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Evaluation of hepatitis C virus RNA RT-PCR qualitative and quantitative second generation assays

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Abstract

Hepatitis C virus (HCV) RNA qualitative and quantitative second generation assays (Amplicor HCV v2.0 and Amplicor HCV Monitor v2.0, respectively) were evaluated by testing serum samples from 132 blood donors anti-HCV positive HCV RNA negative by first generation qualitative assay and 326 viremic patients. An HCV RNA transcript was synthesized and ten-fold dilutions were used to assess sensitivity. Second generation assays were one log more sensitive than their respective first generation tests (10^2 copies per ml vs. 10^3 for the qualitative tests; 10^3 copies per ml vs. 10^4 for the quantitative tests). From the 132 anti-HCV positive RNA negative subjects, 6 (5%) were positive by Amplicor v2.0. Quantification figures by Monitor v2.0 were similar in genotypes 1, 2 and 3, whereas Monitor 1.0 values were higher in genotype 1 than in genotype 2 or 3. In 114 patients, branched-DNA v2.0 obtained higher values than Monitor v2.0 and Monitor v1.0 (6.6 90.6 log RNA copies per ml, 6.490.6, and 5.390.7, respectively, *P*B0.001). HCV RNA qualitative and quantitative second generation assays are more sensitive and genotype independent than first generation assays.

Keywords : HCV RNA; Hepatitis C; RT:PCR

1. Introduction

The hepatitis C virus (HCV), a single-stranded RNA virus, is now the etiologic agent of most cases of post-transfusion and sporadic non-A, non-B hepatitis worldwide (Choo et al., 1989; Kuo et al., 1989).

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This infection has a high rate of persistence and progression to chronic liver dis-ease (Di Bisceglie et al., 1991; Esteban et al., 1991). HCV infection is a leading cause of end-stage liver disease requiring liver transplantation, and is also associated with hepatocellular car-cinoma (Bruix et al., 1989; Read et al., 1991). In recent years, the development and wide-spread use of techniques for detection and quanti-tation of HCV RNA has provided useful information on viral dynamics during natural his-tory of the infection and antiviral treatment, and has permitted the identification of predictors of response to therapy (genotype, viral load and quasiespecies) (Martinot-Peignoux et al., 1995; Pawloski et al., 1996; Hoofnagle and Di

Bisceglie, 1997). Most clinical trials on therapy of chronic hepatitis C are based on 'in-house' RT-PCR methods that are both time consuming and non- tandardized. Furthermore, expert technical per-sonnel and reference laboratories are needed to carry out such techniques. Therefore, it has been difficult to generalize this knowledge to outine clinical practice.

Commercial assays can overcome these difficulties, are easier to use and well standardized. However, first generation tests have technical limitations, such as low sensitivity and:or different levels of efficiency according to HCV genotype.

Recently, two assays based on RT:PCR have been commercialized for research use: Amplicor HCV and Amplicor HCV Monitor, which are qualitative and quantitative tests, respectively (Colucci and Gutekunst, 1997). Amplicor HCV Monitor has been evaluated clinically by several studies and some disadvantages have emerged: lower efficiency of detection of genotypes 2 and 3 in comparison with genotype 1, low reproducibil-ity (Hawkins et al., 1997) and underestimation of HCV viral load in samples with more than 5_10 5 copies per ml (Roth et al., 1996).

The aim of this study was to evaluate the sensitivity, specificity, reproducibility and accu-racy of Roche HCV RNA qualitative and quanti-tative RT:PCR second generation assays, as compared with the respective first generation versions.

2. Materials and methods

2.1. Clinical specimens

Serum samples were obtained from two groups of patients:

1. Anti-HCV positive blood donors (*n*=132): identified at screening for HCV anti-bodies of blood donations. All had undetectable HCV RNA by Amplicor v1.0. They all were anti-HCV positive by ELISA second generation assay, RIBA second generation assay positive or indeterminate, and anti-E2 positive. In case of RIBA second generation indeterminate, samples were retested with RIBA third generation.

2. Chronic hepatitis C patients (*n*_326): the criteria for inclusion in this group were anti-HCV positive by ELISA second generation essay, elevated serum ALT levels, HCV RNA detectable by first generation RT:PCR assay and liver histology compatible with chronic hepatitis. The demographic and clinical characteristics of this group of patients are summarized in Table 1. This group is a representative sample from HCV nfected patients in the Barcelona area.

All blood samples were drawn into Vacutainer tubes with no additives (Becton & Dickinson, Meylan, France) containing a silicone separator and centrifuged within 2 h of collection, and the serum was aliquoted and kept at _80°C until further testing. Two serum samples were taken within 6 months in both groups of patients.

Table 1
Demographic and clinical features of 326 viremic patients

Mean age (year)	47+14
Sex (males)	167(51%)
Percutaneous risk factor	
Transfusion	116 (36)
IVDA [a]	29 (9)
Unknown	181 (55)
Estimated duration of infection (year)	23+12
HCV genotype	
Type 1	265 (81)
Type 2	24 (7)
Type 3	20 (6)
Type 4	17 (5)
Li6er histology	
Necroinflammation grade[b]	6+2.3
Fibrosis stage[c]	2+1.4

a IVDA, intravenous drug abuse.
b Scores range from 0 to 18, with higher scores indicating more severe necroinflammation.
c Scores range from 0 to 6, with higher scores indicating more severe fibrosis and architectural distortion. A value of 6 corresponds to liver cirrhosis.

The viral genotype was determined by InnolipaII (Innogenetics, Zwijndrecht, Belgium) according to the manufacturer's protocol. Non-viremic sam-ples were serotyped with an NS-4 based typing assay (Murex).

2.2. HCV anti-E 2 testing

HCV anti-E2 antibodies were measured using a chemiluminescent assay (IMX, Abbott Laborato-ries, Abbott Park, IL). Results were expressed as the ratio of optical density of the sample to the negative cut-off (*S*:*N*). Samples with a ratio over 1 were considered positive.

2.3. Qualitative RT-PCR

HCV RNA qualitative viremia was undertaken on 200 ml of serum by commercially available second generation RT:PCR (Amplicor HCV v2.0, Roche Molecular Systems, Banchburg, NJ) fol-lowing the manufacturer's instructions. Serum samples were tested in duplicate.

2.4. Quantitative RT-PCR

HCV RNA quantitative viremia was carried out on 100 ml of serum by commercially available second generation RT:PCR (Amplicor HCV Monitor v2.0 Roche Molecular Systems, Banchburg, NJ) according to the manufacturer's instructions. It is based on the same general configuration of the qualitative test, but incorporates an internal standard that serves to assess the efficacy of RNA extraction, amplification and detection, and that is also a reference for calculat-ing the HCV copy number. All results of quanti-tative HCV viremia were expressed as log 10 copies per ml. Samples with viremia greater than 5_10^5 (5.7 log) HCV copies per ml were diluted 1:100 in human HCV RNA negative serum and retested. All serum samples were tested in duplicate.

2.5. Branched-DNA testing

In a subset of 127 patients, HCV RNA was quantified by branched-DNA v2.0 (Quantiplex HCV RNA 2.0, Chiron Diagnostics, Emeryville, CA). This commercial assay is based in hybridization between unamplified HCV RNA and a labeled probe, and detection of the amplified signal.

2.6. Synthesis and quantitation of a standard RNA

An RNA standard representing the 5% NCR of HCV was synthesized in vitro, and the purified transcript was quantified by isotopic tracing. This is a reliable method and has been used to quantify the primary reference standard, but because la-beled RNA is unstable over time, the hot stan-dard was used as a reference to quantify a second stable (nonisotopically labeled) reference standard that can be remade periodically as needed. Four micrograms of a plasmid containing the first half of the HCV genome (genotype 1b) was linearized with *Aat*II, and two in vitro transcriptions, one with [a-32 P] GTP and another without isotopic label, were carried out in parallel. The RNA transcripts were subjected to three successive steps of purification: DNAse I degradation, phenol extraction, and CFII cellulose chromatography to eliminate DNA and the unincorporated nucleotides, followed by polyacrylamide gel electrophoresis to separate the complete RNA transcript from the unfinished transcription fragments and other minor forms. Both labeled and unlabeled RNAs were run in parallel, and bands were eluted from the polyacrylamide gel. All reac-tions were carried out in siliconized-glass tubes
with tRNA as a carrier. The amount of RNA transcript labeled isotopically was measured in a scintillation counter, and the amount of RNA synthesized was calculated from the known incor-poration percentage, as follows: *N* x counts per mn incorporated : total counts per min, where *N* is the quantity of eoxynucleoside triphos-phates (in micrograms) included in the reaction mixture. The following assumptions are made: the synthesized RNA contains equimolar amounts of all four ribonucleotides, the average

molecular weight of a ucleotide is 325, and the contribution of [a-32 P] GTP is negli-gible.

Although, the amounts of all four ribonu-cleotides are not the same, the molecular weight of the RNA synthesized was only 0.2% lower than the estimated molecular weight. Serial dilutions (10^7 –10^3) of the labeled RNA transcript were used as standards to quantify by real-time RT:

PCR (Martell et al., 1999) the HCV RNA in two dilutions containing unknown quantities of the unlabeled transcript.

The integrity of the synthesized transcripts, both labeled and non-labeled, was verified by migration by gel electrophoresis. Two oligonucle-otides (C24 [5%-GGGGAGTGATCTATGGTG-GAG-3%] and C339 [GAGGATCCGGTTTAGG-ATTCGTGCTCATGGT-3%] that are known to hybridize efficiently and specifically with the flanking regions of the amplified fragment were labeled at the at the 5% end with [g-32]ATP and were used to analyze the unlabeled RNA tran-script.

After the annealing reactions, hybrids were subsequently electrophoresed under nondenaturing gel conditions in parallel with an aliquot of the labeled transcript.

Serial 10-fold dilutions from a standard stock of 10 8 copies per ml were used to assess the sensitivity of qualitative and quantitative assays.

2. 7. *Statistical analysis*

Continuous variables are expressed as mean values and standard deviation. Differences in viral load were analyzed by the *T*-test or the Mann– Whitney *U*-test for comparison of means, and the anlysis of variation (ANOVA) or Kruskal–Wallis test when comparing more than two groups. The coefficient of variation (CV) was obtained by dividing the standard deviation by its mean and multiplying that result by 100. Statistical significance was assessed at the $P<0.05$ level. Data processing and analysis were performed with SPSS software version 8.0 (Statistical Package of Services Solutions, SPSS Inc., Chicago, IL).

3. Results

3. 1. *Qualitative RNA assays*

HCV RNA transcript was detected by Am-plicor v2.0 until the dilution corresponding to 10^2 copies per ml, and by Amplicor v1.0 until the dilution of 10^3 copies per ml.

From the 132 anti-HCV positive RNA negative (by Amplicor v1.0) blood donors, 6 (5%) of them were repeatedly positive by Amplicor v2.0.

Table 2 summarizes demographic and serologic charac-teristics of the 132 blood donors grouped accord-ing to Amplicor v2.0 results. Differences between these groups were found for ALT levels (97+120 IU/dl vs. 22+12, P=0.035) and anti-E2 levels (120+54 vs 44+54, P=0.004).

3. 2. Quantitative RNA assays

Lower limits of detection were 10^3 HCV RNA transcript copies per ml for Monitor v2.0 and 10^4 copies per ml for Monitor v1.0. Quantification of HCV RNA transcript ten-fold dilutions have a good correlation in Monitor v2.0 between the dilutions containing 10 3 and 10 6 copies per ml, and in Monitor v1.0 between dilutions containing 10^4 and 10^6 . Monitor v2.0 inter-assay coefficient of variation was of 5.5% (referred to log10) testing repeatedly HCV RNA transcript dilution of 10 3 in 25 runs.

For each viremic patient, two serial samples taken within 6 months were tested by Monitor v2.0. In 90% of the cases, the difference between samples from the same patient was less than 1 log, and in 70% of cases the difference was less than 0.5 log. Fig. 1 summarizes these data.

