down regulation of interferon receptors. If the immune-mediated second phase does not appear before 2-3 days after the first injection, a lag-period of diminished viral suppression is present. Second, the emergence of a viral species for which interferon has a lower efficacy might be another explanation for the hump in viral decline; since the hump was observed in all patients the second explanation is less likely. Research on viral quasispecies, interferon receptors and viral load decline in the liver are necessary to explain convincingly the hump in the viral load curve.

In conclusion, we have demonstrated a high interferon efficacy (>99%) can be reached in difficult to treat patients with a 4-week daily high dose of alpha-interferon, resulting in undetectable virus levels in 77% of the patients. There was no significant difference in viral dynamics parameters between patients receiving twice daily 5 MU interferon or once daily 10 MU interferon. A high clearance of infected cells was found in those patients with a low pre-treatment viral load, reflecting the key role of the host immune activity in the clearance of the virus. Lastly, a hump in the viral load curve was observed in all patients between the already known first and second phase, which calls for a further adjustment of the current viral dynamics models.

7.6 ACKNOWLEDGEMENTS

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Chapter 8

High Sustained Virological Response In Difficult To Treat Hepatitis C Patients By Combination Of Induction And Prolonged Maintenance Interferon-Ribavirin Therapy

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Submitted

8.1 ABSTRACT

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

Methods Twenty-four patients were included in this study with either genotype 1 infection, cirrhosis, high viral load or previous non-response to anti-viral treatment. Patients were treated with 10 million units (MU) of Interferon (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 of less than 500 copies/ml.

Findings Both intention to treat (ITT) and per protocol (PP) analysis showed a high sustained virological response in this difficult to treat population (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. (Patients with advanced cirrhosis had the lowest response rate (50%) and high side-effect rate).

Interpretation With this new treatment regiment that combines induction-, daily- and prolonged treatment it seems possible to cure chronic hepatitis C in the majority of patients that are considered difficult to treat, except those with advanced cirrhosis. Further clinical evaluation of this intensive interferon and ribavirin combination therapy is recommended in centres which can provide close patient monitoring and experienced hepatological support.

8.2 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]. Still, with the current recommended interferon-ribavirin combination therapy, a sustained clearance of the virus is observed in only 40-50% of patients [3-5].

For patients that have unfavourable patient or virus characteristics (cirrhosis, genotype 1 or 4, high viral load) the sustained response rate is much lower and ranges between 17-28% [3-6].

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

This study focuses on patients with a low chance of response to the current recommended schedule of combination therapy. We altered the 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 [7], followed by continued daily interferon and ribavirin combination in order to minimise break-through; 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 [8].

8.3 MATERIALS AND METHODS

8.3.1 Study population

Twenty-four patients with chronic hepatitis C, documented by hepatitis C antibody testing, the presence of plasma HCV RNA and histological findings consistent with fibrosis or cirrhosis, were included in this study. Other clinical relevant concomitant diseases were excluded as previously described [9].

All patients could be classified as "difficult to treat ", based on their baseline characteristics. Twenty-two patients (92%) were either non-responder to Interferon monotherapy or relapsers to combination therapy; 2 patients were treatment-naive cirrhotics. Of the total group 11 (46%) had cirrhosis, of whom 7 could be classified as advanced cirrhosis (Bilirubin >17 µmol/l or platelets $<130x10^6/mm^3$) [10]; thirteen

(46%) had a genotype 1 or 4 [11] infection and the mean viral load in the total group was more than $4x10^6$ copies/ml. Nineteen patients (79%) had two or more unfavourable baseline characteristics.

8.3.2 Treatment schedule

All patients were admitted for the first 7 days of treatment and thereafter intensively monitored at the out-patient clinic. Patients received 10 Million Units of alpha-interferon (Intron A) daily for 4 weeks followed by 5 Million Units daily until week 24, 3 Million Units daily until week 52 and 3 Million Units 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.

8.3.3 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) which were spun directly after collection in order to avoid RNA breakdown. A qualitative HCV RNA assay was used to asses viremia (modified in-house Cobas Amplicor assay). The test was found to have a comparable sensitivity to the NGI assay (100 copies/ml and less than 500 copies/ml when validated by the Eurohep panel) [12].

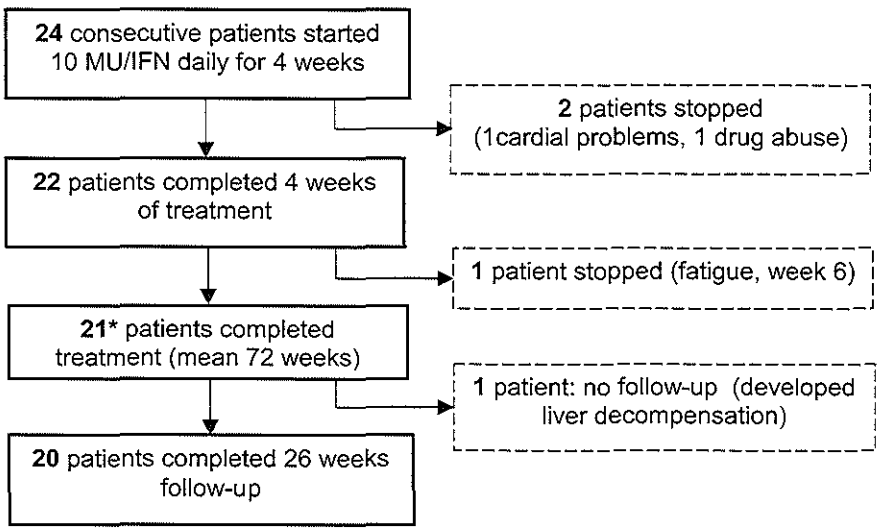
8.3.4 Descriptive analysis

The prevalence of HCV RNA negativity 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 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.

8.4 RESULTS

The intention to treat population (ITT) consisted of 24 patients, 20 completed the study according to protocol (PP) (Fig 1).

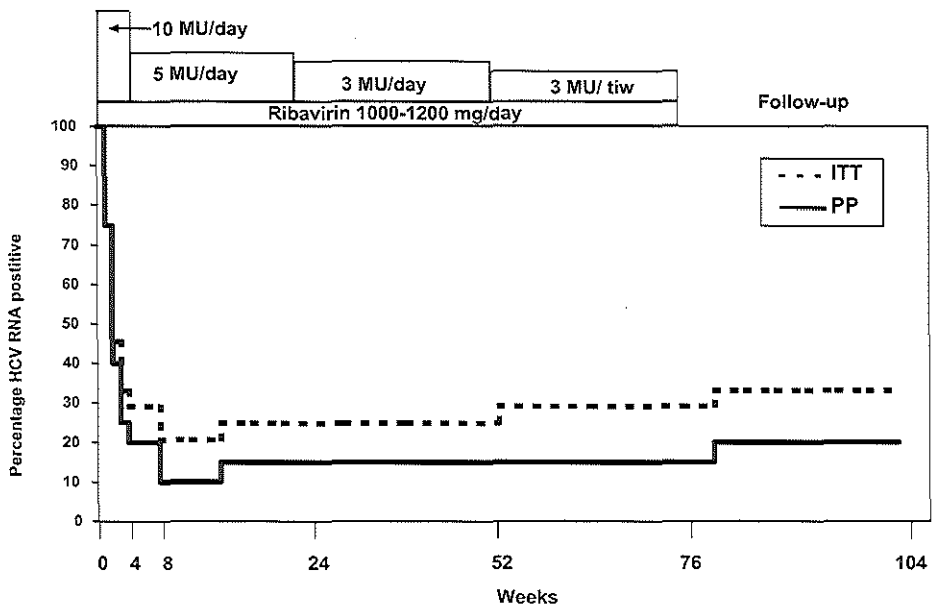
Figure 1: Trial profile



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
Note: 2 patients stopped according to protocol (non-response at week 12-16)

A high initial response rate (ITT 67%, PP 75%) coupled to an additional response of about 10% during treatment and a very low break-through of 5%, led to an end of treatment response of 75% (ITT) - 85% (PP). Due to the very low relapse rate (5%) the sustained response in this group of difficult to treat patients was 67% (ITT) - 80% (PP) (Fig 2).

Figure 2 HCV RNA response during treatment and follow-up of difficult to treat patients with chronic hepatitis C



HCV RNA response during treatment and follow-up of difficult to treat patients with chronic hepatitis C. Note the rapid disappearance of HCV RNA due to daily high dose interferon therapy, and the virtual absence of breakthrough after reduction at 1 month from 10 to 5 Mega Units. Breakthrough and relapse were only seen in 1 patient each of the per protocol cohort

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 one patient characteristic, cirrhosis, and one virus characteristic, genotype, appear to be the key predictors of a sub-optimal response. When the effect of genotype and cirrhosis was analysed by univariate analysis (fisher-exact test), the presence of cirrhosis was a significant factor for not reaching a sustained viral response.

