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Prediction of minimal residual viremia in HCV type 1 infected patients receiving interferon-based therapy

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ABSTRACT

Introduction. Complete suppression of viral replication is crucial in chronic HCV treatment in order to prevent relapse and resistance development. We wanted to find out which factors influence the period from being already HCV RNA negative by bDNA assay (< 615 IU/mL) to become undetectable by the more sensitive TMA test (< 5.3 IU/mL). **Material and methods.** Evaluated were 433 HCV type 1-infected patients. All of them received 1.5 ug/kg Peg-IFNα-2b plus ribavirin for 18-48 weeks. bDNA was performed weekly during the first 8 weeks and thereafter at weeks 12, 24, and 48. Patients who became bDNA undetectable were additionally analysed by TMA. **Results.** Of the 309 patients with on-treatment response (< 615 IU/mL), 289 also reached undetectable HCV RNA levels by TMA. Multivariate analysis revealed that viremia ≤ 400,000 IU/mL (p = 0.001), fast initial virologic decline (p = 0.004) and absence of fibrosis (p = 0.035) were independent predictors of an accelerated on-treatment response by TMA assay in already bDNA negative patients. bDNA negative patients becoming HCV RNA undetectable by TMA within the following 3 weeks had a frequency of relapse of 21%, whereas those showing TMA negativity after 3 weeks relapsed in 38% (p = 0.001). In RVR patients (bDNA < 615 IU/mL at week 4) the corresponding relapse rates were 15.3% *vs.* 37.5%, respectively (p = 0.003). **Conclusion.** Early viral kinetics, baseline viremia and fibrosis stage are important tools to predict persistent minimal viremia during interferon-based therapy. The data have implications for designing a more refined treatment strategy in HCV infection, even in the setting of protease inhibitor-based triple treatment.

Key words. Chronic hepatitis C. Early virologic kinetics. Minimal residual viremia. Treatment strategy.

INTRODUCTION

Chronic infection with hepatitis C virus (HCV) is a major cause of liver cirrhosis and development of hepatocellular carcinoma.[1-4] Protease inhibitor-based triple therapy is the current standard treatment regimen.[5-6] The chance for cure is inversely correlated to the time required to suppress viremia on antiviral therapy. As Peg-Interferon and ribavirin remain the backbone of treatment, at least a partial response to these antiviral agents is essential. Therefore, the previously defined response factors influencing interferon-susceptibility also have an impact on SVR-rate of triple therapy.[7-8] Definition of effective viral suppression is related to the sensitivity of the available virologic assays. They still give only an estimate whether viral replication is completely suppressed or whether there is still a persistent minimal residual viremia in early responder patients. By now, several assays with greater sensitivity, having a detection limit of 5-10 IU/mL or less, are available.[9-13] The sensitive qualitative

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transcription-mediated amplification (TMA) assay has been used in a retrospective analysis by Morishima, *et al*., to detect HCV RNA during treatment course with peginterferon and ribavirin.[14] In a large cohort of patients (n = 1,145) on-treatment response at different time points has been assessed in comparison with a less sensitive PCR-based assay to predict SVR. In a more recent prospective study we compared two HCV RNA tests with different sensitivity (the quantitative branched DNA (bDNA) assay *vs*. TMA assay) with respect to their practicability to tailored treatment strategies as well as to predict long-term therapy outcome.[15] We could show that prediction of treatment outcome was significantly improved when predicated on TMA response instead of bDNA response. Persistence of minimal residual viremia below the detection limit of sensitive assays is considered to be an important factor not only for relapse occurrence but also for the development of drug resistance when using protease inhibitor-based antiviral regimens. High baseline viral load as well as advanced fibrosis could be shown to be associated with residual low-level viremia and consequently with virologic relapse.[16]

The aim of the present analysis based on the patient cohort of the INDIV I Study Group was to find out which factors influence the persistence of a minimal residual viremia by studying the period from being already HCV RNA-undetectable by bDNA assay (detection limit < 615 IU/mL) to become undetectable by the more sensitive TMA test (detection limit 5.3 IU/mL).