From the 326 viremics patients, 265 were geno-type 1, 24 genotype 2, 20 genotype 3 and 17 genotype 4. Quantification of samples by Monitor v2.0 showed a higher HCV RNA viral load than by Monitor v1.0 (6.2+0.9 log RNA copies/ml vs. 5.3+0.9, for all HCV genotypes, P=0.001, 95% CI of difference: [0.8 –1.0]), and

the difference increased in genotypes other than 1 (Fig. 2).

There were no differences in mean viral load between genotypes 1, 2 and 3 measured by Moni-tor v2.0 (6.2+0.9 log RNA copies per ml, 6.4+ 0.7 and 6.3+0.7 log, respectively). Mean viral load of genotype 4 was lower than the mean in

other genotypes tested by Monitor v2.0 (5.7+0.5 log HCV RNA copies per ml vs. 6.2+0.8 log copies per ml, *P*=0.01,

Table 2
Demographic and virologic characteristics of blood donors anti-HCV positive and HCV RNA undetectable by Amplicor 1.0 grouped according to Amplicor 2.0 test results.

Characteristic	Amp2.0 negative (*n*=126)	Amp2.0 positive (*n*=6)	*P*
Age	37+11	48+13	NS
Sex (male)	50%	38%	NS
Risk Factor			
Transfusion	1 (16%)	42 (33%)	
IVDA	1 (16%)	5 (4%)	
Unknown	79(63%)	4(66%)	NS
ALT level (IU/l)	22+12	97+120	0.035
HCV serotype			
1	33 (26%)	5 (83%)	
2	3 (2.4%)	1 (16%)	
3	4 (3.2%)	0	
4	3 (2.4%)	0	
Negative	83 (66%)	0	NS
Anti-E2 O.D.(*S*:*N*)	44+54	120+54	0.004
RIBA 2 *and* 3			
Positive	82 (65%)	6 (100%)	
Indeterminate	44 (35%)	0	NS

IVDA, intravenous drug abuser.
All patients were RIBA2 positive or indeterminate, the last samples were tested by RIBA3.
NS, non-statistical significance.

95% confidence interval of the difference 0.1–1.0 log). See Fig. 2. In a subgroup of 127 HCV infected patients, samples were also quantified by branched-DNA v2.0. From these, 13 (10.2%) had a negative result. The viral load of the remainder 114 patients was higher measured by branched-DNA v2.0 than by Monitor v2.0 (6.6+0.6 log HCV RNA copies per ml vs. 6.4+0.6 log, *P*=0.01, 95% confidence interval of difference 0.14–0.34 log), and lower values than branched-DNA v2.0 and Monitor v2.0 were obtained by Monitor v1.0 (5.3+0.7 log HCV RNA copies per ml, *P*=0.001, Fig. 3).

4. Discussion

Detection and quantitation of HCV RNA has become an essential tool for clinical investigation. However, interpretation of clinical trial data may not be accurate without a previous knowledge of technical limitations of the HCV RNA test used.

Methods for HCV RNA detection and quantitation should be sensitive, specific, reproducible and accurate.

The analytical sensitivity of Amplicor v2.0, i.e., the lower limit of detection, was 100 HCV RNA copies per ml. It improved 1 log the lower limit of Amplicor v1.0, and is comparable with in-house reference methods used in large multicenter HCV treatment trials (McHutchinson et al., 1996; Poy-nard et al., 1998). Amplicor v2.0 was also shown to be a more precise

diagnostic tool in low-risk individuals as blood donors with anti-HCV anti-bodies but RNA undetectable by Amplicor v1.0. A small proportion of these patients had been considered erroneously free of HCV infection, thus hampering their adequate clinical management. Conse- quently, any anti-HCV positive patient should be considered as potentially infected unless viremia has been ruled out by an RNA test capable of detecting 10 2 HCV copies per ml. A correlation between anti-E2 levels and the presence of HCV viremia has been found, suggesting that continu-ous antigenic stimulation is necessary for the production of these antibodies.

Amplicor HCV Monitor v2.0 is the most sensitive commercial quantitative assay available (the lower limit of detection of b-DNA v2.0 has been established at 5.3 log per ml [Detmer et al.]). The lower limit of detection of Monitor v2.0 (10^3 copies per ml) can be complemented by testing negative samples with Amplicor v2.0. Thus, both assays can quantify samples with viral load between 10^2 and 10^6 copies per ml.

As for reproducibility, an interassay CV of a 5.5% was obtained with Monitor v2.0, which represents 0.16 log10, a range of variation that has no clinical significance.

Monitor v1.0 had underestimated viral load in genotype 2 and 3 compared with genotype 1 (Hawkins et al., 1997).

Branched-DNA v2.0 is reported to quantify equally all RNA transcripts analyzed, including sequences of the six main genotypes (Detmer et al., 1996). Primer sequences and RT:PCR conditions have been modified in the second version of Monitor in order to achieve equal quantification of all known genotypes. Our data confirm this fact for genotypes 1, 2, and 3. Furthermore, the difference in quantitation between genotype 4 and genotypes 1–3 has been reduced to one third (from 1.5 to 0.5 log).

Quantitation of a sample by different methods produces different results, as it is showed in this study. Monitor v2.0 values are lower than that of branched-DNA v2.0, but the mean difference is only 0.2 log per ml, which has little clinical value.

However, Monitor v1.0 mean values are about 1 log lower than these of the other two assays.

It is now accepted that qualitative and quantitative HCV RNA tests will soon find a place in the routine management of HCV-infected individ-uals.

Assays available worldwide, easy to carry out and highly standardized are needed. Amplicor and Monitor v2.0 seem to be adequate candidates.

Baseline viral load is one of the powerful

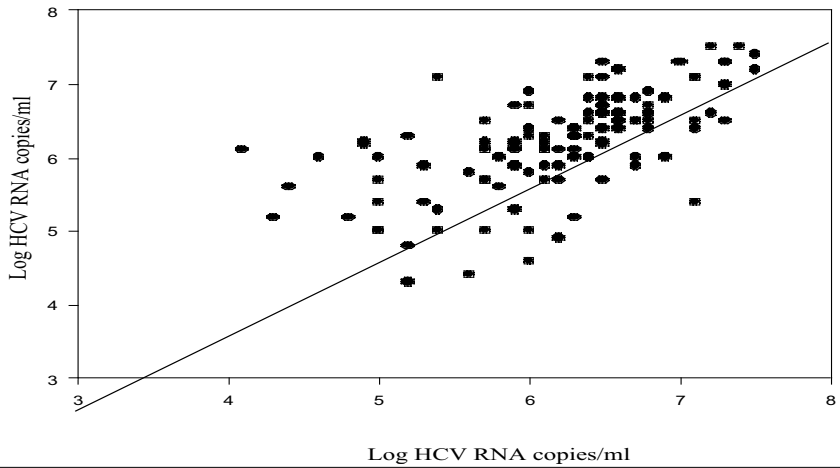

Fig. 1. Correlation between HCV RNA quantitation of two different samples from each patient by Monitor v2.0. The coordinates of each point represent the results of the RNA quantification in the two samples of each patient.

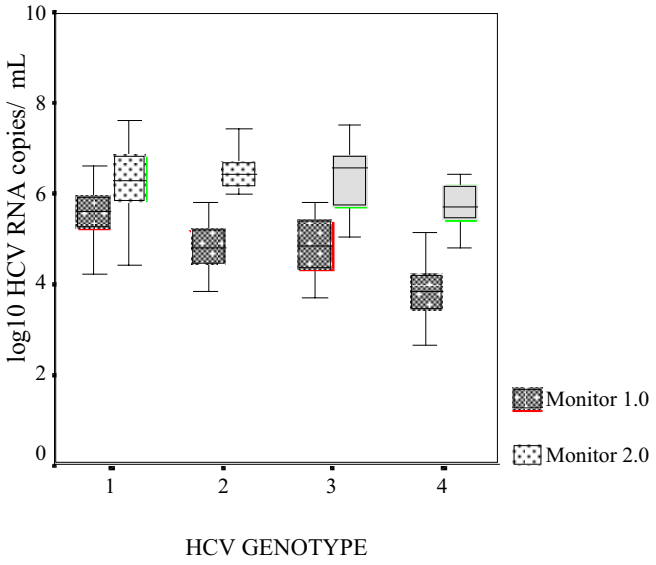

Figure 2. Mean values of HCV viremia (expressed as log 10 HCV RNA copies/ml of serum) measured by Monitor v1.0 and Monitor v2.0, grouped according on HCV genotype. There were differences in each genotype: 5.5 ± 0.7 log RNA copies/ml Vs 6.2 ± 0.9, for genotype 1, 95% CI: [0.7-0.8]; 4.6 ± 1.1 log RNA copies/ml Vs 6.4 ± 0.7, for genotype 2, 95% CI: [1.4-2.1]; 4.8 ± 0.6 log RNA copies/ml Vs 6.3 ± 0.7, for genotype 3, 95% CI: [1.2-1.8]; 3.7 ± 0.8 log RNA copies/ml Vs 5.7 ± 0.5, for genotype 4, 95% CI: [1.6-2.4], P<0.001 for all comparisons)

predictor of sustained response to antiviral therapy (Martinot-Peignoux et al., 1995; Pawloski et al., 1996; Hoofnagle and Di Bisceglie, 1997).

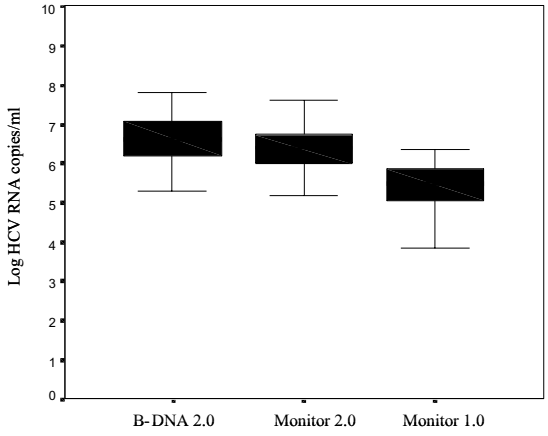

Figure 3. Values of HCV RNA (expressed as log 10 HCV RNA copies/ml of serum) measured by branched-DNA v2.0, Monitor v2.0 and Monitor v1.0 in 114 HCV infected patients. Differences between means were found: 6.6±0.6log HCV RNA copies/ml,6.4±0.6 log and 5.3±0.7log, P<0.01.

It has been reported recently that semiautomatic Amplicor HCV Monitor v2.0 (COBAS v2.0) can classify HCV infected patients as above the defined cut-off level of 2_106 HCV RNA copies per ml with similar results to SuperQuant assay (National Genetic Institute) (Martinot-Peignoux et al., 2000).

Once treatment has begun, assay of viremia can be an early and precise predictor of sustained response. It has been demonstrated recently in a study based on SuperQuant assay (National Genetic Institute) that analyzed data from 1.744 patients, HCV viral load of less than 400.000:ml at week 4 had a negative predictive value of 100% in patients that followed interferon plus ribavirin therapy for 6 months, and 97% in patients that followed a 12 months therapy regimen (Poynard et al., 2000). Monitor v2.0 dynamic range of 10 3 –10 6 copies per ml seems to be appropriate to study viral dynamics of response to antiviral treatment. We have studied viral dynamics during antiviral therapy using Amplicor v2.0 and Moni-tor v2.0. The results support the utility of both assays to predict the long-term outcome (Castro et al., 2000; Sauleda et al., 2000).

Roche HCV RNA qualitative and quantitative second generation assays have appropriately sensitivity, specificity, reproducibility and accuracy and can be very useful in routine clinical manage-ment of HCV-infected patients.