Adverse events led to hospitalization 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 above mentioned patients prior to week 52; it did occur in other patients due to drug abuse, cardiac complaints and fatigue with depression.

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.

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	14	9 (64%)	10 (71%)	10 (71%)	13	9 (69%)	10 (77%)	10 (77%)
Relapser/naïve	10	7 (70%)	8 (80%)	6 (60%)	7	6 (86%)	7 (100%)	6 (86%)

8.5 DISCUSSION

This exploratory study shows that in difficult to treat patients with chronic hepatitis C a high rate of sustained response can be obtained, albeit at the cost of a non-neglectable incidence of clinical relevant adverse effects.

The basis for the high sustained response rate was a high percentage of HCV RNA negativity by PCR at 4 weeks of treatment associated with daily high dose induction therapy, and the very low relapse rate (<10%) due to prolonged treatment up to 1½ years. High initial response rates have been described previously by Japanese investigators [7] and low relapse rates in other clinical studies with prolonged treatment [13]. This study is one of the first in which daily high dose induction for more than 4 weeks was combined with prolonged maintenance treatment bridged by daily interferon therapy to prevent break-through.

These favourable results contrast with the 17-28% sustained response in difficult to treat patients with the current recommended therapy and those with 2-4 weeks induction therapy followed by thrice weekly maintenance therapy in combination with ribavirin for 0.7-1 year [14,15].

When a daily high dose schedule is changed to a standard maintenance schedule of 3 MU Interferon after 2-4 weeks, the percentage of HCVRNA negativity falls to about 50% at the end of treatment [16]. The duration of induction therapy needed to reach viral negativity using daily 5-10 MU combined with ribavirin is estimated to be 45-60 days in previously untreated genotype 1 patients [17].This regimen was applied in our study.

The duration of the maintenance therapy is likely another independent factor affecting the sustained response rate. Relapse rates for patients with undetectable HCVRNA are about 50% for 6 months therapy, 25% for 12 months and preliminary data indicates less than 15% for 18 months of combination therapy [8].

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 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, except for those where cirrhosis has advanced to a stage with elevated bilirubin or low platelets. Having an

effective therapy for those patients with chronic hepatitis C most likely to develop liver-failure or a hepatocellular carcinoma is of major clinical significance for shifting the current trend of progressive morbidity and mortality due to hepatitis C. Further clinical evaluation of intensive interferon/ribavirin combination therapy is recommended in centres which can provide close monitoring and experienced hepatologic support.

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Chapter 9

Changes In Anti-Viral Effectiveness Of Interferon After Dose Reduction In Chronic Hepatitis C Patients: Implications For Treatment Strategy

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Submitted

9.1 ABSTRACT

Although high dose interferon (IFN) induction treatment of hepatitis C viral (HCV) infection blocks viral production over 95%, dose reduction is often necessary due to clinical considerations. The effect of dose reduction on HCV kinetics was studied by allowing longitudinal changes in the parameters of viral dynamics. This model was used in a group of patients (N=15) with dose reduction from 10 to 3 MU of IFN daily in combination with ribavirin, in comparison to a control group (N=9) with no dose reduction. Dose reduction gave rise to a complex viral kinetic pattern, which could be only explained by a decrease in IFN effectiveness in blocking virion production. The benefit of the rapid initial decline following the high induction dose is lost after dose reduction. In addition, in some patients even the second phase viral decline slope, which is highly predictive of success of treatment, was impaired by the dose reduction resulting in smaller percentage of viral clearance in the dose reduction group. A rational strategy for a safe dose reduction is suggested based on individual patient viral kinetics.

9.2 INTRODUCTION

The hepatitis C virus (HCV) causes a slowly progressive liver disease, which may lead to cirrhosis, liver failure and liver cancer. Currently, about 10,000 patients die in the US from HCV related disease yearly and this number is expected to triple in the next 2-3 decades [1]. Anti-viral therapy is successful in arresting the progression of the disease in those patients who reach a sustained clearance of the virus, currently only 40% of treated patients [2]. Response to therapy with alpha Interferon injections thrice a week with or without additional ribavirin is thought to occur gradually over time, and research has focussed on improving efficacy by prolonging treatment up to 1 to 2 years [2-4]. However, reports on viral dynamics analysis show that response to interferon is very fast and that a 10 to 1000 fold decrease in viral load can be reached within 24 hours of treatment [5-7]. The pattern of viral decline seems to be biphasic, with a rapid viral decline within the first 24-48 hours followed by a much slower second phase of viral decline. This biphasic decline is hypothetically caused

by a direct anti-viral effect of interferon in blocking virion production from infected cells [5].

A strong dependence of the viral decline in the first phase on the dose of interferon used has been described [5,8]. Nevertheless, it has been shown that it is the second slope which is the best predictor for response to treatment [5,9,10]. This slower second phase slope of viral decline has large variability between patients, and therefore cross sectional analysis of its dose dependence is hindered. Instead, here we investigated the *longitudinal* changes in viral dynamics in patients going through a dose reduction in order to asses the effect of dose on the second slope. The current model for HCV dynamics, in which the dynamical parameters are fixed during treatment, has been proven to correctly fit the observed biphasic viral decline in patients treated with fixed Interferon dosages [5]. However, in this study we adapted the model such that the dynamical parameters can change over time due to a change in dose. Lastly, we discuss the clinical implications of changes in the dynamics and suggest strategies for managing dose reduction.

9.3 MATERIALS AND METHODS

9.3.1 Study population

All 24 HCV genotype-1 patients enrolled in our high-dose induction studies were evaluated. All patients met the inclusion and exclusion criteria previously described [11]. Note that all patients in these studies were considered "difficult-to-treat", either because they were non-responders to previous treatment or had cirrhosis and/or high baseline viral load. Group 1 patients (N=9) received 10 MU of Interferon-α-2b (IFN, Intron-A, Schering-Plough) daily for 4 weeks. Group 2 patients (N=15) received 10 MU of IFN daily for the first 3 days only, followed by 3 MU IFN daily from day 3 until day 28. In both groups, ribavirin was given orally in divided doses of 1,000-1,200 mg daily (according to weight >75 kg). Subsequently, all patients received a maintenance treatment of minimally 3 MU IFN daily for 52 weeks. The patients baseline characteristics (Table 1) were well balanced, except for a trend for larger number of cirrhotic patients in group 1.

Table 1 Patient baseline characteristics.

Patient characteristics	Group 1	Group 2
Number of patients	9	15
Median age	44	47
Male/Female	7 / 2	11 / 4
Race (Caucasian / Asian)	7 / 2	15 / 0
Pre-treatment RNA HCV	5.5. x 10^6	7.5 x 10^6
Genotype 1	All	All
Median ALT at baseline	89	122
Cirrhosis/No-cirrhosis	4 / 5	1 / 14
Previous NR / other *	6 / 3	11 / 4

*) Non-sustained responder to previous therapy or previously untreated patients with cirrhosis and genotype 1.

9.3.2 Detection of serum HCV RNA

Plasma samples were collected frequently during the first 4 weeks of treatment for HCV RNA detection. Blood samples were collected in PPT tubes (Becton-Dickenson) which were spun directly after collection in order to avoid RNA breakdown. The spun PPT tubes [12] were then transported to the virology department where plasma was alliquoted in 5 separate tubes that were stored at -80°C. Plasma samples were obtained at day 0 (0, 4, 8, 12, 16 hours), day 1 (24, 32 and 40), day 2 (48 and 56 hours), day 3 (72 and 80 hours) day 4, 5, 6 7, 10, 14, 17, 21 and 28 after treatment initiation.

Viral load was quantified using the COBAS AMPLICOR MONITOR™ version 2 (Roche). Since the linearity of quantitative assays for high numbers of viral copies has been questionable [13], we routinely diluted samples and re-tested, if the early quantification of that sample was higher than 10^6 copies/ml.

9.3.3 Mathematical Modelling

Viral kinetics were analyzed using a modification of a previously described mathematical model for viral dynamics [5], for which the analytical solution is,

$$V(t) = V_0\{A \exp[-\lambda_1(t - t_0)] + (1-A) \exp[-\lambda_2(t - t_0)]\} \text{ for } (t > t_0) \quad \text{(Eq. 1)}$$

Where

$$\lambda_{1,2} = \tfrac{1}{2}\{(c + \delta) \pm [(c - \delta)^2 + 4(1 - \varepsilon)(1-\eta)\, c\delta]^{1/2}\} \quad \text{(Eq. 2)}$$

$$A = (\varepsilon c - \lambda_2)/(\lambda_1 - \lambda_2) \quad \text{(Eq. 3)}$$

This formula contains several dynamical parameters (c, δ, η and ε) which may vary per patient according to the best fit of the actual data, but are constant over time. c describes the clearance rate of free virus, with the corresponding virus half-life of ln(2)/c. δ describes the loss rate of productively infected cells, with the corresponding cellular half-life of ln(2)/δ. The effect of IFN can be modelled here either by a block of de-novo cell infection with η effectiveness ($0 \le \eta \le 1$), or block of virion production with effectiveness ε($0 \le \varepsilon \le 1$). The logarithmic drop in viral decline during the first phase (24-48 hours) of treatment can be approximated by log(1-ε). The 2nd phase slope can be approximated by ε times δ when $\eta << 1$, or by δ alone when $\eta \approx 1$.