MATERIAL AND METHODS

Patient selection and treatment

The individual data from 433 treatment-naïve patients with compensated chronic HCV type 1-infection were analysed. The design of the INDIV I Study has been described in detail.[15] All patients received 1.5 ug/kg PEG-IFNα-2b per week plus 800-1,400 mg RBV for at least 18, and up to 48 weeks. Dose adjustments were performed in 7% for PEG-IFNα-2b and in 14% for ribavirin.

For enrollment patients had to fulfill the following entry criteria: positive test for anti-HCV (third-generation enzyme immunoassay); HCV RNA > 1,000 IU/mL by quantitative reverse transcription polymerase chain reaction (Roche AMPLICOR HCV Monitor version 2.0; Roche Diagnostics, Basel, Switzerland); HCV genotype 1 infection; increased serum alanine aminotransferase levels at screening; liver biopsy performed within the preceding 24 months of study enrollment confirming chronic hepatitis; neutrophil and platelet counts of at least 1,500/μL and 80,000/μL, respectively; hemoglobin values of at least 12 g/dL for females and 13 g/dL for males; creatinine levels < 1.5 mg/dL. Exclusion criteria were as follows: decompensated liver disease; hepatitis B virus or human immunodeficiency virus coinfection or other causes of liver disease; autoimmune disorders; concomitant immunosuppressive medication; clinically significant cardiac or cardiovascular abnormalities; organ grafts; systemic infections; preexisting severe psychiatric conditions; evidence of malignant neoplastic diseases; excessive daily intake of alcohol (≥ 40 g/day in women and ≥ 60 g/day in men); drug abuse within the past year; or unwillingness to practice contraception.

Written informed consent was obtained from each patient. Ethics committee approval had been received at each center according to the Declaration of Helsinki and the International Conference on Harmonization/Committee for Proprietary Medicinal Products "Good Clinical Practice" guidelines. All patients were evaluated as outpatients for safety, tolerance, and efficacy.

HCV RNA quantitative and qualitative testing

HCV RNA levels were quantified at baseline and weekly until week 8 and then on weeks 12, 24, and 48 by bDNA assay (Versant 3.0; formerly Bayer Diagnostics, Leverkusen, Germany [now provided by Siemens, Munich, Germany]; detection limit 615 IU/mL). Patients who had HCV RNA levels < 1,000 IU/mL by the bDNA test were additionally analysed by the more sensitive transcription-mediated amplification assay (TMA; Versant qualitative HCV RNA; formerly Bayer Diagnostics, Leverkusen, Germany; detection limit < 5.3 IU/mL, now provided by SIEMENS). The cut-off of 1,000 IU/mL instead of 615 IU/mL was chosen to improve the specificity of the bDNA assay. According to the qualitative HCV RNA results patients were defined as:

- Virologic nonresponders (HCV RNA positive 24 weeks after end of treatment).
- End-of-treatment responders with relapse (reappearance of HCV RNA during follow-up after stopping therapy).
- Breakthrough patients (reappearance of HCV viremia during antiviral treatment period), and

- As virologic sustained responders (HCV RNA negative at the end of follow-up 24 weeks after stopping therapy).

HCV genotyping was performed by way of reverse hybridization (Inno LiPA HCV; Innogenetics, Gent, Belgium).

Liver histology

A liver biopsy was performed in 299 patients before treatment. Histological results were classified by local pathologists according to internationally standardized criteria.[17] For better comparison between the different local pathologists, the individual fibrosis stage was documented ≥ stage 3 or < stage 3 (presence of cirrhosis/transition to cirrhosis or no cirrhosis). Steatosis was graded on a scale of 0-2 (0: absent; 1: < 5% of hepatocytes affected; 2: > 5% of hepatocytes affected).

Statistical analysis

The descriptive data of all relevant dependent variables were presented as absolute and relative frequencies for categorical data and means, standard deviations and ranges for continuous scaled data. Multiple cox regression analysis was used to determine predictive kinetic parameter capturing early viral decline as well as to find variables to be associated with an accelerated on-treatment response. Briefly, an accelerated on-treatment response was defined as minimized period of HCV RNA detection by TMA assay in already bDNA-negative patients. Kaplan Meier survival curves described time from bDNA negativity to TMA negativity of different patient categories. Statistical comparisons were made using the chi-square test. All tests were two-sided, and a P value less than 0.05 was considered significant. Statistical analyses were performed using the statistical software package SPSS for windows 18.0.