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ARTÍCULO 2

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Utility of early testing for HCV viremia as predictive factor of sustained response during interferon or interferon plus ribavirin treatment

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Background/Aim: **To evaluate the utility of early testing for hepatitis C viremia as a predictor of treatment outcome during interferon or combination therapy.**
Methods: **We studied 184 patients with chronic hepatitis C who received interferon and were monitored for HCV RNA. Sixty-two patients received interferon alone for 12 months and 122 patients, who were still HCV RNA positive at 2 months, received an additional 12-month course of interferon and ribavirin combination therapy.**
Results: **Using this strategy, sustained response occurred in a total of 34 patients (18.5%). Independent variables associated with sustained response were HCV genotype (p=0.06), viral load <5.1 logs/ml (p_ 0.005) and negative HCV RNA at 1 month (p<0.0001) in the interferon group, and female sex (p=0.04), genotype (p=0.03), viral load <5.5 logs/ml (p=0.01), normal ALT (p=0.001) and decline in viral load ¯1.2 logs/ml after 2 months of interferon mono-therapy (p<0.001) and negative viremia at 5 months of ribavirin onset (p<0.0001) in the combination therapy group. Persistence of viremia at 1 month of interferon monotherapy and at 5 months of combination therapy were the strongest predictors of non-response (negative predictive value of 100% and 99%, respectively).**
Conclusions: **Qualitative assessment of HCV RNA during treatment is the strongest predictor of sustained response during interferon or combination therapy for chronic hepatitis C.**

Key words: **HCV RNA; Interferon; Predictors of response; Response to therapy; Ribavirin.**

Interferon is the mainstay of treatment for chronic hepatitis C virus (HCV) infection. However, sustained response occurs in less than 30% of treated pa-tients (1–3). The economic cost is very high, and, furthermore, it has several adverse effects. There have therefore been some attempts to choose the best candidates for interferon treatment, in order to improve cost-benefit balance. Some factors have been associated with sustained long-term response to interferon: young age, short duration of infection, absence of cirrhosis, low hepatic iron stores, low HCV RNA levels, and genotype other than 1 or 4 (1–3). Nevertheless, these predictors can hardly determine long-ter out-come in an individual patient.

Several studies have investigated the utility monitoring serum HCV RNA during interferc treatment as a predictor of sustained respons (4–12). In most, early loss of detectable HCV RN (between 1 and 4 months) is the stronge predictor of long-term sustained respons However, most of these studies have been dor using home-made RT/PCR techniques that a not standardized among laboratories and requi complex installations and trained personne Therefore, it seems difficult to generalize the us

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Recently, combination therapy with interferon and ribavirin has been shown to be more effective tha in-terferon alone in both naive patients and thos who have relapsed after interferon treatmer (13–16). Al-though several pre-treatment factors such as HCV genotype other than 1, low base-lin viral load, absence

of cirrhosis and female sex, have been associated with sustained response, the dynamics of viral clearance during combination therapy has not been established, and the utility of monitoring for serum HCV RNA has not been assessed.

We have investigated the utility of testing for serum HCV RNA using a standardized commercially available RT/PCR test to predict therapy outcome in prospectively followed patients treated with interferon or combination therapy.

Subjects and Methods

From 1995 to 1997 we enrolled 184 chronic hepatitis C patients with indications for IFN treatment (elevated ALT values, detectable HCV RNA in serum and histological evidence of chronic hepatitis). Exclusion criteria were HIV infection, hemophilia, HBV infection, alcohol abuse, other causes of liver disease, and major contraindications to interferon therapy.

An initial observation period of 6 months was established to accomplish a clinical evaluation, monthly ALT determination, HCV genotyping and quantification of HCV baseline viremia. All patients began interferon alfa-2b (Intron-A, Schering-Plough, Kenilworth, NV, USA) treatment at standard doses (3 MU/tiw). Alanine amino-transferase (ALT) and qualitative RT/PCR for serum HCV RNA were tested every week during the first month, and biweekly during the second month of IFN therapy. At week 8, patients who had be-come serum HCV RNA negative were continued on IFN for another 10 months, with monthly ALT and HCV RNA determination. Those in whom HCV RNA was still detectable were offered combination treatment with IFN and ribavirin for 1 year. ALT and qualitative HCV viremia were assessed monthly.

Post-treatment follow-up period consisted of a minimum of 6 months, with monthly determination of ALT. Qualitative HCV viremia was tested 6 months after the end of therapy. A post-treatment liver biopsy was also obtained after informed consent in a subset of patients, 6 months to 1 year after completion of therapy. In addition, quantitation of HCV viremia after the first 2 months of IFN treatment was performed in serum samples from a subset of 43 patients who had completed a year of combination treatment. In this group there was a similar proportion of sustained responders (15), relapsers (14) or non-responders (14).

HCV RNA testing

In all cases blood samples were centrifuged within 2 h of collection and the serum aliquoted and stored at –80º until further testing.

Qualitative RT/PCR for HCV RNA was performed using a microwell plate-based detection test, Amplicor A HCV v1.0 (Roche Diagnostic Systems Inc., Branchburg, NJ, USA) according to the manufacturer's instructions. In all patients the serum sample obtained at 4 weeks of treatment was also assessed with an improved second-generation RT/ PCR test (Amplicor HCV v2.0). Similarly, monthly samples from patients who received combination therapy were tested with Amplicor HCV v2.0. The lower detection limit of these techniques is 1000 copies/ ml for Amplicor HCV v1.0 and less than 100 copies/ml for Amplicor HCV v2.0 (17). Quantitative RT-PCR for HCV was performed using Amplicor HCV Monitor A v2.0 (Roche Diagnostic Systems Inc., Branchburg, NJ, USA) according to the manufacturer's instructions (18). All results of quantitative HCV viremia are expressed as log10 copies/ml.

Viral genotype was determined with a commercial strip hybridiza-tion assay (Inno-lipa II Innogenetics, Zwijndrecht, Belgium).

Liver biopsies were examined by an expert pathologist, who gave a numeric score referring to necroinflammatory activity (Grade, from 0 to 18) and another score referring to fibrosis and architectural distortion (Stage, from 1 to 6), following the method defined by Ishaaket al. (19).

Response definitions

A patient was considered to have a sustained response when ALT values remained normal and HCV RNA undetectable for 6 months after the end of therapy. Relapse was defined as the normalization of ALT values during treatment, followed by elevation of ALT or detectable HCV RNA after stopping therapy. All other patients were considered non-responders.

Statistical analysis

Baseline data were descriptively summarized. Differences between groups were analyzed using Student's *t*-test and X^2 methods, for quantitative and qualitative variables, respectively. The positive and nega-tive predictive value, specificity, andsensitivity were assessed as previously described (20). Multivariate analysis was performed by multiple logistic regression, and stepwise discriminant-function analysis was used to predict sustained response. Differences in the histological indexes before and after therapy were analyzed by the paired *t*-test or the Wilcoxon rank-sum test. All statistical significance was assessed at the $p<0.05$ level. All data analyses were carried out using the SPSS for Windows, version 7.5 (Statistical Package of Services Solutions, SPSS Inc., Chicago, IL, USA).

Results

After 8 weeks of interferon treatment, HCV viremia was undetectable in 27 (15%) patients, and all of them continued interferon for 10 more months. Of the remaining 157 viremic patients, 122 were given combination treatment for a year, and 35 completed a year of interferon monotherapy (the latter did not receive RBV because they did not consent, there was a contraindication to treatment or because of discontinuity in RBV supply). Baseline charac-teristics of patients are summarized in Table 1.

IFN group

Of the 62 patients who received interferon alone, 13 (21%) patients achieved a sustained response, 33 (53%) became relapsers, 13 (21%) did not respond, and 3 (5%) discontinued therapy because of side-effects.

Pretreatment factors analyzed were sex, age, age at infection, duration of infection, risk factor, previous interferon therapy, baseline ALT, genotype, baseline HCV viremia, and histological indexes (Grade and Stage). Previous transfusion was associated with a lower response rate (4% *versus* 35%, $p=0.004$), whereas an HCV genotype other than 1 or 4 and a viral load of 5.1 log copies/ml or less were associated with a higher response rate (47% *versus* 12%, $p=0.006$ and 60% *versus* 14%, $p=0.005$, respec-tively). Other basal factors had no relation with sustained response ($p\pm0.05$).

Multivariate analysis of pretreatment factors by step-ise logistic regression identified absence of transfusion, genotype other than 1 or 4 and viral load of 5.1 log/ml or less as independent predictors of sustained response.

Results of qualitative HCV viremia using Amplicor HCV v1.0 during IFN treatment showed that all sustained responders were serum HCV RNA negative by

Table1. Baseline characteristics of patients according to treatment group.*

Characteristic	Interferon (n=62)	Combination therapy (n=122)
Age-years	39±13	46±13
Sex-males	39(63%)	79(65%)
Age at infection-years	21±12	22±14
Duration of infection-years†	17±9	21±13
Parenteral risk factor		
Transfusion	25(40%)	44(36%)
Intravenous drug use	12(19%)	10(8%)
Unknown	23(37%)	68(56%)
Naive patients	52(84%)	81(66%)
Baseline ALT-U/ml	92±63	107±61
Genotype		
1	41(66%)	109(89%)
2	7(11%)	4(3%)
3	10(16%)	4(3%)
4	4(6%)	5(4%)
Serum HCV RNA (log 10 copies/ml)	6.0±0.8	6.3±0.7
Liver histology		
Grade‡	5.8±2	6.1±2.4
Stage§	2.4±1.3	3±1.6

*Plus-minus values are means ±SD. Because of rounding, percentages may not total 100.
†The duration of infection was estimated from the date of transfusion or initial exposure to parenteral sources, and it could not be calculated for patients in whom the source of infection was unknown.
‡Scores could range from 0 to 18, with higher scores indicating more severe necroinflammation.
§Scores could range from 0 to 6, with higher scores indicating more severe fibrosis and architectural distorsion. A value of 6 corresponds to liver cirrhosis.

week 4. Serum HCV RNA at week 4 as measured by Amplicor HCV v1.0 was a strong predictor of sus-tained response with a sensitivity (that is the pro-portion of sustained responders who had undetectable HCV viremia) of 100%, specificity (that is the pro-portion of relapsers and non-responders who had de-tectable viremia) of 74%, positive predictive value (that is the proportion of non-viremic patients who achieved a sustained response) of 52%, and negative predictive value (that is the proportion of viremic patients who did not achieve a sustained response) of 100%. When tested by the more sensitive Amplicor HCV v2.0, loss of detectable HCV RNA at 1 month was an even stronger predictor of sustained response (sensitivity of 100%, specificity of 93%, positive and negative predic-tive value of 81% and 100%, respectively). Fig. 1 sum-marizes Amplicor HCV v2.0 results at 1 month of in-terferon therapy. The maximum sensitivity and speci-ficity to predict sustained response was for the associ-ation of a normal ALT value and undetectable HCV RNA by Amplicor HCV v2.0 at 4 weeks (sensitivity of 100%, specificity of 98%, positive and negative predic-tive value of 93% and of 100%, respectively) (Table 2).

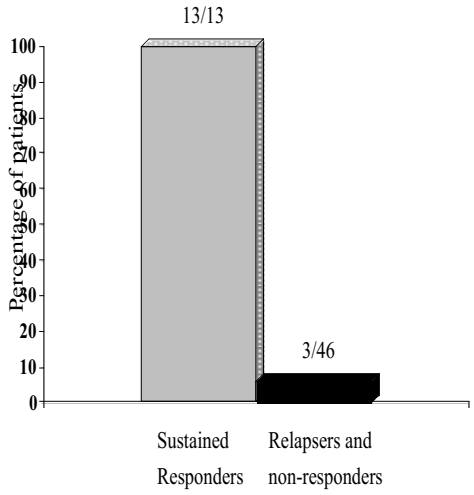

Fig. 1. Proportion of patients with undetectable serum HCV RNA by Amplicor HCV v2.0 at 1 month in the interferon monotherapy group according to treatment outcome.

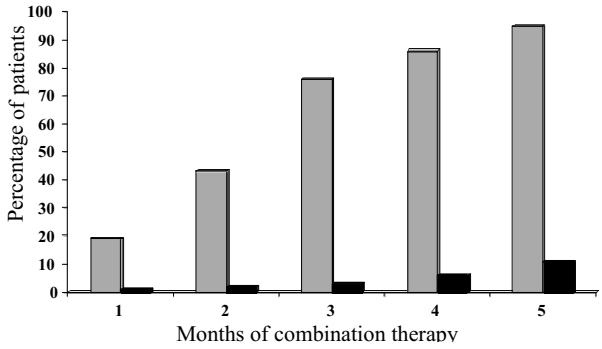

Fig. 2. Cumulative proportion of patients with a negative Amplicor HCV v2.0 test by month after onset of ribavirin in the combination therapy group according to treatment response. Grey bars summarize sustained responders' re-sults and black bars non-sustained responders' results.