To investigate the effect of reducing treatment dose, *all* the above dynamical parameters were allowed to change over time in the solution, e.g. for IFN effectiveness in blocking virion production, ε, we use the function $\varepsilon(t)$:

$$\text{for } t \le t_1\text{: } \varepsilon(t) = \varepsilon_1 \quad \text{(Eq. 4)}$$

$$\text{for } t > t_1\text{: } \varepsilon(t) = (\varepsilon_1 - \varepsilon_2 \exp(-k(t-t_1)) + \varepsilon_2$$

where t_1 is the time of dose change and k is a exponential rate representing how rapid does the change in IFN dose effect the change in the parameter. Thus, the blocking effectiveness starts at ε_1 (for $t \le t_1$), and changes with an exponential transition to ε_2 (after $(t-t_1) >> 1/k$). The same functional form was used to investigate also changes in η (from η_1 to η_2), δ (from δ_1 to δ_2), and c (from c_1 to c_2).

It is important to note that we do not explicitly model in Eq. 1 the dynamics of viral replication after the dose reduction, but rather replace the fixed parameters by time

dependent parameters in the original analytical solution obtained with fixed parameters. Nevertheless, we have tested this approximation by simulating a modification of the original differential equation model [5] where changes in the dynamical parameters were allowed to change at the time of dose reduction. We found no significant difference between the simulation of the full modified differential equation model and the modified analytical solution. Since we only have 2-3 viral measurements immediately after dose reduction, we can not estimate the appropriate replication parameters and thus chose to use the simple approximation given in Eq 1.

To estimate HCV viral kinetic parameters for each patient, the logarithm of V(t) in Eq. 1 (using $\varepsilon(t)$ from Eq. 4) was fit to the logarithm of the viral load data by a non-linear least squares method using the Madonna software (R.I. Macey and G.F. Oster, Berkeley, CA, USA). Two patients in group 2 were missing viral load data during the first week of treatment and one patient had a null response (less than 3 fold change in viral load during treatment) and therefore their viral kinetics could not be fitted.

9.3.4 Statistical analysis

The Fisher-exact test (2x2 tables) and the Chi-square test (NxN tables) were used to determine the statistical significance of the distribution of categorical variables between groups. The non-parametric independent (or related) Mann-Whitney rank sum test was used to determine the statistical significance of differences in continuous variables between the two groups (or of changes in the parameters within the same patients). Correlation among parameters, or between parameters and baseline values, was evaluated using the Spearman non-parametric test. Significance was established at $P<0.05$.

9.4 RESULTS

The bi-phasic decline previously reported [5-7,14] describes the viral kinetics during the first month for all patients in group 1 (see Figure 1 and Figure 2a), for whom IFN dose was kept constant. In contrast, we observed a complex dynamic pattern for 11 out of 12 evaluable patients in group 2 (see Figure 1 and Figure 2b-d). In these patients, a rapid decline occurred during the first day, followed by a slower decline on the second and third days, at which point a rapid increase in viral load (mean 0.8 [range 0-1.3] log copies/ml) is observed within 24-48 hours after the reduction in IFN dose. Thereafter, viral load again declined with a mean exponential slope comparable to the second phase slope in group 1.

We have tried to fit the viral kinetics of the patients in group 2 with several models, in each one of them allowing to change one parameter (ε, η, c or δ) at the time of dose reduction. The only model that was able to qualitatively reproduce the observed kinetics was the one which allowed a longitudinal change in the IFN effectiveness in blocking virion production (ε) as function of the IFN dose (see Eq 4). By only allowing to change the IFN effectiveness in blocking de-novo infection (η), death rate of infected cells (δ) or the clearance rate of free virions (c), it was not possible to fit the observed data. When assuming the major effect of IFN is to block virion production in a dose dependent way ($1>\varepsilon_1>\varepsilon_2>0$), it was possible to fit the data both with $\eta=0$ or $\eta=1$. Thus it was not possible to determine if IFN also blocks de-novo infection, in addition to blocking virion production, or not. Varying η between 0 and 1 only gives rise to minimal changes in the estimate of ε and c, while somewhat affecting the estimate of δ when ε is smaller than 0.98 (minimal estimate of δ obtained for $\eta=1$, and maximal estimate for $\eta=0$). Moreover, when allowing ε to change at the time of dose reduction, it was not possible to rule out that the other parameters also change at the same time.

For simplicity sake, and since our data only implies minute effects due to changes in the other parameters, we have assumed a change occurs only in ε when estimating the dynamical parameters. In addition, we needed to estimate the transition rate k (see Eq. 4) from ε_1 to ε_2. It was not possible to get a unique estimate of k for each patient individually, with only 2-3 measurements during the rebound. Since using k=1 up to 20 did not significantly affect the estimate of the other parameters, we have

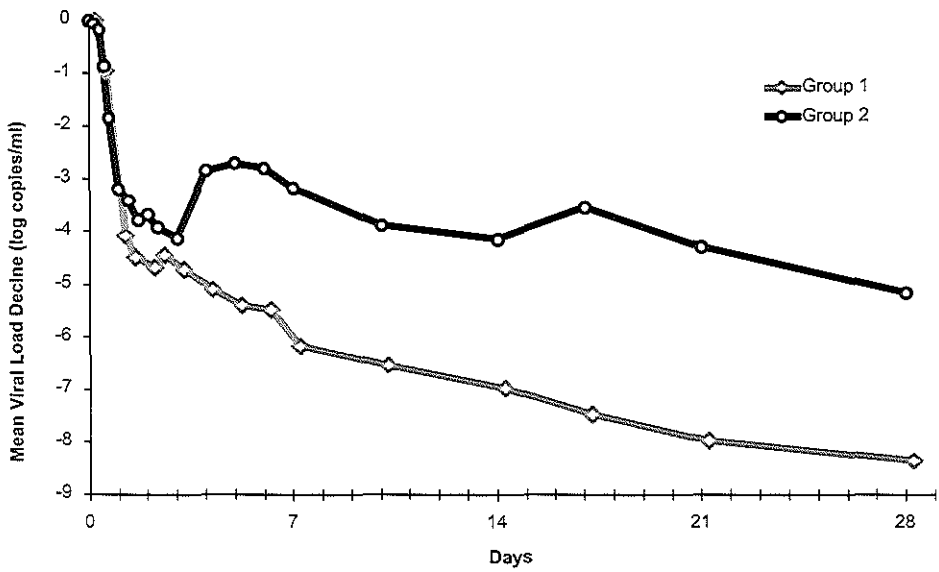
assumed k=2 for all patients such that the effect of ε_1 vanishes within 24-48 hours after the dose reduction in accordance to the observed data.

The estimates obtained by non-linear fitting of each patient's viral kinetics individually are given in Table 2. As expected the baseline viral load, the half-life of free virions and the initial effectiveness in blocking virion production, all related to the first phase decline, were similar between the 2 groups. Mean half-life of free virions for all patients was 3.0 hours ± standard deviation of 1.6 hours, similar to that derived in previous studies for IFN-α monotherapy (2.7 hours) [5]. The mean effectiveness in blocking virion production for all patients was 95.6% ± 6.5% for the 10 MU IFN dose. For group 1 the non-linear fit (with Eq. 4) did not give rise to a significant change in ε over time, in accordance to the constant daily IFN dose used in these patients (see insert in Figure 2a). For group 2, however, a significant ($p<0.005$) decrease in effectiveness of blocking production was observed (see inserts in Figure 2b-d and Figure 3a) from an average of ε_1=94% ± 8% to ε_2=69% ± 27%. The effectiveness of IFN with 10 MU (ε_1) and with 3 MU (ε_2) were pair wise correlated ($R=0.8$, $P<0.001$).

Cross-sectionally there was no difference observed between the 2 groups in the death rate of infected cells (see Table 2) or in the second phase slope (see Figure 3b). However, longitudinal analysis of group 2 patients revealed a significant decrease from the predicted second phase slope, which would have occurred without a dose reduction, to the actual slope after dose reduction in 4 out of 12 patients (see Figure 4a). These are the patients with the largest decrease (range 20% to 68%) from ε_1 to ε_2, since the second slope is a linear function of the IFN effectiveness in blocking production (ε).