RESULTS

Patient profile

Of the 433 patients enrolled in the INDIV I study who were treated with pegylated interferon-alpha 2b (Peg-IFNα-2b) plus ribavirin, 335 (77%) became bDNA undetectable (Figure 1). 26 patients were excluded from our analysis due to incomplete data by TMA assay. This finally led to a total of 309 evaluable data. Table 1 represents baseline characteristics of the included patients. Stage of fibrosis and degree of steatosis were assessed in 299 and 255 patients, respectively.

Virologic response pattern

Of the 309 patients with on-treatment response by bDNA (< 615 IU/mL), 289 (93.5%) also reached undetectable HCV RNA levels by the more sensitive TMA assay throughout the whole study period. 193 (62.5%) had a baseline viral load ≤ 800,000 IU/mL and even 133 (43%) were tested ≤ 400,000 IU/mL

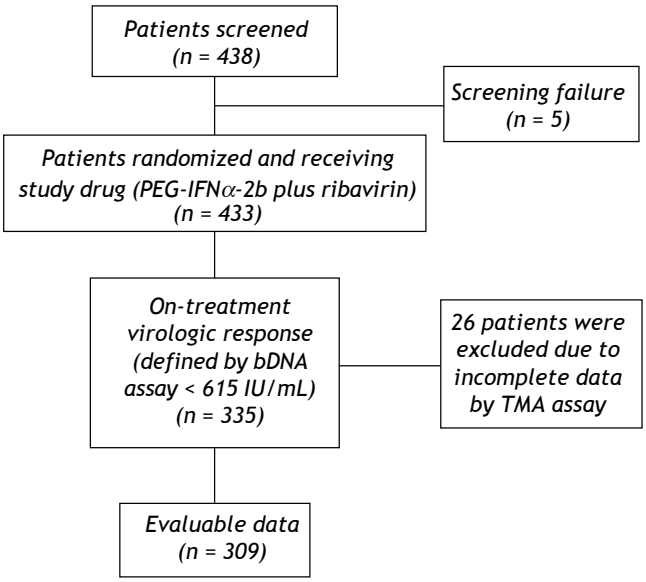

***Figure 1**. Trial profile diagram.*

Table 1. Summary of demographic, biochemical, serological and histological characteristics of patients at baseline.

Patient characteristics	n = 309
Sex, n (%):	
Male	164 (53.1)
Female	145 (46.9)
Age (years)*	43 ± 0.63 (18-68)
BMI (kg/m^2)*	25.5 ± 0.25 (17.7-40)
ALT levels x ULN (IU/L)*	2.5 ± 0.12 (0.4-16.2)
GGT levels x ULN (IU/L)*	1.5 ± 0.1 (0.17-18.1)
HCV RNA (log IU/mL)*	5.7 ± 0.04 (2.79-7.82)
Fibrosis stage, n (%):	
Stage 0-2	250 (85.6)
Stage 3-4	42 (14.4)
Steatosis (> 5%), n (%):	
Yes	38 (15.3)
No	211 (84.7)

* Data were given as mean ± SD (range).