In multivariate analysis, a stepwise logistic re-gression identified loss of detectable HCV viremia at 1 month of treatment by Amplicor HCV v2.0 (p °0.001) as the stronger predictor of sustained response. Fur-thermore, when this variable was entered in the predic-tive equation, no other variable improved the predic-tion (Table 3).

A liver biopsy was obtained 6 to 12 months after end of therapy in 22 patients (10 sustained responders and 12 relapsers). While no significant differences on fibrosis were observed with respect to the pre-treatment biopsy in any group, a significant decrease in necroinflammatory activity was observed in sustained re-sponders (mean -4.5, 95% confidence interval (-5.6,-3.4), p<0.001).

Table 2. Utility of HCV RNA testing at one month of interferon monotherapy to predict sustained response. Comparison of two versions of the qualitative Amplicor HCV assay.

Assay version	Sensitivity	Specificity	PPV	NPV
Amplicor v1.0	100%	72%	52%	100%
Amplicor v2.0	100%	94%	81%	100%

PPV: positive predictive value. NPV: negative predictive value.

Combination treatment group

Of the 122 patients who received IFN and RBV ther- apy, 21 (17%) achieved a sustained response, 66 (54%) became relapsers, 22 (18%) did not respond, and 13 (11%) discontinued treatment due to side-effects.

We evaluated pretreatment factors: sex, age, dur- ation of infection, age of infection, risk factor, previous interferon therapy, baseline ALT, genotype, baseline HCV viremia, and histological indexes (grade and stage) as predictors of sustained response. In univariate analysis, an HCV genotype other than 1 or 4 (57% versus 17%, p=0.035), a baseline viral load of 5.5 log per ml or less (44% versus 14%, p=0.007), female sex (29% versus 13%, p=0.04), and a histological grade higher than 6 (31% versus 12%, p=0.03) were associ-ated with a higher likelihood of sustained response. Other basal factors had no relation with sustained re-spc ($p\pm0.05$). Logistic regression analysis of base-line varia identified: genotype other than 1 or 4, a baseline viral load of 5.5 log per ml or less, and female sex as independent predictors of sustained response.

In addition to baseline factors, three additional vari-a obtained at 2 months of IFN monotherapy were also associated with a higher sustained response rate after combination therapy: normalization of ALT level (42% *versus* 14%, $p=0.02$), a viral load of 5.1 log/ml or (70% *versus* 13%, $p<0.001$) and a decline in viral load v respect to pretreatment values of 1.2 log/ml or more (80% *versus* 12%, $p<0.001$). When these vari-a were entered in a multivariate analysis along with pretreatment variables, a decline in viral load of 1.2 log/ml or more ($p<0.001$) was the strongest predictor of a sustained response, followed by normalization of ALT level ($p=0.001$).

Fig. 2 shows the dynamics of viral clearance during combination treatment, in patients who had a sus-tained response and those who had a relapse or non-respo All but one of the sustained responders had undetecta HCV RNA 5 months after ribavirin onset.

Serum HVC RNA as measured by Amplicor HCV v2.(5 months of combination therapy was a strong predictor of sustained response ($p°0.001$), with a sensi-

Table 3.
Distribution of predictors in the group of IFN monotherapy according to response to treatment.*

Characteristic	Sustained Response (n=13)	Relapse or Non Response (n=46)	P
Age-years	36±13	39±12	0.35
Sex: males	7(19%)	30(81%)	0.5
females	6(27%)	16(73%)	
Age at infection-years	19±3	20±14	0.8
Duration of infection-y	14±7	18±9	0.3
Parenteral risk factor:			
Transfusion	1(4%)	24(96%)	0.004
Intravenous drug use	4(33%)	8(67%)	0.5
Unknown	8(36%)	14(64%)	0.5
Naive	12(24.5%)	37(75.5%)	
Treated	1(10%)	9(90%)	0.5
ALT-U/ml	94±63	88±64	0.7
Genotype:			
1 or 4	5(12%)	37(88%)	0.006
2 or 3	8(47%)	9(53%)	
Serum HCV RNA (log 10 copies/ml)			
≤5.1	6(60%)	4(40%)	0.005
>5.1	7(14%)	42(86%)	
Grade	6.2±2	5.9±2	0.8
Stage	2.5±1	2.5±1	0.9
HCV RNA at week 4 measured by AMPLICOR HCV v2.0			
Negative	13(81%)	3(19%)	<0.001
Positive	0	43(100%)	

*Plus-minus values are means ±SD. Percentages are referred to the characteristic that defines the row.

Table 4. Predictive values for sustained response of qualitative HCV RNA testing at months 1 to 5 after ribavirine onset. Comparison of two versions of the Amplicor HCV test.

	Month	1	2	3	4	5
Amplicor v1.0	PPV	67%	50%	53%	44%	38%
	NPV	85%	88%	95%	96%	98%
Amplicor v2.0	PPV	80%	82%	84%	78%	67%
	NPV	85%	90%	94%	96%	99%

PPV: positive predictive value. NPV: negative predictive value.

tivity of 95%, specificity of 89%, positive predictive value of 67% and negative predictive value of 99%. The corresponding figures for a negative Amplicor HCV v1.0 test result at five months of combination therapy were 95%, 64%, 38%, and 98%, respectively (Table 4).

A stepwise logistic regression including all the sig-nificant variables identified qualitative HCV viremia at the fifth month of combination therapy (p°0.001) as the most powerful predictor of sustained response, followed by a decline in viral load of 1.2 log/ml or more after 2 IFN months (p°0.001). After these two variables were entered in the logistic equation, the addition of other variables could not improve the prediction (Table 5). Fifty-six post-treatment liver biopsies were available in the combination therapy group, 13 from sustained responders, 38 from relapsers and 5 from non-re-spond There was no difference in relation to stage pre- and p treatment in any group. Grade improved significantly sustained responders after therapy (mean –3.6, 9 confidence interval (»4.6,»2.7), p°0.001), while there w no differences in relapsers and non-responders.

Discussion

In order to improve the efficacy of antiviral therapy for chronic hepatitis C, several studies have investigated pretreatment factors associated with a higher likeli-hoo response (21–24). In our series two pretreat-ment fact low baseline viral load and HCV geno-type other than 1 were associated with a higher sustained response rate, bot patients treated with

Table 5. Distribution of predictors in the group of combination therapy according to response to treatment.

Characteristic		Sustained Response (n=21)	Relapse or Non Response (n=88)	P
Age-years		47±12	44±12	0.4
Sex: males		9(13%)	59(87%)	0.04
females		12(29%)	29(71%)	
Age at infection-ys		23±9	20±16	0.6
Duration of infection-ys		23±12	22±13	0.9
Parenteral risk factor				
Transfusion		8(19%)	34(81%)	
Intravenous drug use		1(11%)	8(89%)	0.5
Unknown		12(21%)	46(79%)	
Naïve		16(22%)	58(78%)	0.5
Treated		5(14%)	30(86%)	
ALT-U/ml		99±69	100±58	0.9
Genotype:	1 or 4	17(16%)	85(84%)	0.035
	2 or 3	4(57%)	3(43%)	
Serum HCV RNA (log 10 copies/ml)				
	≤5.5	8(44%)	10(56%)	0.007
	>5.5	13(14%)	78(86%)	
Grade: >6		15(32%)	32(68%)	0.03
<6		8(13%)	53(87%)	
Stage		3.1±1.5	2.9±1.5	0.6
ALT after IFNx2m: Normal		13(42%)	18(58%)	0.02
Elevated		11(14%)	67(86%)	
HCV RNA decline after IFNx2m-log10 copies/ml				
≥1.2		12(80%)	3(20%)	<0.001
<1.2		3(12%)	25(88%)	
HCV RNA at fifth m by AMPLICOR HCV v2.0:				
Negative		20(65%)	11(35%)	<0.001
Positive		1(1%)	77(99%)	

*Plus-minus values are means +SD. Percentages are referred to the characteristic that defines the row.

interferon alone and in patients treated with interferon and ribavirin. However, as in other published studies, the prognostic value of these factors is of limited value in individual cases (25).

In contrast, our study confirms previous obser-vations that qualitative HCV viremia at 1 month of interferon therapy is a very strong predictor of treat-ment outcome. Using a standardized commercially available RT/PCR test with a lower limit of detection of less than 100 copies per ml, all sustained responders had undetectable viremia by 4 weeks of interferon treatment. The fact that none of the patients who were HCV RNA positive at 8 weeks and continued inter-feron monotherapy for 12 months became sustained responders, further supports the utility of early moni-toring for HCV viremia. The predictive value of vi-remia could be improved when combined with ALT value, so that 93% of the patients who had normal ALT level and undetectable HCV RNA at 4 weeks be-came sustained responders.

In the present study the predictive value for sus-tained response of a negative Amplicor v2.0 test result at 1 month, was in fact higher than that reported for other qualitative RT/PCR assays and similar to that reported for a quantitative decline in HCV RNA level of 3 or more logs/ml at 4 weeks of interferon mono-therapy (26).

Based on these results, management of HCV-in- fected patients following interferon monotherapy could be improved by monitoring HCV RNA at 1 month by Amplicor v2.0. For those with a negative result, a complete 12-month course of interferon monotherapy would clear infection in more than 80% of patients. For patients with a positive test result at 1 month, addition of ribavirin should be considered.

Because of the study design we were able to analyze early predictive factors of eventual response in those patients who were given additional therapy with ribavi-rin after 2 months of interferon monotherapy. Al-though by definition none of these patients cleared HCV viremia during the first 2 months, those who had a decline in viral load of 1.2 log/ml or more during initial therapy were more likely to become sustained responders after addition of ribavirin. The likelihood was even higher for those who had also normalized ALT levels. These data suggest that, in fact, response to interferon plus ribavirin therapy could be better pre-dicted on the basis of early response to interferon treat-ment. This finding would be in agreement with the ob-servation that relapsers after interferon monotherapy are more likely to respond to combination treatment than naive patients.

During combination treatment, viremia was not as early a predictor of sustained response as in interferon monotherapy. Sustained responders in the combination therapy group took an average of 2.9 months to clear viremia after initiation of ribavirin. The reason for this slower clearance is probably related to different modes of action of interferon and ribavirin. These results, howeve cannot be generalized to all patients on com-binatio therapy, since our patients were given ribavir-in precisel because they had failed to clear HCV RNA after 2 months of interferon monotherapy. Hence, the precise timing at which persistence of a positive Ampl-ico v2.0 test may have the strongest negative predictive valu might be different in patients given combination treatmer from the start. Nonetheless, it seems evident that, irrespectiv of treatment schedule, sustained re-sponders afte combination therapy may take longer to clear viremia tha those treated with interferon monotherapy. Additional studie should be carried out to establish the most cost-effectiv strategy to monitor patients on standard combination therapy.

In addition it must be remembered that most (93%) of our patients on combination therapy were infected with HCV genotypes 1 or 4, and the dynamics of viral clearance among patients infected with other geno-type might be different.

In summary we have shown that monitoring for serum HCV RNA with a readily-available and simple-to-us qualitative RT/PCR test may be useful to tailor antiviral treatment in patients with chronic hepatitis C.

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ARTÍCULO 3

EARLY DETECTION OF NON RESPONSE TO INTERFERON PLUS RIBAVIRIN COMBINATION TREATMENT OF CHRONIC HEPATITIS C.

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[2]Roche Molecular Systems, Barcelona, Spain.

Abstract

The dynamics of hepatitis C virus RNA during treatment with interferon plus ribavirin are not well known. We evaluated the predictive value of HCV RNA early decline during combination therapy. Thirty chronic hepatitis C patients that had followed interferon plus ribavirin for twelve months were included. Serum HCV RNA was measured in sera obtained at baseline and at 4, 8 and 12 weeks after initiation of treatment using a second generation commercially available quantitative RT/PCR assay. After six months of postherapy follow-up 10 (33%) patients were considered sustained responders and 20 (66%) non responders. While in non responders HCV RNA levels remained stable during the first three months of treatment (6.3±0.7 versus 6±0.9, 5,7±1 and 5,9±0.9 log10 HCV RNA IU/mL at baseline and at weeks 4, 8 and 12 respectively) sustained responders showed significant declines in viral load at each subsequent sample (❒HCV RNA of 3.5±1.6 log10 IU/mL, 5.1±1.7, and 6±0.9 at weeks 4,8 and 12 respectively). At week 12 all sustained responders had HCV RNA levels below 600 IU/mL, as compared to only one of 20 non responders (P<0.001). ROC curves of HCV RNA level for sustained response prediction identified sensitivity peaks (5 log at 4 weeks, 4 log at 8 weeks and 3 log at 12 weeks) with 100% negative predictive value. Our results suggest that HCV RNA levels during the first three months of combination therapy for chronic hepatitis C are the strongest predictors of response.