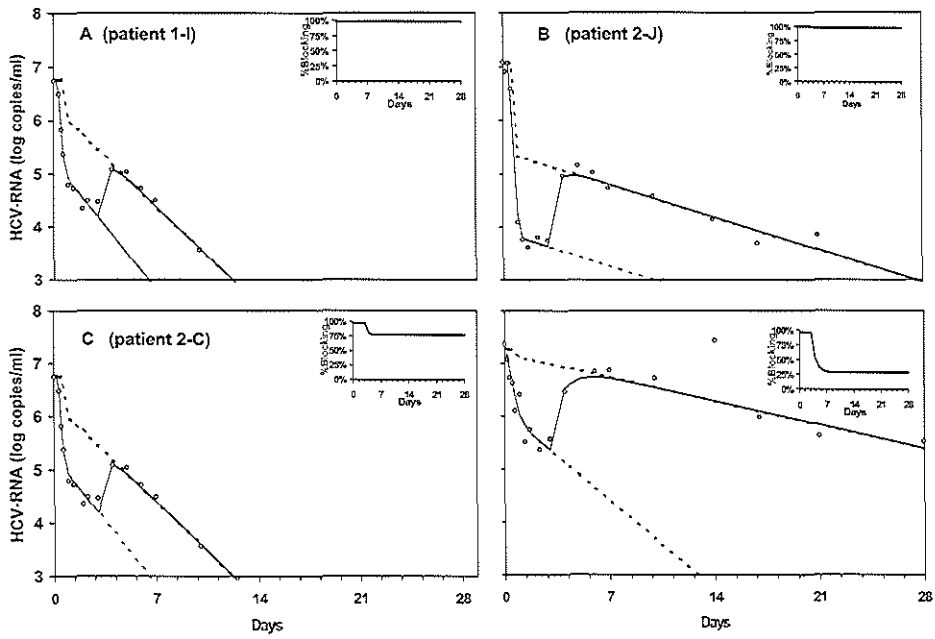
The clinical consequences of the viral dynamics data relate to the predicted time of HCV RNA negativity, which is strongly dependent on the second phase slope. The predicted time to HCV-negativity with the bi-phasic model (median 3.5 weeks) was confirmed by the observed individual data of group 1 patients (median 4 weeks), and the dose-reduction model (with the reduced 3 MU dose) predicted the time to HCV-negativity in group 2 patients (medians 12 and 14.5 weeks, see Table 3). Reducing the IFN dose to 3 MU daily decreased the predicted number of patients that would become HCV-negative within 12 weeks from 12/15 with the 10 MU dose to only 7/15 with the reduced 3 MU dose, as indeed confirmed by the actual individual data (8/15).

Figure 1



Group 1 (N=9) received a continues dose of 10 MU IFN daily for 28 days. Group 2 (dose reduction group, N=15) reduced from 10 MU IFN daily to 3 MU IFN daily after 3 days of treatment. After the viral load data was log-transformed, the geometric means were calculated for the 2 groups for each time-point of viral load measurement. The created mean viral for group 1 clearly shows a biphasic decline in viral load as previously described for chronic HCV. Viral decline in the patients with a IFN dose reduction (see line Group 2) is more complex: a rebound in viral load is observed coinciding with the time of IFN dose reduction.

Figure 2 A-D



In figure 2a the actual viral load measurements (see black dots) and the fitted viral decline curve using Eq. 1 (see black line) is presented of one of the patients (No 1-I) who receive 10 MU IFN daily for 28 days. The actual decline in viral load in this patient is best described by the original bi-phasic model.

In figure 2 b-d the actual viral load (black dots) and the fitted viral decline using the modified model allowing for a change in IFN efficacy as described in Eq.5 is presented (see black line). The actual viral decline could only be correctly fitted by using our modified model.

The striped black lines represent the calculated bi-phasic decline curve for a continues 10 MU IFN regimen in the patients of group 2 by fitting only the observed viral load during the treatment period of 10 MU daily in the original model.

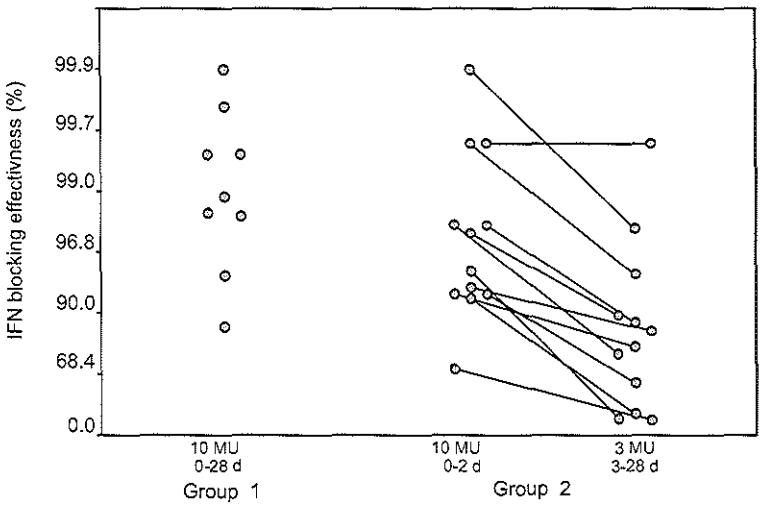
The grey line represents the calculated viral decline curve as if a continues treatment of 3 MU IFN daily was given from the start of treatment (only the viral load during 3 MU IFN are fitted). Note that the difference in the slope between the calculated decline curves for 10 MU versus 3 MU (black stripes versus grey lines) is dependent on the change in interferon efficacy after dose reduction (see inserts). Patients 2-H was calculated to become HCV RNA negative within 14 days if 10 MU IFN was continued (grey line figure 2-d), but after dose reduction the actual viral decline curved shifted dramatically due to a strong decline in IFN efficacy (see black striped line).

Table 2: Results of non-linear fitting of viral dynamics

Patient [1]	*Initial viral load* (log copies/ml)	*% blocking production*		Half-life [2] of free virions (hours)	Half-life of infected cells (days)
		During 10 MU qd	After reduction to 3 MU qd		minimal and maximal estimates[3]
1-A	6.9	98.4	---	2.3	8.7 - 8.8
1-B	6.9	99.9	---	2.5	3.0 - 3.0
1-C	6.7	99.5	---	2.2	1.7 - 1.7
1-D	6.1	86.9	---	1.9	7.8 - 9.0
1-E	5.8	99.5	---	5.3	4.4 - 4.5
1-F	6.4	98.5	---	6.4	3.2 - 3.2
1-G	7.0	95.1	---	1.8	6.1 - 6.4
1-H	7.1	99.8	---	1.7	1.1 - 1.1
1-I	7.4	98.9	---	3.3	4.9 - 4.9
Group 1[4] mean (std)	6.7 (0.5)	97.4 % (0.04)	---	3.0 hours (1.7)	4.5 - 4.7 days (2.6 - 2.8)
2-A	7.3	93.0	62.5	1.8	4.8 - 7.7
2-C	6.8	98.1	78.5	3.3	0.9 - 1.2
2-E	6.7	93.8	86.0	3.3	7.1 - 8.2
2-F	6.5	99.6	95.2	1.9	1.0 - 1.0
2-G	7.2	97.8	85.0	3.3	4.0 - 4.7
2-H	7.3	95.5	27.8	3.9	1.2 - 4.7
2-I	6.1	98.1	88.0	1.1	11.8 - 13.5
2-J	7.1	99.9	98.0	1.6	3.4 - 3.4
2-K	7.1	71.4	25.2	2.1	2.6 - 2.7
2-L	6.3	99.6	99.6	6.9	2.2 - 2.3
2-M	7.4	93.0	81.1	2.1	12.0 -14.8
2-N	6.8	92.4	33.2	3.6	2.5 - 7.7
Group 2 [4] mean (std)	6.9 (0.4)	94.3% (0.08)	69.1% [**] (26.6)	2.9 hours (1.6)	4.4 - 6.7 days (3.9 - 4.6)

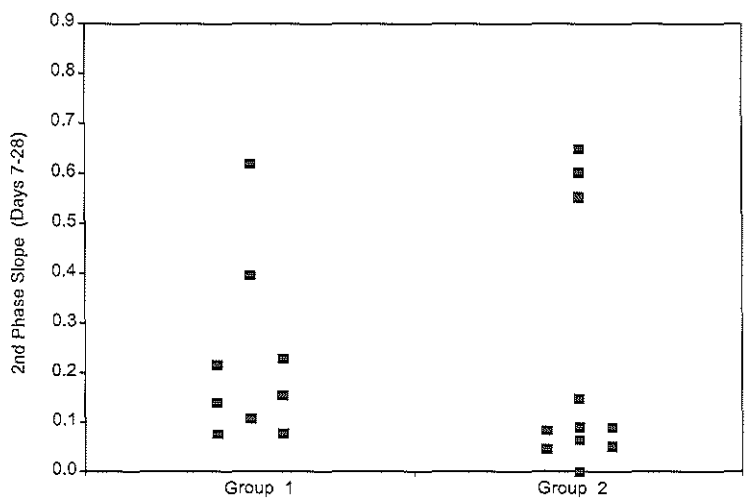
1) Fitting was not done for: patients 2-B (non-detectable at day 2 on), 2-D (Null-response), 2-O (missing data and rebound).
2) For group 1 half-life of free virions is only a maximal estimate because only samples from 0, 8 and 24 hours were available.
3) Minimal and maximal estimates of the half-life of infected cells was estimated assuming $\eta=1$ and $\eta=0$ respectively in Eq 2.
4) No statistically significant differences in any parameter between the two groups.
**) Statistically significant ($p<0.002$) difference in percentage blocking production before and after dose reduction in group 2.