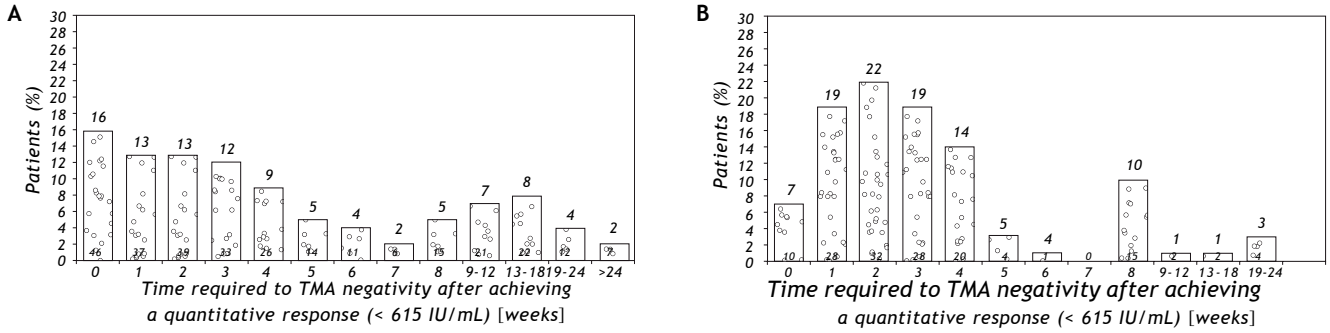

Figure 2. A. *Time to complete virologic response (< 5.3 IU/mL by TMA) in weeks in patients who reached non-quantifiable levels (< 615 IU/mL by bDNA). In each column the percentage of patients is given according to the time category (in weeks) required to reach TMA negativity after achieving a quantitative response by bDNA. The total number of patients in each subgroup is given at the bottom of each subgroup.* ***B.*** *Time to complete virologic response (< 5.3 IU/mL by TMA) in weeks in patients with RVR who reached non-quantifiable levels (< 615 IU/mL by bDNA). In each column the percentage of patients is given according to the time category (in weeks) required to reach TMA negativity after achieving a quantitative response by bDNA. The total number of patients in each subgroup is given at the bottom of each subgroup.*

before treatment. 47% of the patients (146/309) became bDNA-undetectable within the first 4 treatment weeks i.e. showing a rapid quantitative virologic response (RVR) pattern, and all of them also achieved qualitative virologic response (TMA negativity) during the following treatment period.

Figure 2A shows the time required to complete virologic response (undetectable TMA levels, < 5.3 IU/mL) after achieving a quantitative response (< 615 IU/mL) by bDNA assay. The median time from bDNA negativity to TMA negativity was 3 weeks with a range of 0 to 48 weeks. In 2% (7/189) of the bDNA responders more than 24 weeks of treatment were required to achieve a qualitative response. Figure 2B illustrates time to TMA response in the subgroup of RVR patients with undetectable HCV RNA by means of bDNA assay at week 4. All of them became TMA negative within the following 24 weeks of treatment.

Variables associated with an accelerated on-treatment response

Multivariate regression analysis revealed that low baseline viral load (≤ 400,000 IU/mL), a rapid initial virologic decline (defined as virologic decline after one week of treatment; slope > 0.88) as well as low fibrosis stage were significantly associated with an accelerated on-treatment response by TMA assay in already bDNA negative patients (Table 2). ALT levels, steatosis as well as dose reductions of PEG-IFNα-2b and ribavirin were shown to have no significant effect on the period of minimal viremia. However, the number of patients in whom dose adjustments were performed was small.

Next, Kaplan-Meier curve analysis was performed to illustrate how the time to complete treatment response (defined as TMA negativity in already bDNA-negative patients) was influenced by early viral kinetics as well as the other above mentioned baseline characteristics (i.e. fibrosis stage and, baseline viremia). The duration of the observation period ended in TMA-nonresponders representing those who remained TMA-positive.

In figure 3A the Kaplan–Meier Survival curve demonstrates time to TMA negativity in patients with undetectable HCV RNA by bDNA in relationship to rapid virologic response status (defined as undetectable HCV RNA level at treatment week 4 by

Table 2. Independent predictors associated with an accelerated on-treatment response by TMA assay in already bDNA negative patients.

Variables	Exp (B)	p-value
Pretreatment HCV RNA ≤ vs. > 400,000 IU/mL.	0.546	0.001
Initial virologic decline.	1.723	0.004
Fibrosis stage (< stage 3 vs. ≥ stage 3).	0.606	0.035
Steatosis.	0.680	0.073
ALT (x ULN).	1.074	0.106
Dose reduction of PEG-IFNα-2b.	0.670	0.249
Dose reduction of ribavirin.	1.109	0.641