Introduction

Two recent multicenter randomized trials (1,2) have shown that the combination of interferon-alfa plus ribavirin is more effective than interferon alone in the treatment of previously untreated (naive) patients with chronic hepatitis C. Unfortunately, even with combination therapy, close to 60% of patients did not achieve a sustained response. In an attempt to select the best candidates for treatment, several baseline characteristics associated with the likelihood of response such as age, gender, histology, genotype and baseline viral load have been identified (1,2). However, in clinical practice none of this response factors, either singly or in combination, has enough predictive value to establish indication of treatment in a given patient, they only can help to decide therapy duration (3).

Several studies have shown a strong association between clearance of HCV RNA from serum at 4 weeks of therapy and sustained response to interferon (4-8). However, viral dynamics during combination therapy seems to be different, in McHutchison et al HCV RNA was detectable until 12 or 24 weeks in 59% of sustained responders (1).

Most of these studies are based on non standardized qualitative or quantitative RT/PCR assays. A collaborative study established the World Health Organization (WHO) International Standard for HCV RNA quantification, to allow comparisons between results obtained with different assays(9). Roche Molecular Systems have released the Amplicor HCV Monitor v2.0 assay giving results in IU/mL.

Recently, Poynard et al (10) in a reanalysis of database from two large multicenter trials of interferon and ribavirin combination therapy, found that patients who still have more than 400.000 RNA copies/mL (measured by Superquant) at 4 weeks of therapy have virtually no chance of becoming sustained responders, strongly suggesting that viral load monitoring could be very useful for early identification of non responders. If the goal of therapy is sustained viral clearance, then prediction of sustained or non response early in the course of treatment would be helpful in avoiding unnecessary therapy, thus reducing side effects and costs.

In the present study we have retrospectively investigated the utility of early monitoring of HCV viral load using a standardized quantitative RT/PCR test to predict sustained response in naive chronic hepatitis C patients treated with interferon-alpha plus ribavirin for twelve months.

Patients and Methods

Between 1996 and 1998, 109 consecutive chronic hepatitis C patients received combination treatment with interferon-alpha plus ribavirin at our Hospital. For the purpose of this study we selected 30 naive patients who had completed 48 weeks of standard therapy with interferon and ribavirin and from whom sera obtained at monthly intervals during the first three months of treatment were available. The remaining 79 patients were excluded because ribavirin had been started two months after onset of interferon therapy (40 patients), had received previous treatment (30 patients), or because therapy had been discontinued due to side effects (9 patients).

All 30 patients selected for this study received 3 MU three times per week of interferon alfa-2b (Intron A, Schering-Plough) and 800-1200 mg/day, depending on body weight, of ribavirin (Rebetrol, Schering-Plough) for 48 weeks and were followed for an additional 24 weeks after stopping therapy. Diagnosis of chronic hepatitis C was based on elevated serum alanine aminotransferase levels (ALT) for at least six months, consistent detection of serum HCV RNA and, in most patients, histological evidence of chronic hepatitis. All patients were negative for hepatitis B surface antigen and human inmunodeficiency viruses.

Monitoring of patients. Blood samples were obtained immediately before treatment and at monthly intervals thereafter. All samples were drawn into SST Vacutainer tubes (Becton & Dickinson), centrifuged within 2 h of collection, tested fresh for ALT levels and aliquoted and stored at –80ºC until further testing. Patients were monitored monthly during treatment for liver function test and hematological parameters.

Definition of response. Sustained response to treatment was considered when serum HCV RNA was undetectable by second generation qualitative RT/PCR assay (lower limit of detection <100 copies/mL) at the end of treatment and six months thereafter. All other patients (primary non-responders and relapsers) were considered non-responders.

Histological evaluation. In 23 of the 30 patients liver biopsies were performed before beginning of treatment. Seven patients did not undergo biopsies because of haemophilia. Biopsy specimens were stained with hematoxiline/eosine and Picro Sirius red and evaluated by an experienced pathologist. Necroinflamation and fibrosis

in each specimen were scored separately according to a modified Knodell score proposed by Ishak et al (11).

Detection of HCV RNA. Stored sera obtained at twelve months of therapy and at the end of follow-up were tested for the presence of HCV RNA by commercially available second generation qualitative RT/PCR test (Amplicor HCV v2.0, Roche Molecular Systems, Pleasanton, CA) with a lower limit of detection of less than 100 copies/mL (12), as recommended by the manufacturer.

Quantitation of HCV RNA. Serum samples corresponding to baseline, month 1, 2 and 3 after beginning of treatment were tested by quantitative RT/PCR for HCV. Quantitation was performed on 100µL of serum by Amplicor HCV Monitor v2.0 (Roche Molecular Systems, Pleasanton, CA) following manufacturer's instructions. The assay is similar to the first generation test (13) but modifications have been made to the master mix in order to increase the assay sensitivity and allow an equal quantification of all HCV genotypes. The estimated sensitivity of the assay is 2.8 log IU/mL. Samples with viremia greater than 850000 (5.9 log) IU/mL were diluted 1:100 in HCV RNA-negative human serum and retested. All results of quantitative HCV viremia are expressed as log10 IU/mL.

HCV genotyping. Viral genotype was determined with a commercial strip hybridization assay (Inno-Lipa II, Innogenetics, Zwijndrecht, Belgium), according to manufacturer's instructions.

Statistical analysis. Continuous variables are expressed as mean values and standard deviations. In case of serum samples with HCV RNA undetectable by Monitor 2.0, a value of zero was assigned for some calculations. Categorical variables were compared with the Fisher's exact test. Normal distribution of quantitative variables was assessed by the Kolmogorov-Smirnoff test, and means were compared with Student's t-test or Mann-Whitney Rank Sum test. Stepwise logistic regression was performed with significant variables by univariate analysis. All P values are two-tailed and differences are considered significant when $P<0.05$. Sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) were calculated as described elsewhere (14). Receiver operating characteristics (ROC) plots were constructed with every cutoff point of predicted probability of viral loads corresponding to months 1, 2 and 3 after treatment onset. The area under the ROC curves and their 95% confidence intervals were measured to assess the degree of discrimination provided by these variables. All data analysis was conducted using the SPSS for Windows, version 8.0 (Statistical Package of Services Solutions, SPSS Inc., Chicago, IL).

Results

Baseline characteristics of the 30 included patients are summarized in table1. At the end of the 24-week follow up period 10 (33%) patients achieved a sustained response and 20 were considered non responders. As shown in table 2, sustained responders were not significantly different from non responders with regard to demographic, virological, biochemical or histological parameters.

However, significant differences were found in viral dynamics during the first three months of treatment between both groups (see figure 1). Among non responders mean

HCV RNA levels did not change significantly from baseline (6.3±0.7 versus 6±0.9, 5,7±1 and 5,9±0.9 log10 HCV RNA IU/mL at baseline and at weeks 4, 8 and 12 respectively), although one patient had undetectable HCV RNA by Amplicor HCV Monitor v2.0 (less than 600 IU/mL) at 12 weeks. In contrast, among sustained responders, four had undetectable HCV RNA at week 4 and the mean viral load of those still viremic had decreased to 3.9±1 log IU/mL, corresponding to a mean decrease in viral load from baseline (❒HCV RNA) of 3.5±1.6 log10 IU/mL (P<0.001).

At week 8, only three of the sustained responders had detectable HCV RNA (mean 3.4±0.6 log IU/mL, mean ❒HCV RNA 5.1±1.7, P<0.001). At 12 weeks all sustained responders had HCV RNA levels below 600 IU/mL as compared to only one of non responders (P<0.001). See table 3.

ROC curves of 4, 8 and 12 weeks of serum HCV RNA load (in log IU/mL) and 4, 8 and 12 week serum HCV RNA differences from baseline (❒HCV RNA) for sustained response prediction were constructed. The areas under the ROC curves were 0.977 (95% CI, 0.932-1.023) for week 4 viral load, 1.0 for week 8 viral load and 0.969 (95% CI, 0.897-1.041) for week 12 viral load. Sensitivity peaks were observed for 5.0 logs (100.000 IU/mL) at 4 weeks (Se=1.00, Sp=0.90, PPV=0.83, NPV=1.00), for 4.0 logs (10.000 IU/mL) at 8 weeks (Se=1.00, Sp=1.00, PPV=1.00, NPV=1.00) and for 3 logs (1000 IU/mL) at 12 weeks (Se=1.00, Sp=0.95, PPV=0.91, NPV=1.00). The ROC curves of ❒HCV RNA values at 4, 8 and 12 weeks had similar areas and sensitivity peaks of one, two and three logs, respectively. See table 4.

A stepwise logistic regression was performed including absolute viral loads and ❒HCV RNAs at weeks 4, 8 and 12; absolute viral load at week 12 was identified as the most powerful predictor of sustained response. Indeed, when this variable was entered in the predictive equation, no other variable could improve the prediction.

Discussion

Our data suggest that kinetics of viral load during the first three months of therapy is the strongest predictor of sustained response to a 48-weeks course of interferon plus ribavirin combination treatment, and may permit taking very early therapeutic decisions. Indeed, as in all previous studies (4-8), sustained responders cleared HCV RNA early in treatment so that differences in viral load with non responders were clear-cut by 4 weeks, the earliest time point measured, and became larger at each subsequent sample because of stabilization of viral load in non responders. In fact, in all sustained responders viral load decreased at least 1 log IU/mL per month from baseline and absolute viral load cut-off points with 100% negative predictive value for sustained response could be obtained for all three monthly samples (5 log IU/mL at 4 weeks; 4 log IU/mL at 8 weeks; 3 log IU/mL at 12 weeks).

Our patient population was characterized by having a low prevalence of favorable response factors. Patients were mostly middle-age males with long standing infection, predominantly infected with genotype 1 and had high viral load and significant liver fibrosis. Hence our results do not seem to be conditioned by host or viral factors associated with better responsiveness. In addition, despite the small number of patients involved, our results are consistent with the data reported by Poynard et al (10) in studies involving more than one thousand patients.

Our results argue for the implementation of viral load monitoring to tailor duration of combination therapy. The use of a quantitative test to monitor viral response has several advantages. First, as long as the assay is readily available and standardized, viral load may be less subject to specificity problems than qualitative tests. Second, especially in patients under combination therapy, the quantitative results appear more sensitive for early detection of non responders. And third, the magnitude of viral load decrease has higher predictive value for sustained response in single testing than qualitative test results.

The quantitative test used in this study has been shown to be reproducible (12) and to be able to classify patients before therapy as above or below the $2x10^6$ copies/mL (SuperQuant^{TM}. National Genetics Institute, Culver City, CA) cut-off level defined in large multicenter trials (15). Recently, a semi-automated version of Amplicor HCV Monitor v2.0 (Cobas v2.0, Roche Molecular Systems, Pleasanton, CA) with results in IU has been evaluated (16). The values obtained correlated with that of Superquant ($r=0.932;P<0.0001$) and linear regression analysis yielded a conversion equation between copies/mL (measured by Superquant) and IU/mL. Applying this formula, the 5.6 log copies/mL cut-off at 4 weeks reported by Poynard et al (10) converts to 5.3 log IU/mL, which is very similar to our 5 log IU/mL cut-off at 4 weeks.

If our results were confirmed by studies in larger groups of patients, treatment could be stopped early in patients who fail to show a consistent decline in HCV RNA levels within 12 weeks of treatment onset. This could not only reduce side effects and costs but would also allow alternative treatment options (i.e.: high dose induction, or the use of pegilated interferon) to be pursued sooner in the course of treatment. In addition, such strategy might encourage many reluctant patients to try therapy. These

patients would be more likely to accept a rather low chance of sustained response and take a potentially toxic drug with unpleasant side effects if they knew that potential non response would be established early and further unnecessary treatment avoided. We can conclude that early monitoring of viral load during interferon plus ribavirin therapy is a useful predictor of non response. Further studies involving a larger number of patients and the use of semiautomatic technology are warranted.