Figure 3A



Interferon efficacy in blocking production (%) is displayed for group 1 and group 2. For the patients that received a dose reduction after 3 days of treatment (group 2), both the efficacy for the initial, 10 MU IFN daily period, is displayed as well as the reduced efficacy after the dose reduction to 3 MU daily. Note that the initial interferon efficacy between group 1 and 2 does not differ. After the dose reduction the interferon efficacy is reduced in 11/12 patients in group 2 (see connected black dots).

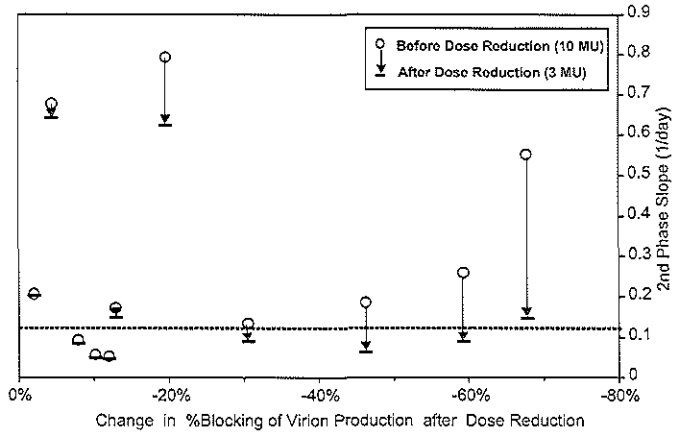
Figure 3B



The slopes of the second phase decline ($days^{-1}$) is represented per patient (black squares) for both groups. No differences between both groups could be observed in this study.

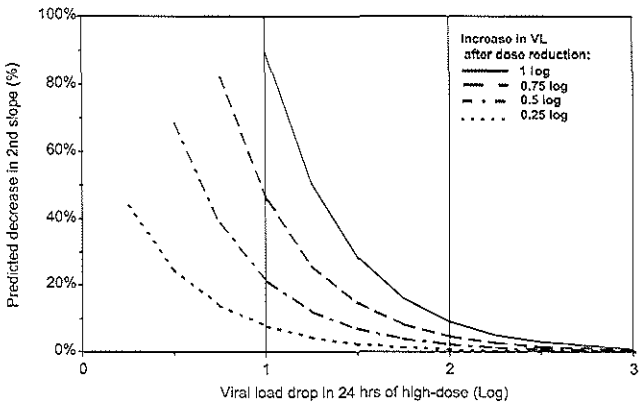
Figure 4 A and B

A



The effect of the change in interferon efficacy after the dose reduction (x-axis) for the patients in group 2 is related to the change in slope of the second phase viral decline (open circles: second phase slope *before* dose reduction; horizontal lines: second phase slope *after* dose reduction).
In 4 patients the slope of the second phase reduces drastically, note that 3/4 of these patients have the largest reduction in interferon efficacy.The dashed line reflects the threshold of 0.13 $days^{-1}$ below which no sustained response was observed.

B



The relation between the initial drop in viral load in log (x-axis) and the predicted change in the slope of the second phase (y-axis) due to dose reduction is displayed. In this figure the four different lines represent 4 possible responses in the viral load after dose reduction (ranging from a 0.25 up to 1 log increase in viral load as actually observed in this study).In patients with a very steep initial drop in viral load (>2 log drop, corresponding with an IFN efficacy of more than 99%), the effect of dose reduction is minimal. Even is there would be a rebound in viral load of 1 log after dose reduction, the interferon efficacy would only be reduced from 99% to 90%, corresponding with a minimal change of the second phase slope. Patients with a more modest (1-2 log) initial drop, could experience a large change in the second phase slope if the increase of viral load is more than 0.5 log. In patients with <1 log initial decline, any change increase in viral load after dose reduction will lead to large changes in the second slope.

Table 3: Predicted and observed time to HCV RNA negativity

Patient	Predicted time (weeks) to HCV RNA negativity[1] with 10 MU/daily	Predicted time (weeks) to HCV RNA negativity[1] with 3 MU/daily	First observedHCV RNA negativity[1] (weeks)
1-A	11	---	12[2]
1-B	2	---	2
1-C	1.5	---	2
1-D	12	---	4 – 8
1-E	2.5	---	2
1-F	3.5	---	4
1-G	10.5	---	4 – 8
1-H	1	---	1
1-I	9	---	Positive at 24 weeks [3] [4]
Group 1: HCV neg before 12 weeks	9 / 9 patients	---	8 / 9 patients
2-A	9	16	Positive at 24 weeks [3]
2-B	1	1	1
2-C	1	2	1.5
2-D	Never	Never	Positive at 24 weeks [3]
2-E	11	13	4 – 8
2-F	1	1.5	1.5
2-G	5.5	7	4 – 8
2-H	3	10	12 – 16
2-I	11	18	Positive at 24 weeks [3]
2-J	3	5	4
2-K	7.5	24	Positive at 24 weeks [3]
2-L	1.5	1.5	1.5
2-M	23	30	8 – 12
2-N	4	16	Positive at 24 weeks [3]
2-O	Never	Never	Positive at 24 weeks [3]
Group 2: HCV neg before 12 weeks	12 / 15 patients	7 / 15 patients	8 / 15 patients

Detection level for HCV RNA negativity is at < 500 copies/ml. 2) Patient 1-D had a viral breakthrough after 12 weeks and was HCV RNA positive at 24 weeks of treatment. 3) Patients with positive HCV RNA at 24 weeks stopped treatment according to protocol. 4) Patient 1-I was non-compliant from 8 weeks on according to self-report.

9.5 DISCUSSION

Our results indicate that the effect of IFN dose reduction on viral dynamics can be completely attributed to decrease in the effectiveness of IFN in blocking virion production (ε). Changes in other parameters, such as blocking de-novo infection (η) loss rate of infected cells (δ) and clearance rate of free virions (c), without a change in blocking production, can not reproduce the observed kinetics. The longitudinal dose dependence of the IFN anti-viral effectiveness observed here corroborates the dose dependence of IFN effectiveness previously described only cross-sectionally [5]. The strength of the current results is that the dose dependence of the effectiveness cannot be attributed to baseline differences between the patients. Since longitudinal changes in the other parameters of this model (such as η, δ and c) do not give rise to significant differences in the kinetics, we can not rule out combined effects of changes in the other parameters concomitantly with the change in effectiveness of blocking production (ε).

In turn, the dose dependency of the IFN effectiveness determines the result of both the 1st phase and the 2nd phase kinetics. The effectiveness in blocking virion production with 10 MU before dose reduction (mean 95.6% and a mean viral decline of 1.8 log cp/ml) was similar to that of a previous study with 10 MU IFN (96%) [5]. However, we show that the benefit of the initial viral decline due to 3 days of high induction dose was lost after the dose reduction in almost all patients (see Figure 1). The effectiveness in blocking virion production with 3 MU after the dose reduction (mean 69.1% and a mean viral decline of 0.7 log cp/ml) was similar to that estimated in a previous study with 3 MU IFN initially (70%) [8]. As a consequence, the viral kinetics after the dose reduction in group 2 patients is similar to the kinetics that would have been obtained if the patients had started with the reduced dose to begin with (see grey line in Figure 2).

In contrast to the 1st phase viral decline, which exponentially depends on the IFN effectiveness, the 2nd phase slope is a linear function of the effectiveness in blocking virion production. This slope is the most predictive parameter for treatment outcome, with a threshold of 0.13 $days^{-1}$ below which no sustained response was observed [5,10]. While the increase observed in viral load immediately after dose reduction can delay the time to negativity by several weeks at the most (see patients 2-J and 2-C in

Figure 2), the decrease in the second phase slope can radically reduce the chance for HCV-RNA negativity (see patient 2-H in Figure 2). Therefore the decrease in the second slope to below the 0.13 threshold in some patients (Figure 4a) could be crucial for their success of treatment. Indeed, the number of patients predicted to become HCV-negative within 12 weeks with the high induction dose was drastically reduced due to the dose reduction (compare the 10 MU column versus 3 MU column in Table 3).