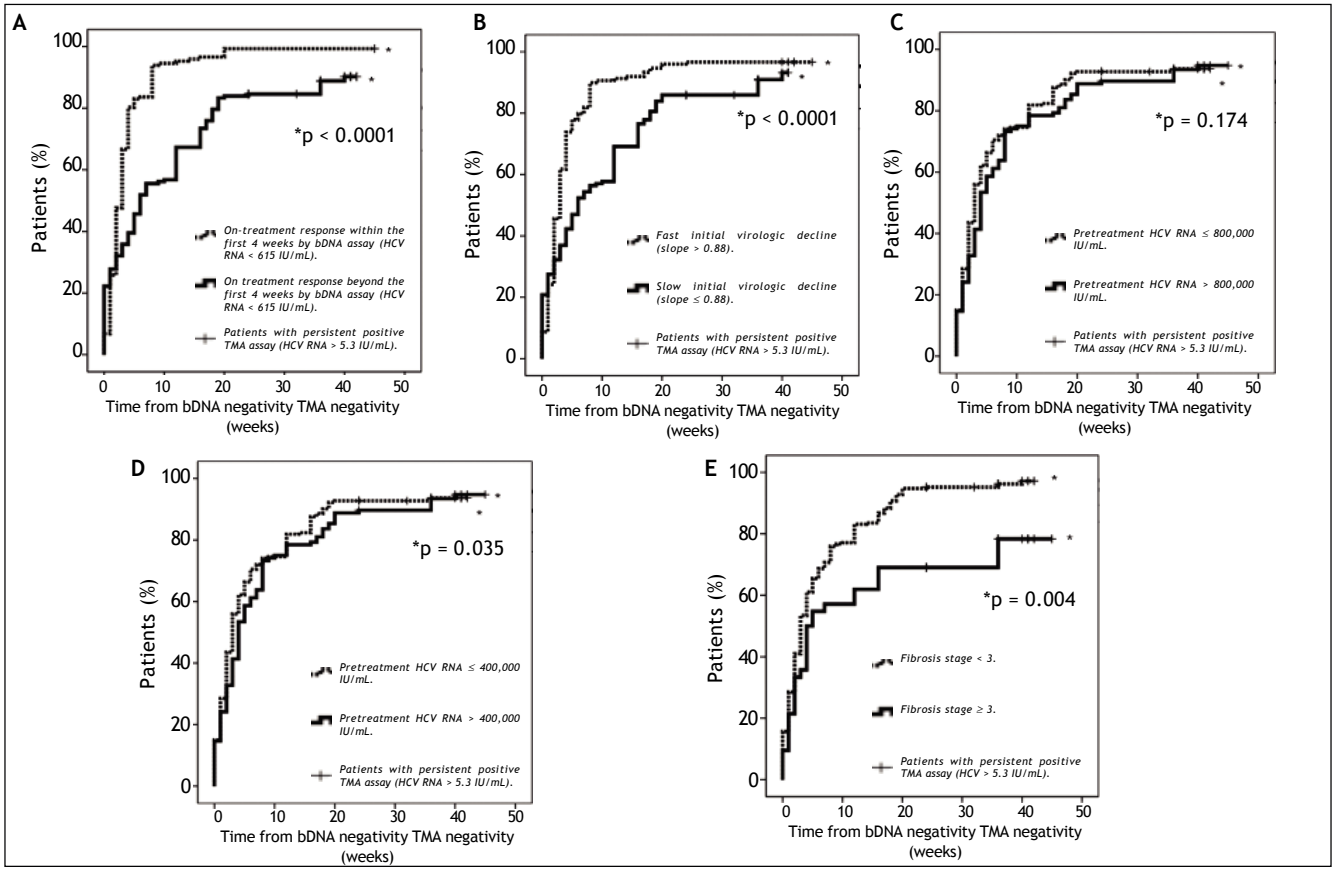

***Figure 3**. **A** Time (in weeks) to TMA negativity in patients with undetectable HCV RNA by bDNA in relationship to the rapid virologic response (RVR) status (defined as undetectable HCV RNA level at treatment week 4 by bDNA). 20/309 patients (+++) remained TMA positive throughout the whole observation period. **B**. Time (in weeks) to TMA negativity in patients with undetectable HCV RNA by bDNA in relationship to early virologic slope of decline at week 1 (defined as slope > 0.88). 20/309 patients (+++) remained TMA positive throughout the whole observation period. **C**. Time (in weeks) to TMA negativity in patients with undetectable HCV RNA by bDNA in relationship to level of baseline viremia. 800,000 IU/mL was chosen as cut-off viral concentrations, respectively. 20/309 patients (+++) remained TMA positive throughout the whole observation period. **D**. Time (in weeks) to TMA negativity in patients with undetectable HCV RNA by bDNA in relationship to level of baseline viremia. 400.000 IU/mL was chosen as cut-off viral concentrations, respectively. 20/309 patients (+++) remained TMA positive throughout the whole observation period. **E**. Time (in weeks) to TMA negativity in 292 patients with undetectable HCV RNA by bDNA in relationship to fibrosis stage. 19/292 patients (+++) remained TMA positive throughout the whole observation period.*

bDNA). Patients with on-treatment response within the first 4 weeks by bDNA showed a more rapid TMA negativity than patients with on-treatment response beyond week 4. The difference was statistically significant ($p < 0.0001$).

Early slope of decline in hepatitis C viremia was shown to be highly predictive for the time required to reach TMA negativity in patients with bDNA response. A cut-off value of 0.88 log decline differentiated patients with short or prolonged periods of minimal residual viremia most effectively.