Acknowledgements

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Table 1. Demographic and virologic features of HCV infected patients (n=30).

Feature	Value
Age (yr)	40±13
Sex (male)	23(77%)
Percutaneous risk factor	
Transfusion	9(30%)
IVDA*	1(3%)
Haemophilia	7(23%)
Unknown	13(43%)
Age at infection	15±6
Duration of infection	20±12
Genotype: Type 1	27(90%)
Type 2	0
Type 3	2 (7%)
Type 4	1 (3%)
Baseline viral load[#]	6,2±0,9
Baseline ALT	97±67
Liver histology	
Grade[‡]	6±3
Stage[§]	3±1

IVDA, intravenous drug abuser. # Viral load is expressed as log10 HCV RNA IU/ml.[‡] Scores could range from 0 to 18, with higher scores indicating more severe necroinflammation. [§] Scores could range from 0 to 6, with higher scores indicating more severe fibrosis and architectural distortion. A value of 6 corresponds to liver cirrhosis.

Table 2. Demographic and virologic characteristics of HCV infected patients according to response to therapy.

	sustained responders	non responders	P
Age (years)	34±15	43±11	0.06
Sex (males)	8(80%)	15(75%)	0.7
Risk factor:			
Transfusión	2(20%)	7(35%)	
IVDA*	1(5%)	0	
Haemophilia	4(40%)	3(15%)	
Unknown	3(30%)	10(50%)	0.2
Age at infection	6.2±9.3	19±16	0.2
Duration of infection	16.5±2.6	21.6±14	0.3
Genotype: 1 or 4	8(80%)	20(100%)	
2 or 3	2(20%)	0	0.1
Baseline viral load[#]	6.2±0.9	6.3±0.7	0.6
Baseline ALT	90±24	101±81	0.5
Liver histology:			
Grade[‡]	5.1±1.2	6.1±3	0.3
Stage[§]	2.7±1.5	2.6±1.2	0.9

IVDA, intravenous drug abuser. # Viral load is expressed as log 10 HCV RNA IU/ml.[‡] Scores could range from 0 to 18, with higher scores indicating more severe necroinflammation. [§] Scores could range from 0 to 6, with higher scores indicating more severe fibrosis and architectural distortion. A value of 6 corresponds to liver cirrhosis.

Table 3. Viral dynamics during the first three months of combination therapy.

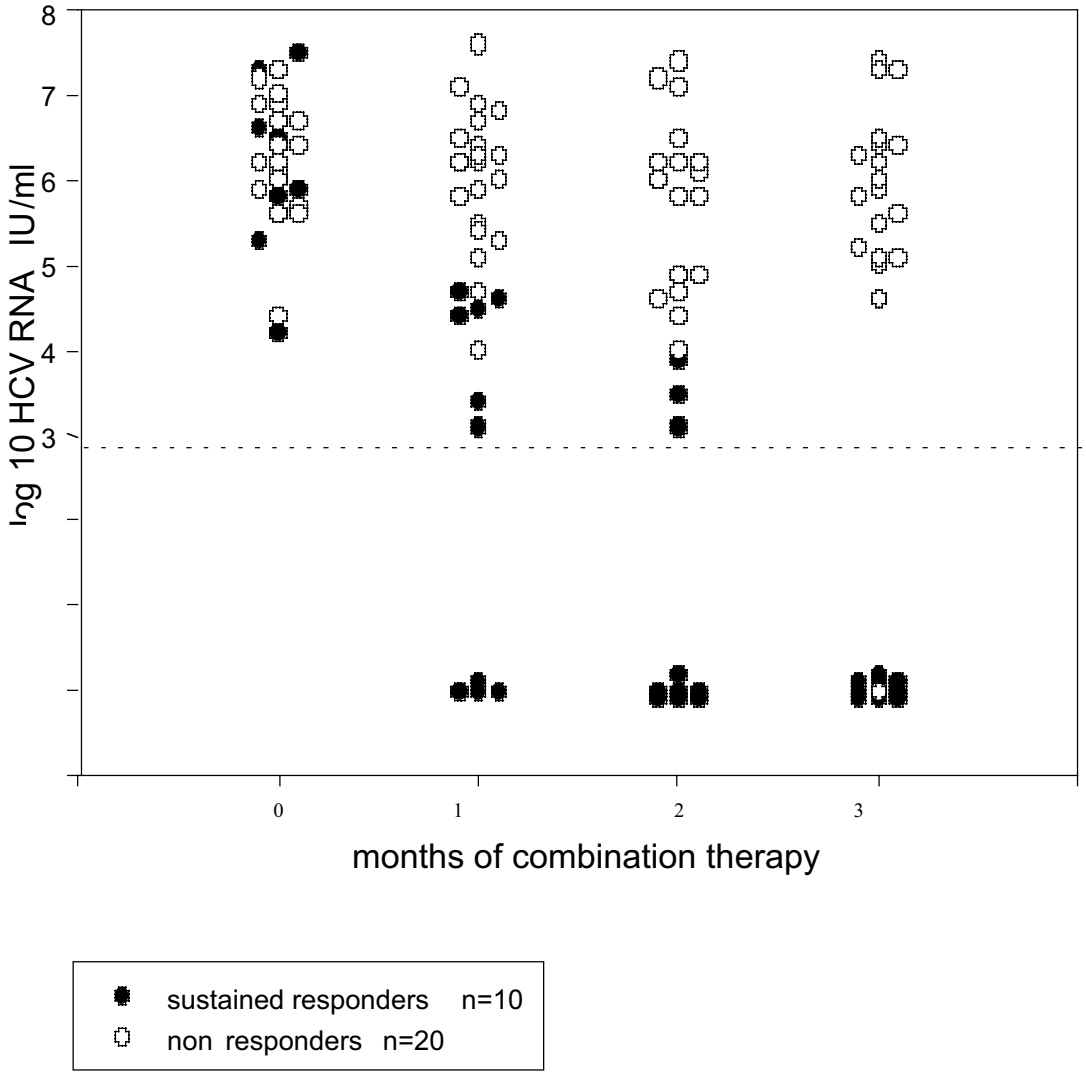
	Month 1			Month 2			Month 3		
	SR	NR	P	SR	NR	P	SR	NR	P
ARN-VHC negative*	4/10	0/20	0.02	7/10	0/20	0.002	10/10	1/20	0.004
Viral load	3.9±1	6±1	0.001	3.4±0.6	5.7±1	0.002	<3	5.9±1	<0.001
r RNA HCV	3.5±2	0.2±1	<0.001	5.1±2	0.5±1	<0.001	6±1	0.8±1	<0.001

*The inferior limit of detection of Amplicor HCV Monitor v2.0 is 600 IU/mL. SR: sustained response.NR: non response. r RNA HCV: decrease in viral load from baseline. Viral load is expressed as log IU/mL.

Table 4.Sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) for sustained response of viral load during the first three months of combination therapy

		Se	Sp	PPV	NPV
Month 1	Viral load< 5 log	100%	90%	83%	100%
	r HCVRNA> 1 log	100%	90%	83%	100%
Month 2	Viral load< 4 log	100%	100%	100%	10 0%
	r HCVRNA≥2 log	100%	90%	83%	100%
Month 3	Viral load< 3 log	100%	95%	91%	100%
	r HCVRNA> 3 log	100%	95%	91%	100%

r HCVRNA: decline in viral load from baseline . Viral load is expressed as log 10 HCV RNA IU/mL. PPV is the ability to predict sustained response, and NPV is the ability to predict non response.



HCV viral load levels of each patient measured by Amplicor HCV Monitor v2.0 at baseline (0), month 1, 2 and 3 after interferon plus ribavirin therapy onset. The inferior limit of detection of Amplicor HCV Monitor v2.0 is 600 (2.8 log) IU/mL.

RESULTADOS Y DISCUSIÓN

1-EVALUACIÓN DE LA SEGUNDA VERSIÓN DE UN MÉTODO COMERCIAL DE RT/PCR CUALITATIVA Y CUANTITATIVA PARA VHC.

1.1-RT/PCR cualitativa:

El límite inferior de detección fue de 100 copias de transcrito (un estándard que representa la porción 5'UTR cuantificado mediante marcaje con isótopos, para mayor información lease artículo 1) del VHC /mL para Amplicor v2.0 y de 1000 copias/mL para Amplicor v1.0.

De los 132 donantes de sangre con serología anti-VHC positiva y ARN del VHC indetectable por Amplicor v1.0, en 6 (5%) casos se detectó ARN del VHC utilizando Amplicor v2.0. Tras clasificar estos individuos como virémicos o no virémicos según Amplicor v2.0, la comparación de ambos grupos mostró diferencias con respecto al nivel de ALT (97$\pm$120 versus 22$\pm$12, P=0.035) y el de anti-E2 (120$\pm$54 versus 44$\pm$54). Ver tabla 1.

En conjunto Amplicor v2.0 ha mejorado en un logaritmo la sensibilidad con respecto a la versión anterior, y ha mostrado ser más útil en el diagnóstico de pacientes con anticuerpos contra el VHC en plasma y niveles bajos de carga viral. Creemos que no se debería descartar la presencia de viremia en pacientes con serología positiva para VHC si no ha sido utilizado un método de RT/PCR con un límite de detección inferior o igual a 100 copias/mL.

Tabla 1. Características demográficas y virológicas de los donantes de sangre anti-VHC positivo y ARN del VHC negativo por Amplicor v1.0, en función del resultado de Amplicor v2.0.

Variable		Amp2.0 ✚ (n=6)	Amp2.0 ━ (n=126)	P
Edad		37±11	48±13	0.6
Sexo (varón)		50%	38%	0.5
Factor de riesgo				
Transfusión		1(16%)	42(33%)	
ADVP*		1(16%)	5(4%)	
Desconocido		4(66%)	79(63%)	0.4
ALT (UI/L)		97±120	22±12	0.035
HCV serotipo:	1	5(83%)	33(26%)	
	2	1(16%)	3(2.4%)	
	3	0	4(3.2%)	
	4	0	3(2.4%)	
	Negativo	0	83(66%)	0.7
Anti-E2 D:O. (S/N)		120±54	44±54	0.004
RIBA-2 y 3†:	Positivo	6(100%)	82(65%)	
	Indet.	0	44(35%)	0.6

*ADVP, adicto a drogas por vía parenteral. †Todos los pacientes eran RIBA-2 positivo o indeterminado, los últimos casos se testaron por RIBA-3. Indet.: indeterminado.

1.2-RT/PCR cuantitativa:

Con respecto a la reproducibilidad, Monitor v2.0 obtuvo un coeficiente de variación inter-ensayo del 5.5%. Dos muestras de cada paciente virémico fueron testadas por Monitor v2.0, en el 90% de los casos la diferencia entre muestras fue inferior a 1 log y en el 70% de los casos fue inferior a 0.5 log. En la práctica clínica se acepta que una diferencia inferior a 0.5 log es atribuible a a variabilidad de la técnica por precisa que esta sea.

La carga viral según Monitor v2.0 fue mayor que la obtenida mediante Monitor v1.0 para todos los genotipos. No hubo diferencias entre las cargas virales medias de los genotipos 1, 2 y 3 al utilizar Monitor v2.0, mientras que la carga viral del genotipo 4 fue inferior al resto. Ver figura 15. Se ha descrito que Monitor 1.0 subestima la carga viral en los genotipos 2 y 3 con respecto al genotipo 1[73]. En cambio Monitor v2.0 cuantifica por igual los genotipos 1, 2 y 3. Además ha reducido la diferencia entre el genotipo 4 y el resto de 1.5 log a 0.5 log.

De los 127 pacientes en los cuales se determinó la carga viral mediante B-DNA 2.0, en 13(10.2%) no se detectó ARN del VHC. En el resto la carga viral media fue superior a la medida por Monitor v2.0 (6.6$\pm$0.6 log copias/mL versus 6.4$\pm$0.6 log copias/ml, $P<0.001$).