Interestingly, the results obtained here are for IFN and ribavirin combination treatment, while the results from previous studies [5,8,10] are for IFN monotherapy. On the other hand, in this study most patients are non-responders or cirrhotic rather than normal naïve patients as in the previous studies [5,8]. Thus, we can not conclude if ribavirin has an additive effect on initial viral decline or not.

Is induction treatment beneficial at all considering that following dose reduction the virus rebounds back to the level it would reach anyway with the reduced dose? Previous studies with a somewhat longer period of induction treatment (14 days) do not show a consistent viral rebound as observed here [15]. Moreover, studies of prolonged induction treatment (up to 24 weeks with daily high dose of IFN) show a substantial decrease in relapse rate [16]. Therefore, it could be suggested that an induction period of 3 days is too short, but longer induction periods, which continue until viral clearance, might give rise to continuous suppression of viral replication. However, since dose reduction is then applied when virus is undetectable it is not possible to monitor the changes in the viral decline slope.

Alternatively, based on our observation that the second phase viral decline slope is related to the IFN effectiveness, we suggest a new treatment strategy for a safe dose reduction protocol. Patients with an initial (within 24-48 hours) viral load decline of 2 log or more with 10 MU IFN treatment could reduce the dose to 3 MU already at 3 days without a major loss of chance for successful treatment. This is due to the fact that even if the increase in viral load following dose reduction would be of 1 log, still the change in effectiveness and hence the change in the second slope would be only of 10% (Figure 4b). However, patients whose initial viral decline was only of 1-2 logs with 10 MU IFN induction treatment, could suffer from a significant decrease in their second phase slope if the viral load increases more than 0.5 log after the dose reduction. Therefore in these patients we would recommend to postpone the dose reduction until 2-4 weeks of treatment and monitor the viral load rebound carefully

during the dose reduction. Furthermore, patients with an initial viral decline of less than 1 log with 10 MU would most probably loose any chance for rapid second phase decline if the dose is reduced. In those patients we would recommend to continue the daily high dose as long as clinically possible.

The results from this study allow to predict in which patients is dose reduction safe. In addition, the slope of the second phase viral decline can determine the actual probability for sustained response. Altogether, these results indicate that individual monitoring of viral kinetics could be used for rational modification of treatment resulting in a higher success rate and lower unnecessary therapy burden on the patients.

9.6 ACKNOWLEDGMENT

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Chapter 10

General Discussion

Implications Of Viral Kinetics Studies For The Treatment Of Hepatitis C, Today And Tomorrow

F.C Bekkering and S.W.Schalm

10.1 CHRONIC HEPATITIS C TREATMENT TODAY

In the decade that hepatitis C virus infection (HCV) has been identified as a major cause of chronic liver disease, successive therapeutic regimens have resulted in clearance of virus in increasing proportion. The first effective treatment of chronic hepatitis C was alpha-Interferon, given as monotherapy 3 million units (MU) thrice a week (tiw) for 6 months [1,2] Such interferon monotherapy induces a sustained virological response only in a minority of patients (genotype 1: 2-9%, genotype non-1: 16-21%) [3-5] Strategies to increase the antiviral activity included the adjustment of interferon dose, the frequency of administration, and the duration of therapy as well as combination of interferon with ribavirin. More recently a new form of alpha-interferon (pegylated interferon) has been introduced.

Prolongation of interferon therapy tiw till 12 months reduces the relapse rate and thereby increases the sustained response rate (genotype 1: 9%, genotype non-1: 36%) [4,5]. The effects of increasing the dose of interferon from 3 MU upwards or increasing the frequency of administration from thrice weekly to daily have been less clear [6,7].

Ribavirin, a guanosine analogue, lowers serum transaminases in many patients with chronic hepatitis C, but has no effect on serum HCV RNA levels [8,9]. When used in combination with interferon, it increases the end of treatment response and reduces post treatment relapse [10-13]. Recent large randomized placebo-controlled trials in the U.S. and abroad have confirmed the enhanced efficacy of combination interferon-ribavirin therapy (sustained response in genotype 1: 17 to 29% for 6 vs 12 months therapy , genotype non-1: around 65% for both 6 and 12 months) [3,4].

Table 1: Sustained virological response percentage (6 months after end of treatment)

	IFN monotherapy 6 months	**IFN** monotherapy 12 months	**IFN-ribavirin** combination 6 months	**IFN-ribavirin** combination 12 months
Genotype 1	2%	9%	17%	29%
Genotype non-1*	16%	36%	66%	65%

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

Pegylated interferon is a slow release preparation of alpha-interferon, that maintains virus suppressive interferon levels for about a week; this formulation increases the end of treatment response and reduces breakthrough (sustained response in genotype 1: 27-28% for 6 vs 12 months monotherapy; genotype non-1: 36-58% for 6 vs 12 months monotherapy) [14,15]. Pegylated interferon in combination with ribavirin increases the sustained response rate significantly, but final outcome is similar to interferon plus ribavirin for 6 months therapy; it is improved for 12 months therapy (sustained response in genotype 1: 22-42% for 6 vs 12 months combination therapy; genotype non-1: 62-82% for 6 vs 12 months combination therapy) [16]

Table 2: sustained virological response rates for pegylated interferon therapy

	Peg IFN monotherapy 6 months	**Peg IFN** monotherapy 12 months	**Peg IFN-ribavirin** combination 6 months	**Peg IFN-ribavirin** combination 12 months
Genotype 1	27%	28%	22%	42%
Genotype non-1	36%	58%	62%	82%

Although combination therapy and Peg-interferon represent marked improvements in our ability to treat chronic HCV infection, there are still approximately 50% of patients who will not respond to antiviral therapy. Currently, much attention is focused on patient or virus characteristics that can identify those patients that will or will not benefit from treatment. Large multi-center trials have identified variables that influence response to combination interferon-ribavirin therapy. The major pre-treatment factors influencing the response rates in a clinically relevant way are the HCV genotype and the degree of fibrosis; with combination therapy, the viremia level, age and gender are of lesser significance [3,4,12].

Since genotype 1 is the predominant genotype in the western world and the sustained response rate with combination therapy is only 28%-42%, more effective treatment for this large group is desirable. Another group of patients that currently is in need of better treatment is the patient with cirrhosis, in view of the fact that morbidity and mortality of chronic hepatitis C is predominantly in this category of patients [17].

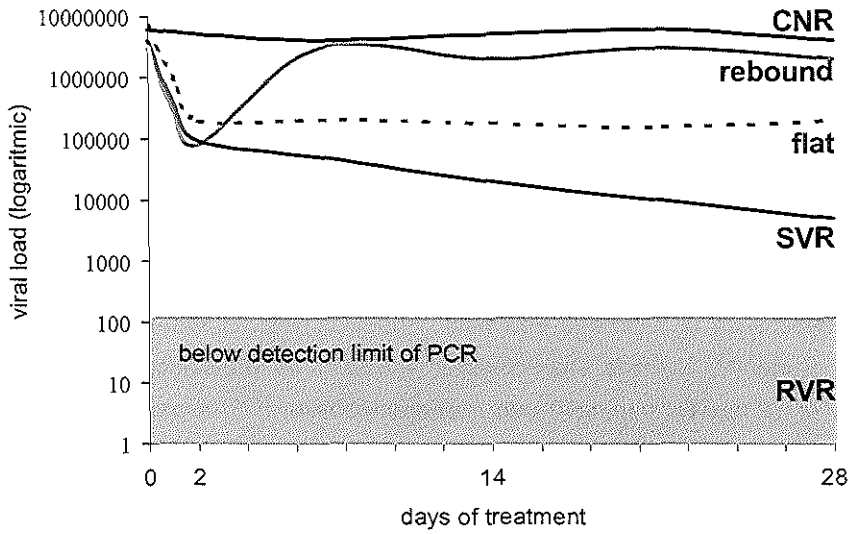
Daily high doses of IFN have been used for years In Japan, resulting in high (more than 80%) initial response rates. In Rotterdam, treatment with daily high dose IFN was evaluated for the "difficult to treat" patients with genotype 1 or cirrhosis. Since the introduction of validated quantitative PCR assays for HCV RNA, monitoring the viral load during the initial treatment phase has become feasible [18,19]. Therefore, in order to evaluate the effect of the high daily dose regime the initial viral decline pattern was closely examined. Analysis of this viral decline during the first 4 weeks of treatment revealed a bi-phasic decline in nearly all patients [20-22]. Twenty out of the 24 included patients completed the study period [23]. The first phase showed a mean drop in viral load of 2.3 log in 2 days (range 0.8–3.2). Fifteen patients declined rapidly during the first phase and were negative at 4 weeks. Three declined slowly and were expected to become HCV RNA seronegative before 26 weeks and 2 patients were unlikely to become seronegative within 26 weeks. In summary, treatment with daily high dose interferon in Rotterdam was also found to result in 75% early virological response.