Figure 3B illustrates the time to become TMA undetectable dependent on initial viral decline at week 1. Patients with an early virologic slope of decline (defined as slope > 0.88) showed a shorter period to achieve TMA negativity than patients having a virologic slope of decline ≤ 0.88). The difference was statistically significant ($p < 0.0001$).

Additionally we tried to figure out to what extent the level of baseline viremia add additional predictive information to the proposed model. Stratification according to baseline viral load was demonstrated in figures 3C and 3D. Taken HCV RNA of 800,000 IU/mL as cut-off (Figure 3C) the difference was not significant in both groups. However, by using a more strict cut-off of 400.000 IU/mL (Figure 3D) the difference was statistically significant ($p = 0.035$) with regard to an accelerated on-treatment response by TMA assay.

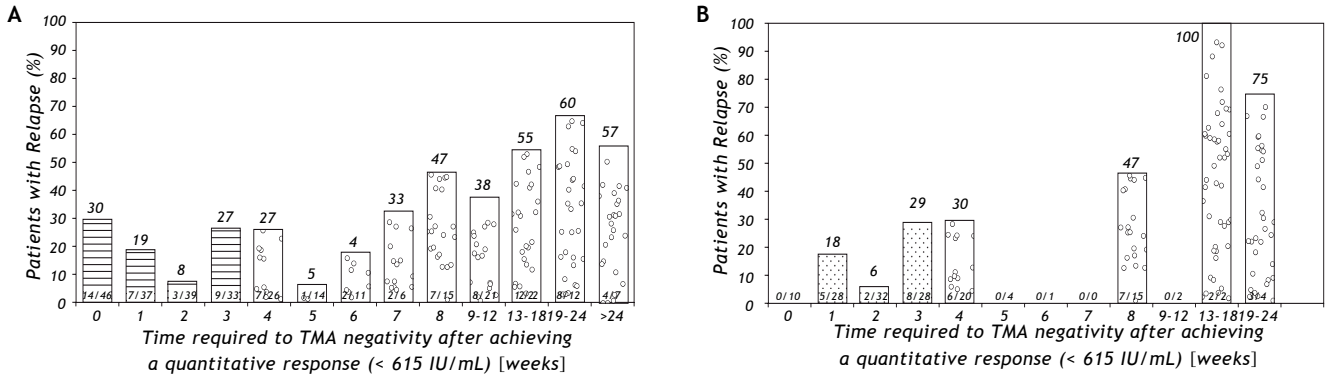

Figure 4. A. *Time to complete virologic response (< 5.3 IU/mL by TMA) in weeks in patients who reached non-quantifiable levels (< 615 IU/mL by bDNA). In each column the percentage of patients with virologic relapse is given according to the time category (in weeks) required to reach TMA negativity after achieving a quantitative response by bDNA. The total number of patients in each subgroup is given at the bottom of each subgroup.* ***B.*** *Time to complete virologic response (< 5.3 IU/mL by TMA) in weeks in patients with RVR who reached non-quantifiable levels (< 615 IU/mL by bDNA). In each column the percentage of patients with virologic relapse is given according to the time category (in weeks) required to reach TMA negativity after achieving a quantitative response by bDNA. The total number of patients in each subgroup is given at the bottom of each subgroup.*