El rango dinámico de Monitor v2.0 es de 10^3 a 10^6 copias/mL sin diluir las muestras. Este rango parece muy apropiado para la valoración de la dinámica viral durante el tratamiento antiviral. Añadiendo una dilución 1:100 previa, el rango se convierte en 10^5 a 10^8, más conveniente para la medición de la carga viral basal. De hecho, este

test ha mostrado ser capaz de clasificar a los pacientes infectados por VHC como con carga viral superior o inferior al umbral de $2x10^6$ copias/mL (según SuperQuant) definido en ensayos clínicos multicéntricos[120].

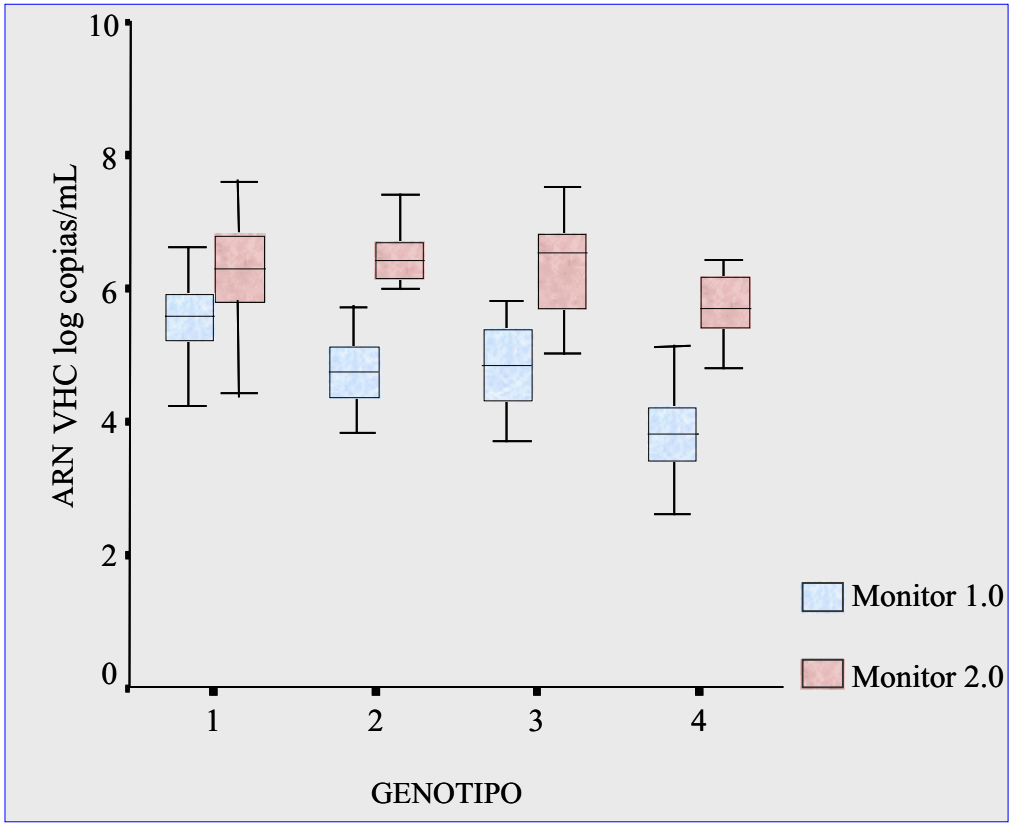


Figura 15. Comparación de los resultados de la determinación de la carga viral de los 326 pacientes infectados por el VHC en función de la versión de Monitor utilizada.

2-VALOR PREDICTIVO DE LA RT/PCR CUALITATIVA DURANTE EL TRATAMIENTO DE LA HEPATITIS C CON INTERFERON O TRATAMIENTO COMBINADO.

En este estudio se incluyeron 184 pacientes afectos de hepatitis crónica C, los cuales recibieron interferon-✂2b durante las primeras 8 semanas. Entonces se llevó a cabo una RT/PCR cualitativa de primera generación (límite de detección de 1000 copias/mL). Los pacientes con ARN del VHC indetectable continuaron terapia con interferon durante 10 meses más, mientras que a los pacientes con ARN detectable se les ofreció tratamiento combinado (interferón más ribavirina) durante 12 meses más.

En 27 pacientes (15%) la viremia fue negativa en la semana 8 y continuaron tratamiento con interferón. De los 157 pacientes virémicos, 122 iniciaron tratamiento combinado, en los 35 restantes no se añadió ribavirina a la terapia por contraindicación, ausencia de consentimiento o problemas en el suministro del fármaco.

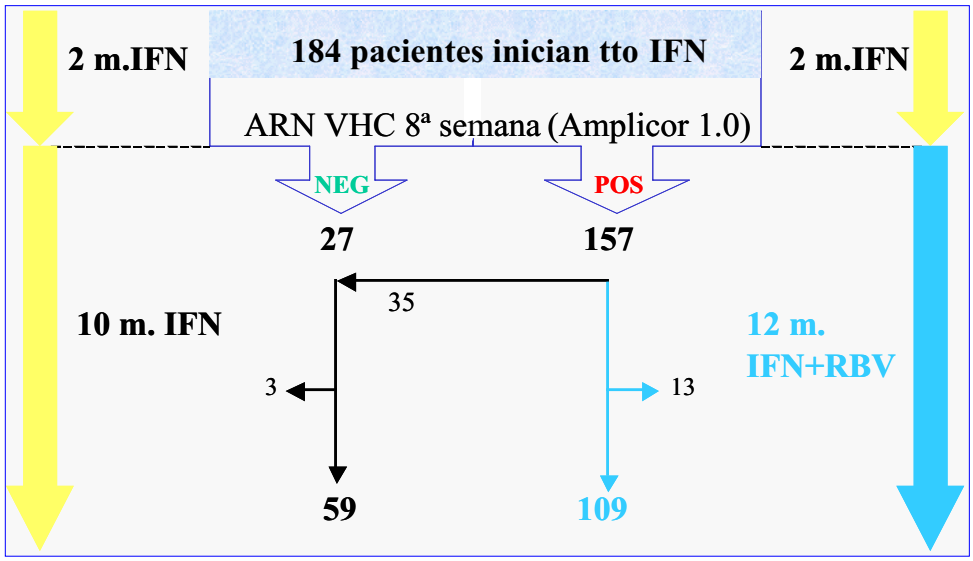


Figura 16. Asignación de la terapia en función del resultado de la RT/PCR cualitativa de primera generación en la 8ª semana de tratamiento.

2.1-Grupo de monoterapia con interferón-✂2b:

Tras 12 meses de tratamiento y 6 de control posterior se observó que 13 pacientes (21%) presentaron una respuesta mantenida, 33 (53%) una recidiva, 13(21%) no respondieron y 3(5%) abandonaron la terapia a raíz de efectos secundarios.

Se analizó la relación entre las siguientes características basales y la respuesta mantenida: edad, sexo, edad de la infección, duración de la infección, factor de riesgo parenteral, tratamiento previo con interferón, ALT, genotipo, carga viral, necroinflamación y fibrosis en la biopsia hepática. El antecedente de transfusión de hemoderivados se asoció con una menor tasa de respuesta mantenida (4% versus 35%, P=0.004). El genotipo no 1 no 4 y la carga viral inferior a 5.1 log copias/mL fueron asociados con una mayor tasa de respuesta mantenida (47% versus 12%, P=0.006 y 60% versus 14%, P=0.005, respectivamente). Ver tabla 2.

La detección de ARN del VHC a la 4ª semana de tratamiento con interferón se relacionó con la ausencia de respuesta mantenida, con un valor predictivo negativo del 100% tanto al utilizar la RT/PCR de primera generación como la de segunda generación. La no detección de ARN del VHC se relacionó con una mayor tasa de respuesta mantenida, con un valor predictivo positivo del 52% con RT/PCR de 1ª generación y del 81% con RT/PCR de 2ª generación. Este valor predictivo positivo era aún más alto si considerábamos la asociación ALT normal y RT/PCR de 2ª generación negativa, llegando al 93%. Ver tabla 3.

Tabla 2. Distribución de las características basales en el grupo de monoterapia con IFN en función de la respuesta al tratamiento.

Característica	Respuesta mantenida	Recidiva/no respuesta	P
	(n=13)	(n=46)	
Edad	36±13	39±12	0.35
Sexo: varones	7(19%)	30(81%)	0.5
mujeres	6(27%)	16(73%)	
Edad de infección	19±3	20±14	0.8
Duration de infección	14±7	18±9	0.3
Factor de riesgo:			
Transfusión	1(4%)	24(96%)	0.004
ADVP	4(33%)	8(67%)	
Desconocido	8(36%)	14(64%)	
No tratado	12(24.5%)	37(75.5%)	
Trat.previo	1(10%)	9(90%)	
ALT	94±63	88±64	0.7
Genotipo:			
1 ó 4	5(12%)	37(88%)	0.006
2 ó 3	8(47%)	9(53%)	
Carga viral:			
≤5.1	6(60%)	4(40%)	0.005
>5.1	7(14%)	42(86%)	
Grado	6.2±2	5.9±2	0.8
Estadío	2.5±1	2.5±1	0.9
ARN del VHC a la 4ª semana según Amplicor 2.0			
Negativo	13(81%)	3(19%)	<0.001
Positivo	0	43(100%)	

Los porcentajes se refieren a la característica que define la fila. ADVP: adicto a drogas por vía parenteral. La carga viral es expresada en log copias/mL.

El análisis estadístico multivariado (regresión logística) identificó a la viremia cualitativa a la 4ª semana de terapia como el predictor de respuesta mantenida más potente. Tras la entrada de esta variable en la ecuación predictiva, las demás variables no podían mejorar la predicción.

Tabla 3. Predicción de la respuesta mantenida mediante la detección de viremia del VHC a la 4ª semana de tratamiento con interferon.

Test utilizado	Sensibilidad	Especificidad	VPP	VPN
Amplicor v1.0	100%	72%	52%	100%
Amplicor v2.0	100%	94%	81%	100%
Amplicor v2.0 +ALT	100%	98%	93%	100%

VPP: valor predictivo positivo, la capacidad para predecir una respuesta mantenida.
VPN: valor predictivo negativo, la capacidad para predecir la no respuesta.

Se llevó a cabo biopsia hepática postratamiento (entre 6 y 12 meses tras finalizar la terapia) en 22 pacientes, 10 habían presentado una respuesta mantenida y los otros 12 una recidiva de la infección. Al compararla con la biopsia basal, no hubo diferencias con respecto a la fibrosis en ningún grupo, mientras que la actividad necroinflamatoria mejoró significativamente en los pacientes con respuesta mantenida (media 4.5, IC del 95%[3.4,5.6], P<0.001) y no hubo cambios significativos en los pacientes con recidiva.

Nuestros resultados son concordantes con los estudios previos al identificar a la viremia cualitativa a la 4ª semana del tratamiento de la hepatitis C con interferón

como el predictor de respuesta más potente descrito[104-112]. En el estudio hemos utilizado una técnica de RT/PCR cualitativa comercial, por lo que es accesible y fácil de llevar a cabo. Esto permitiría llevar a la práctica clínica este potente predictor de respuesta, hasta ahora relegado a los ensayos clínicos.

Según nuestros hallazgos, en los pacientes que sigan monoterapia con interferón, a la 4ª semana se debería llevar a cabo una determinación de viremia cualitativa con un test capaz de detectar 100 copias/mL. Si la viremia fuese detectable, la terapia debería ser modificada (abandono o añadir ribavirina) ; y si la viremia no fuese detectable, al completar los 12 meses de terapia con interferón más del 80% de los pacientes presentarían una respuesta mantenida.

2.1-Grupo de tratamiento combinado (interferón-✂2b más ribavirina):

De los 122 pacientes que siguieron tratamiento combinado, 21 (17%) presentaron una respuesta mantenida, 66 (54%) una recidiva, 22 (18%) no respondieron y 13 (11%) abandonaron la terapia a raíz de efectos adversos.