Daily high dose treatment has not been accepted in Western Europe and the USA since no lasting beneficial effect on sustained virological response has been shown. However, in Japan treatment duration has been limited to 6 months in all studies7; also in other studies the high relapse rate might be explained by insufficient treatment duration or by too rapidly lowering the dose [24] According to a large multicenter Benelux study [25], reductions in relapse rates to less than 10% can be obtained by prolonging combination therapy to 18 months. Therefore, the daily high dose interferon therapy (10 MU/d) in Rotterdam was combined with ribavirin, daily interferon was maintained for 12 months, the dose of interferon was lowered only after one month and again after 6 months and the total duration of therapy was prolonged to 18 months. As a result, this single centre study in "difficult to treat" patients showed marked improvements in inducing an end-of-treatment virological response up to 75%. (genotype 1: 67%, genotype non-1: 91%; per protocol analysis) [23]. With an overall sustained response of about 70% with high dose prolonged therapy, it emerges that most patients with chronic hepatitis C, except those with advanced cirrhosis, can probably be cured of their illness.

10.2 TOMORROW: INTERFERON – RIBAVIRIN ADAPTED TO INDIVIDUAL VIRAL KINETICS

Maximal results in viral clearance seem to be induced by a therapy that combines the high initial response rates, observed with high dose interferon (daily or pegylated), with the reduced relapse rates observed with prolonged (>12 months) therapy. However, due to side-effects and costs such a treatment regime is not feasible nor desirable for all patients. Since (pegylated) interferon and ribavirin therapy has a number of side effects that increase with dose and duration [4,5,6,16,26], and since not all patients need such an intensive treatment schedule, patients should be carefully selected. Currently, baseline characteristics are used to pre-select patients that receive 6 or 12 months treatment with interferon-ribavirin [27,28]. A problem with this concept is that some patients with a slow decline in viral load might not reach a durable viral clearance within the pre-defined treatment period [28,29]. Some patients with 6 months of therapy might have benefit from 12 months of treatment [28,29] and patients with genotype 1, high viral load and 12 months of therapy might have foregone a relapse by extending treatment to 18 months [24]. Furthermore, about 60% of patients with genotype 1 or cirrhosis will not reach an sustained response. Early identification of these nonresponders could save considerably on side-effects and costs. Analysis of the viral decline of the first 4 weeks of treatment will likely identify all these patients (figure 1).

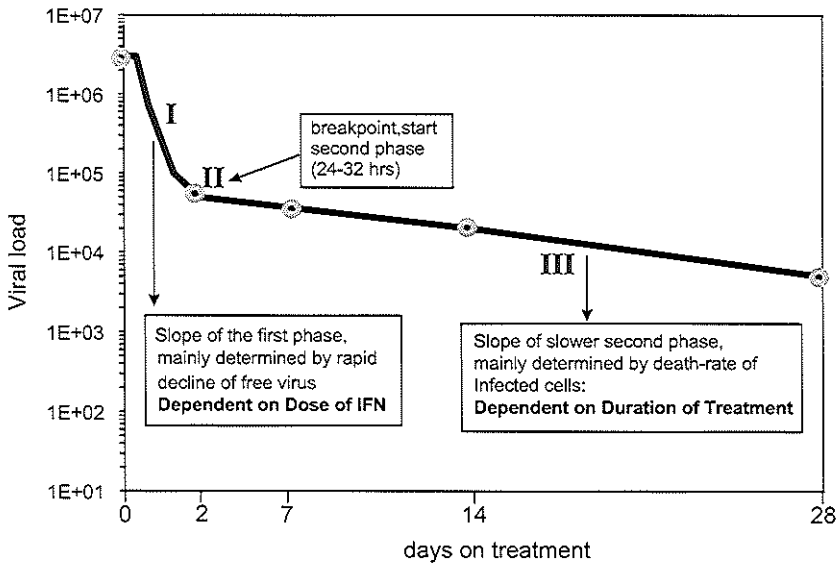
Figure 1 Response patterns in viral load decline



When looking at the individual decline patterns it appears that three different patterns of response are present: Nonresponse (NR), rapid viral response (RVR) and slow viral response (SVR) Nonresponse can further be divided in: patients with no drop in viral load at all (complete nonresponse), patients who have an initial decline in viral load but remain flat thereafter (flat) and patients who rebound after an initial decline (rebounders).

We propose to start with standard or high dose therapy based on base-line characteristics. All patients with genotype 1 and a viral load of more than 2 million copies/ml or early, compensated cirrhosis could be advised to receive high dose (daily interferon 10MU or an equivalent pegylated interferon dose) combined with ribavirin. All other patients could start with standard dose combination therapy. Quantitative measurement at 5 time-points (start of treatment, day 2, week 1, week 2 and week 4) would reveal the viral kinetics of each individual patient (figure 2).

Figure 2 Individual viral decline curve, constructed out of 5 quantitative HCV RNA measurements



According to the individual virus decline treatment could be altered within 6 weeks:

a) Patients who show a rebound, flat or complete nonresponse pattern could either stop treatment (if they have been treated with a high daily dose) or change to a high daily dose (if they have been treated with the standard dose).

b) All patients who show a slow viral response (projected serum HCV RNA negativity within 2-6 months) could be advised to continue treatment for 12-18 months.

c) Patients with a rapid viral response (serum HCV RNA negative within 1 month) could stop treatment after 6 months.

With the average cost of $150 per quantitative HCVRNA test, an individual analysis of the viral decline pattern will cost about $600-750. Compared to the cost of 6 months additional combination treatment ($6000 - 8000) [30], an early treatment discontinuation in patients with a rapid decline pattern could prove not only to lower side-effects but be cost effective as well. Instead of prescribing all patients a standard 48 weeks pegylated interferon and ribavirin therapy, that is simply not

effective enough for most genotype 1 patients and too intensive for most genotype non-1 patients, individual tailoring of antiviral therapy based on the initial viral decline will be a logic step forward in the treatment for chronic HCV patients.

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Chapter 11

Summary

11.1 INTRODUCTION

Treatment for chronic hepatitis C emerged only a decade ago. Since the first treatment strategies the aim of the treatment shifted from lowering the ALT level, towards clearance of the Hepatitis C RNA in serum as the major endpoint of treatment. With the availability of standardised quantitative assays for the detection of HCV RNA in serum, the actual decline pattern of the virus itself could be monitored during treatment. With these data it became possible to conduct the viral kinetic and dynamic studies as presented in this thesis. These studies provided the basis for new treatment strategies for "difficult to treat" patients with chronic hepatitis C.

11.2 ASSESSMENT OF VIRAL LOAD

Qualitative HCV RNA analysis has been shown to be more informative than ALT levels for assessing the response to treatment, during treatment, at the end of treatment and after 6 months of follow-up. HCV RNA positivity after 4 weeks of therapy predicts uniformly nonresponse. However, a problem with qualitative assays is the constantly declining level of detection of HCV RNA; with new, more sensitive assays that are able to detect only a very small amount of circulating HCV RNA, early testing for HCV RNA will be more frequently positive than with older, less sensitive assays. This could lead to postponing the decision whether or not to stop treatment because of non-response. Quantitative HCV RNA assays can accurately assess the actual amount of virus at any given time during treatment and allow for early analysis of the drop in viral load. The actual drop in viral load will be suitable for the decision to stop or continue anti-viral treatment than one qualitative virus measurement. (*chapter 2*)

In *chapter 3* the validation of the newly available quantitative HCV RNA assays is described. Plasma samples frozen at –80C of patients with chronic hepatitis C were sent to NGI laboratories in Los Angeles California, where they were tested with the Superquant™ assay. All samples were blinded, and test samples from a validated standard dilution series (EUROHEP dilution series for hepatitis C RNA, genotype 1 and 3) were included. During the test phase of the samples, NGI laboratories was

visited by the investigator to observe the process. Besides testing all samples with the NGI assay, they were also analysed with a second quantitative assay (Roche Amplicor assay) in Rotterdam. Both assays performed equally well and the detection limit of the assays was calculated from the dilution series to be around 500 copies per milliliter plasma. This study was able to show that quantitative were both sensitive and specific and test results were linear over a wide range of viral load.