Finally, figure 3E depicts time to TMA negativity according to fibrosis stage (≥ 3 *vs.* < 3) in 292 patients with evaluable biopsy results. Patients with transition to cirrhosis/presence of cirrhosis had a longer period of minimal viremia than patients with lower degree of fibrosis. The difference was statistically significant (p = 0.004).

Minimal residual viral replication as prediction of virologic relapse

In figure 4A the frequency of relapse of the entire study population is shown according to the time required to TMA negativity after achieving a quantitative response. As the median time from bDNA negativity to TMA negativity were 3 weeks this was found to be optimal for use as cut-off for short *vs.* prolonged period of minimal residual viral replication. With respect to the experience of a relapse we could show that 33/155 (21%) (≤ 3 weeks) *vs.* 51/134 (38%) (> 3 weeks) relapsed after 48 weeks of treatment (p = 0.001). Figure 4B illustrates the frequency of relapse in the subgroup of patients achieving a quantitative RVR after 4 weeks (by bDNA) of treatment. The corresponding relapse rates relative to the 3 week cut-off were 15/98 (15.3%) (≤ 3 weeks) *vs.* 18/48 (37.5%) (> 3 weeks) (p = 0.003).

DISCUSSION

Exact determination of early virologic response has great implication for designing a more refined treatment strategy in HCV infection. To achieve this goal accurate information is necessary to find parameters govering the time to HCV RNA negativation. The available virologic tests differ in their sensitivity and they only give an estimate whether viral replication is completely suppressed.[13]

In the present multicenter study we used 2 assays of different sensitivity to evaluate long-term persistence of minimal viremia. Although bDNA assay is not part of clinical practise anymore it is used in the present study as tool to illustrate residual viral replication. Residual low-level viremia was defined as viral load ≤ 615 IU/mL, but > 5.3 IU/mL.

We could demonstrate that the median time from bDNA negativity to become also HCV RNA undetectable by the more sensitive TMA assay took 3 weeks (with a wide range of 0 to 48 weeks). Multivariate regression analysis identified baseline viral load ≤ 400,000 IU/mL, a rapid initial virologic decline as well as fibrosis stage < 3 as predictive parameters associated with an accelerated on-treatment response by TMA assay in already bDNA negative patients. In earlier studies with different settings these baseline parameters were shown to be reliable in the prediction of SVR or non-SVR without considering end-of-treatment response.[18-20] Concerning telaprevir-based combination therapy it could be demonstrated that high viremia as well as advanced fibrosis were associated with treatment failure.[7] Our analyses clearly indicate that these parameters predominantly affect viral kinetics, in particular complete viral suppression. Thus, the development of viral resistance and relapse is promoted.

In a couple of recent studies it has been suggested that treatment outcome correlated well with viral kinetics based on mathematical and statistical models reflecting the biphasic decay of viremia.[21-22] The first-phase slope within the first 24-48 h after initiation of IFN therapy is rapid and mainly determined by the free virion clearance rate and treatment efficacy whereas the second-phase slope is supposed to be influenced by the infected cell death rate and the efficacy and has large interpatient variation.[23-24] Layden, *et al.*[22] demonstrated a strong correlation between the degree of viral load reduction during the first phase and the subsequent second-phase decline slope. Based on the fact that viral clearance in the second phase results from elimination of infected liver cells by the immune system it is postulated that reaching a critical serum viral load is necessary to activate the immune system. In our study we did not have the chance to assess viral decline during the first 48 h of treatment corresponding to the first phase as, according to the protocol, patient visits were at least once a week. However, we postulate that the initial viral decline may have a strong influence on viral response and is highly predictive to dispose of minimal residual viral replication rather quickly. We could also show that baseline viral load was significantly correlated to the period from being already HCV RNA negative by bDNA assay to become undetectable by the more sensitive TMA test. The distinction of high and low viral load defined as above or below 400,000 IU/mL turned out to be much more crucial than stratification between ≤ 800,000 *vs.* > 800,000 IU/mL. Moreover, patients with rapid on-treatment response (defined as HCV RNA < 615 IU/mL within the first 4 treatment weeks) required a significant shorter period from bDNA negativity to TMA negativity compared to those with bDNA viral response beyond week 4. We already know that these parameters are predictive for achieving a sustained viral response, but it hasn't documented so far that they also influence very significantly the interval of minimal residual viremia.[25-29] Earlier studies with standard treatment time revealed the importance of HCV RNA negativity by more sensitive tests during or at the end of treatment in order to achieve SVR.[14,30] Harrington, *et al.*, pointed out the clinical relevance of detectable, but not quantifiable HCV RNA (HCV RNA < 25 IU/mL) during boceprevir or telaprevir treatment. Patients with on-treatment HCV RNA results of detectable (HCV RNA < 25 IU/mL, but > 9.3 IU/mL) had a reduced SVR-rate compared to those with undetectable HCV RNA at the same time point.[13] Our results supported the fact that minimal residual viral replication was associated with virologic relapse. It was clarified that not only the time point of becoming HCV RNA undetectable by sensitive assay is important, but also the period of minimal residual viral replication. Whether relapse could have been prevented by continuing treatment for a longer duration in those patients remains speculative. According to the study protocol there hadn't been a more stringent measuring of bDNA and TMA beyond week 8 so we abstained from defining exact time points for those patients who became HCV RNA undetectable thereafter. The TMA assay allows in comparison to the less sensitive bDNA test an approximately 2 $\log_{10}$ improvement in sensitivity. It would be quite interesting to assess the effect of an assay with even lower detection limit, because it remains unknown how rapidly viremia is completely suppressed and whether one could calculate the time point of complete suppression by less sensitive tests. As noted above our findings supported the concept that relapse is mainly due to long-term persistence of a minimal residual viremia, i.e. relapse patients may be in fact non responders on a minimal replication level. Patients with a short period of minimal residual viral replication had a higher likelihood to achieve sustained virological response and to prevent a virological relapse.

In conclusion our study clearly indicates that the period of minimal residual viremia was of prognostic relevance for the prediction of relapse. Early viral kinetics as well as knowledge of certain baseline predictors are important tools to assess the likelihood of persistent minimal replication in responder patients during interferon-based therapy. The data have implications for designing a more refined treatment strategy in HCV infection, even in the setting of current triple therapy.

ABBREVIATIONS

- **HCV:** hepatitis C virus.
- **IFN:** interferon.
- **RBV:** ribavirin.
- **ALT:** alanine aminotransferase.
- **GGT:** gamma-glutamyltranspepdidase.
- **BMI:** body mass index.
- **SVR:** sustained virologic response.
- **NR:** nonresponse.
- **RNA:** ribonucleic acid.
- **DNA:** deoxyribonucleic acid.
- **bDNA:** branched DNA.
- **TMA:** transcription-mediated amplification.

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FINANCIAL DISCLOSURES

- Viola Knop has no conflict of interest.
- Gerlinde Teuber has no conflict of interest.
- Hartwig Klinker has no conflict of interest.
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- Tilman Gerlach has no conflict of interest.
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- Peter Buggisch has no conflict of interest.
- Konrad Neumann has no conflict of interest.
- Christoph Sarrazin provides research support for Schering-Plough/Merck and Roche pharma.
- Stefan Zeuzem is a clinical investigator, consultant and member of the speaker,s bureau of Schering-Plough/Merck and Roche pharma.
- Thomas Berg received an unrestricted grant and is an investigator of Essex Pharma, a subsidiary of Schering-Plough/Merck (Kenilworth, NJ, jetzt Merck, MSD USA), and has served on speakers bureaus for Schering-Plough/Merck (Kenilworth, NJ, USA), Essex Pharma (Munich, Germany) and Roche pharma.

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