11.3 DESCRIPTION OF THE DECLINE IN VIRAL LOAD

(first aim of this thesis)

With the Superquant™ assay for quantitative HCV RNA detection validated, all plasma samples of chronic HCV patients that were re-treated according to a protocol designed in 1991 were analysed. All patients in this study had previously received the standard treatment (3 mega-units Interferon thrice weekly for 6 months) without a response; re-treatment consisted of 10 mega units interferon daily for 5 days, thereafter 10 mega units was give thrice weekly until week 4. A gradual decline in viral load was expected to occur; however, nearly all decline in viral load occurred within 2 weeks of treatment. Close examination of the viral load decline within the first days showed a bi-phasic decline, with a rapid initial decline in all patients (alpha phase) and a much slower decline or even an increase thereafter (beta phase). Viral kinetics analysis revealed that the rapid decline in viral load corresponded with a half-life of free virus of approximately 5 hours. Three out of 11 patients became sustained responders in this study: those 3 patients revealed a very fast clearance of the virus and no virus could be detected in plasma after 2 weeks of treatment. (*chapter 4*)
In *chapter 5*, we responded to an article that stated that the viral load declined exponentially in chronic hepatitis C patients infected with genotype 1. However this study used only viral load data from the first days after treatment. In our study viral load data was available beyond 2 days of treatment. From this data we concluded that the viral load declines in at least 2 stages in genotype 1 patients. The rapid alpha phase and the slower beta phase were described for the first time in this article. Calculations on this second slower phase revealed that the viral decline was so slow in genotype 1 patients that virus-negativity could not be reached within 6 months.

Prolonged treatment was therefore advised for all chronic hepatitis C patients infected with genotype 1.

11.4 INFLUENCE OF DOSE AND FREQUENCY OF INTERFERON ON VIRAL DECLINE

(second aim of this thesis)

In order to analyse the influence of changes to the standard treatment regimen, a study had to be conducted where a relative homogeneous group of HCV patients received a standard treatment, a higher and a more frequent interferon dose. The study displayed in *chapter 6* included 4 groups of patients that had never been treated before, were all infected with genotype 1 and had no cirrhosis. The average viral decline was analysed for all groups. The group of patients that received the current standard dose of 3 mega units thrice weekly revealed the slowest decline, within the 4 weeks of treatment. 3 MU given daily improved the average speed of viral decline, but the fastest overall drop in viral load was observed when patients received 10 MU of interferon daily. This study indicated that daily dosage reveals a more gradual, faster decline in viral load, and that an increase of Interferon dose is followed by an increase in viral load decline. Theoretically, a faster decline in viral load could prevent the formation of mutations in the hepatitis C virus. A very rapid clearance of the virus leaves no time for mutations to emerge that are resistant to the given treatment. Since daily administration of interferon resulted in a more rapid decline of viral load, the next step was to evaluate the efficacy of twice daily administration of interferon. The rationale behind this schedule is the relative short half-life of Interferon of about 10 hours. However, viral kinetics analysis showed no benefit of a twice daily 5 mega units regimen when compared to daily 10 mega units of Interferon. (*chapter 7*)

11.5 MATHEMATICAL MODELLING OF VIRAL DECLINE

(third aim of this thesis)

After it became apparent that a bi-phasic pattern in viral decline exists for hepatitis C, a way to mathematically model this pattern was examined. In the first studies

(*chapter 4 and 5*) a simple exponential curve was created for both phases of viral decline. Theoretically, from the alpha phase, the clearance of virus particles from the blood could be estimated, and from the beta phase the clearance of infected cells.
In order to create a simple mathematical model that would describe the average decline in groups of patient (*chapter 6*) a linear regression analysis was performed on log transformed data. This model can be used by any physician and is a simplification of the actual decline pattern.
Neumann et al. created a mathematical model that did incorporate the dynamical parameters that could explain the observed viral decline. Those parameters are: virus particle clearance (a), efficacy of Interferon in blocking the production of virus particles (ε) and the death rate of infected cells. These parameters are all combined in one equation which provides information for individual patients. In *chapter 7* this equation is used to obtain the individual viral dynamics parameters but additionally, a biostatistical mixed model with random effects was used that allowed us to analyse and compare groups rather than individuals. The benefit of individual modelling is that the exact decline pattern of the patient can be analysed. The benefit of the group analysis is the possibility to compare the viral decline between 2 groups of patients.

11.6 CAN VIRAL KINETICS ANALYSES HELP US TO OPTIMISE THE TREATMENT FOR CHRONIC HEPATITIS C ?

(forth aim of this thesis)

Ultimately, the goal of the viral kinetics research is to improve the current treatment results for chronic hepatitis C. Despite the introduction of ribavirin as addition to interferon and the prolongation of treatment to 1 year instead of 6 months, more than 50% of the patients that are treated do not clear the virus. Moreover, those patients that need treatment the most (cirrhotics) have a sustained response rate below 20%; manay such patients have failed to clear the virus when previously treated. In *chapter 8* a study is displayed that focuses on improving treatment results in the "difficult to treat" patient group. Treatment has been optimised according to the results of previous viral kinetics research. First, the initial dose of interferon is increased to 10

MU daily for one month (optimise initial decline), second daily therapy was continued thereafter for 1 year (suppress break-through) and finally treatment was continued for 1,5 year (prevent return of virus after stopping). In this pilot study this regimen resulted in promising results: Initial response was improved, recurrence of virus during treatment was minimal and all but one patient that cleared the virus at the end of treatment remained negative for HCV. This led to an overall sustained clearance of the virus in nearly 70% of these "difficult to treat" patients. Downside of this treatment strategy was however the high incidence of side-effects that was observed in this group. Twenty-fife percent of the patients had to be hospitalised. Adverse events occurred mainly in patients with advanced cirrhosis.

After this, a study was conducted to analyse whether Interferon dosage could be lowered earlier without losing the improved viral clearance results. Rationale was that 90 to 99.9% of the decline in viral load occurs within 2 days, therefore the new treatment schedule as described in *chapter 9* lowered the dose of 10 MU interferon after 3 days to 3 MU Interferon daily. Unfortunately, the viral load rebounded directly after the treatment dose was lowered to 3 MU, resulting in a significant lower clearance rate of the virus within 4 weeks when compared to the patients that continued 10 MU of interferon daily.

11.7 POSSIBLE BIOLOGICAL MECHANISMS OF INTERFERON

(fifth aim of this thesis)

Since the dose of interferon was lowered in each individual early after treatment initiation, the effect of this dose lowering on the level of viremia could be observed. In all other studies this analysis was hampered by the fact that the majority of patients reached undetectable levels of HCV RNA within 4 weeks, so dose reduction after that point did not allow for viral kinetic analyses.

When the viral kinetic patterns were analysed it became apparent that the curves were not bi-phasic. Therefore the model described by Neumann had to be modified. The only modification that could correctly fit the observed data was the model that allowed for a continues change in the efficacy of interferon to block the production of the hepatitis C virus. Furthermore, individual analysis showed that in patients with a

very rapid decline (corresponding with a high interferon efficacy in those patients) within the first days, a dose reduction after 3 days did not really influence the viral decline curve. Contrary, in those patients with a slow initial decline the drop in viral load was reduced dramatically after the dose reduction. Individual monitoring of the viral load during treatment would allow for individual treatment schedules. Such a strategy would lead to early lowering of medication in those with a high interferon efficacy and continuing high dose treatment in those with a low interferon efficacy.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 25 april 1969 te Katwijk aan Zee. In 1987 behaalde hij het V.W.O. diploma aan de R.S.G. de Drie Waarden te Schoonhoven. Vervolgens begon hij na uitloting aan de studie economie aan de Erasmus Universiteit te Rotterdam. Na het propedeutisch examen economie behaald te hebben werd hij ingeloot voor de studie geneeskunde in 1988. Tijdens zijn studie was hij actief in het bestuur van de Medische faculteitsvereniging Rotterdam. Als student participeerde hij in een promotie onderzoek naar constitutioneel lange gestalte (onder begeleiding van prof. dr. S.L.S. Drop) en deed hij een half jaar onderzoek naar hepatitis C bij transplantatiepatiënten op de afdeling gastroenterologie van de University of Alabama, U.S.A. onder begeleiding van dr. D.J van Leeuwen.

Hij behaalde het artsexamen op 16 februari 1996 (Cum Laude). Van maart 1996 tot januari 2000 was hij werkzaam als arts-onderzoeker op de afdeling Maag-, Darm- en Leverziekten van het Academisch Ziekenhuis Rotterdam (Dijkzigt). Tijdens deze periode werd onder begeleiding van prof. dr. S.W. Schalm onderzoek verricht naar de virale kinetiek van het hepatitis C virus, hetgeen de basis vormde voor dit proefschrift. Sinds januari 2000 is hij in opleiding tot gastroenteroloog. Momenteel is hij werkzaam in het Havenziekenhuis te Rotterdam (opleider dr. A.G.C. Bauer).