Se analizó la relación entre las siguientes características basales y la respuesta mantenida: edad, sexo, edad de la infección, duración de la infección, factor de riesgo parenteral, tratamiento previo con interferon, ALT, genotipo, carga viral, necroinflamación y fibrosis en la biopsia hepática. En el análisis estadístico univariado se detectó asociación entre una mayor tasa de respuesta mantenida y las siguientes variables: Genotipo no 1 no 4 (57% versus 17%, P=0.035), carga viral

inferior a 5.5 log copias/mL (44% versus 14%), sexo femenino (29% versus 13%, P=0.04), y necroinflamación superior a 6 (31% versus 12%, P=0.03).
El análisis multivariado mediante regresión logística identificó el genotipo, la carga viral y el sexo femenino como predictores independientes de respuesta mantenida.

Tras los dos meses de monoterapia con interferón fueron definidas tres variables que resultaron asociadas a una mayor tasa de respuesta mantenida: normalización de los niveles de ALT (42% versus 14%, P=0.02), carga viral a la 8ª semana inferior o igual a 5.1 log copias/mL (70% versus 13%, P<0.001) y descenso de la carga viral con respecto a la basal mayor o igual a 1.2 log copias/ml (80% versus 12%, P<0.001).

El análisis multivariado de los factores basales más las variables definidas tras dos meses de tratamiento con interferón identificó al descenso de la carga viral con respecto a la basal ≥1.2 log copias/mL (P<0.001) como el predictor más potente de respuesta mantenida. Tras la incorporación de esta variable a la ecuación predictiva solo la normalización de ALT a los dos meses podía mejorar la predicción.

Con respecto a la dinámica viral durante el tratamiento combinado, la no detección del ARN del VHC tras 5 meses de terapia combinada mediante la RT/PCR cualitativa de segunda generación (Amplicor v2.0) resultó ser un potente predictor de respuesta mantenida (81% versus 19%, P<0.001) con una sensibilidad del 95%, especificidad del 89%, valor predictivo positivo del 67% y valor predictivo negativo del 99%. Ver tabla 4 y figura 17.

Tabla 4. Valores predictivos para respuesta mantenida de la RT/PCR cualitativa durante los primeros 5 meses de terapia combinada en función del test utilizado.

	Mes	1	2	3	4	5
Amplicor v1.0	VPP	67%	50%	53%	44%	38%
	VPN	85%	88%	95%	96%	98%
Amplicor v2.0	VPP	80%	82%	84%	78%	67%
	VPN	85%	90%	94%	96%	99%

VPP: valor predictivo positivo. VPN: valor predictivo negativo.

El análisis multivariado mediante regresión logística incluyendo todas las variables significativas identificó a la viremia cualitativa tras 5 meses de terapia combinada como el predictor más potente de respuesta mantenida, y tras este al descenso de carga viral ≥1.2 log copias/mL a la 8ª semana de tratamiento con interferón. Una vez

que estas dos variables se habían incorporado a la ecuación predictiva, ninguna otra podía mejorar la predicción. Ver tabla 5.

Se llevaron a cabo 62 biopsias postratamiento, 13 en pacientes con respuesta mantenida, 38 en pacientes con recidiva y 5 en no respondedores. Al comparar los parámetros histológicos con la biopsia basal, no hubo diferencias con respecto a la fibrosis en ningún grupo, mientras que la necroinflamación solo mejoró en el grupo de respuesta mantenida (media 3.6, IC 95% [2.7,4.6], P<0.001).

Tabla 5. Distribución de las características significativas en el grupo de terapia combinada (interferón más ribavirina) en función de la respuesta al tratamiento.

Característica		Respuesta Mantenida (n=21)	Recidiva/no respuesta (n=88)	P
Sexo:	varones	9(13%)	59(87%)	0.04
	Mujeres	12(29%)	29(71%)	
Genotipo:				
	1 ó 4	17(16%)	85(84%)	0.035
	2 ó 3	4(57%)	3(43%)	
Carga viral basal:				
	≤5.5	8(44%)	10(56%)	0.007
	>5.5	13(14%)	78(86%)	
Necroinflamación:				
	>6	15(32%)	32(68%)	0.03
	<6	8(13%)	53(87%)	
ALT tras IFNx2meses				
	Normal	13(42%)	18(58%)	0.02
	Elevada	11(14%)	67(86%)	
Descenso de carga viral tras IFNx2meses				
	≥1.2	12(80%)	3(20%)	<0.001
	<1.2	3(12%)	25(88%)	
ARN del VHC al 5ª mes según Amplicor v2.0:	Negativo	20(65%)	11(35%)	<0.001
	Positivo	1(1%)	77(99%)	

Los porcentajes son referidos a la característica que define la fila. ADVP: adicto a drogas por vía parenteral. La carga viral es expresada en log copias/mL. IFN: interferón-✂2b.

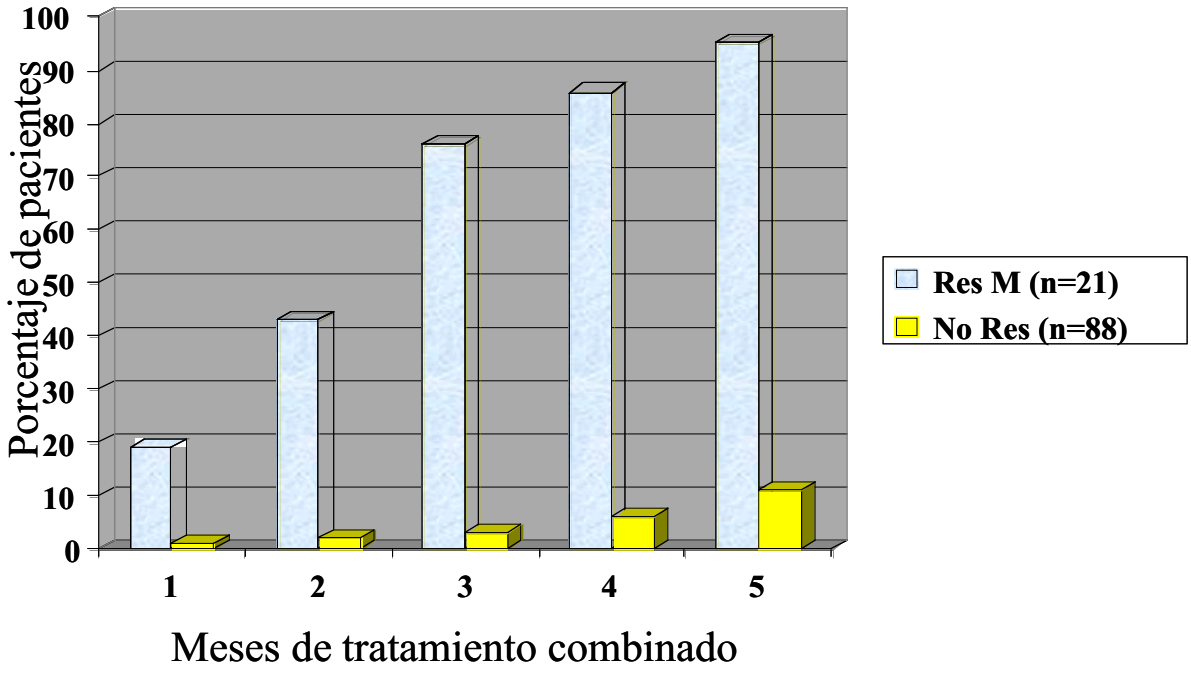


Figura 17. Porcentaje acumulativo de pacientes con ARN del VHC indetectable por Amplicor v2.0 durante los primeros 5 meses de terapia combinada (interferón más ribavirina) agrupados según la respuesta. Res M: respuesta mantenida. No Res: no respuesta mantenida (se considera en el mismo grupo la recidiva y la no respuesta).

El diseño del estudio nos ha permitido analizar dos variables relacionadas con la respuesta al tratamiento con interferón en monoterapia durante dos meses, la normalización de los niveles de ALT y el descenso de la carga viral ≥1.2 log/mL, que han resultado ser predictores de respuesta al tratamiento combinado que se llevó a cabo posteriormente. En el análisis multivariado mostraron ser predictores más potentes que los factores basales (sexo, genotipo y viremia). Estos hallazgos sugieren que la respuesta a la terapia combinada está estrechamente relacionada con la respuesta al tratamiento con interferón, y que la primera puede ser predecida en función de la segunda. Apoyan esta teoría el hecho de que los pacientes que habían

presentado una recidiva tras tratamiento con interferón tengan una tasa de respuesta mantenida tras terapia combinada del 49%[119] , mientras que los pacientes con no respuesta a interferón en monoterapia presentan una tasa de respuesta mantenida al tratamiento combinado inferior al 15%[121].

La ausencia de detección del ARN del VHC durante el tratamiento combinado parece ser un predictor de respuesta más tardío que en el caso de la terapia con interferón. En nuestro estudio los pacientes que presentaron una respuesta mantenida tras tratamiento combinado tardaron en negativizar el ARN del VHC una media de 2.9 meses más los 2 meses iniciales de terapia con interferón. Esta cifra no es extrapolable a los paciente que siguen una pauta de tratamiento combinado estándar (inicio simultáneo de interferón-✂ y ribavirina, duración del tratamiento 6-12 meses).

Otra precaución a tener en cuenta al interpretar los resultados es que el 93% de los pacientes del grupo de tratamiento combinado estaba infectado por el VHC perteneciente al genotipo 1. Por lo tanto, la dinámica viral durante el tratamiento combinado de los otros genotipos del VHC puede ser diferente.

Aún así, nuestros resultados son concordantes con los de un análisis retrospectivo de una base de datos que incluye más de 1500 pacientes que han seguido tratamiento combinado[122]. Dicho análisis muestra que los pacientes con viremia detectable tras tres meses de tratamiento combinado tienen una probabilidad del 10% de conseguir una respuesta mantenida si continuan la terapia hasta completar 12 meses, mientras que tras 6 meses de terapia la detección de viremia del VHC supone una probabilidad

de respuesta mantenida del 2%. La recomendación actual en cuanto al uso de la RT/PCR cualitativa de VHC en la monitorización de la respuesta al tratamiento combinado consiste en llevar a cabo la detección del ARN del VHC tras 6 meses de terapia, si la detección es positiva se debe abandonar el tratamiento, dadas las escasas posibilidades de erradicación de la infección.

3-VALOR PREDICTIVO DE LA RT/PCR CUANTITATIVA DURANTE LOS PRIMEROS MESES DE TRATAMIENTO COMBINADO.

Se incluyeron 30 pacientes que habían seguido tratamiento con interferón-✂2b más ribavirina durante 12 meses. Seis meses tras la retirada de la terapia 10 pacientes presentaron una respuesta mantenida y los otros 20 fueron considerados no respondedores. La tabla 6 muestra las características basales de los 30 pacientes.

Se analizó la relación entre las siguientes características basales y la respuesta mantenida: edad, sexo, edad de la infección, duración de la infección, factor de riesgo parenteral, tratamiento previo con interferón, ALT, genotipo, carga viral, necroinflamación y fibrosis en la biopsia hepática. Ninguna variable obtuvo una asociación significativa con la respuesta mantenida. En el caso del genotipo y la carga viral es muy probable que esto se deba a un insuficiente tamaño de muestra, ya que estas variables han sido descritas como los predictores basales más potentes de respuesta[122].

Tabla 6. Datos iniciales de los pacientes afectos de hepatitis crónica C (n=30).

Característica		Valor
Edad		40±13
Sexo (hombres)		23(77%)
Factor de riesgo:	Transfusión	9(30%)
	ADVP*	1(3%)
	Hemofilia	7(23%)
	Desconocido	13(43%)
Edad de infección		15±6
Duración de infección		20±12
Genotipo:	1	27(90%)
	2	0
	3	2 (7%)
	4	1 (3%)
Carga viral⁻		6,2±0,9
ALT		97±67
Histología hepática:	Grado‡	6±3
	Estadío§	3±1

*.ADVP: adicto a drogas por vía parenteral. ⁻La carga viral se expresa en log UI/mL. ‡Puntuación referente a la necroinflamación, con valores de 0 a 18. §Puntuación referente a la fibrosis hepática, con valores de 0 a 6.

Para analizar la dinámica viral durante los primeros meses de terapia combinada se determinó la carga viral basal, al mes, dos meses y tres meses de tratamiento mediante una RT/PCR cuantitativa calibrada en unidades internacionales (Amplicor HCV Monitor v2.0). La figura 18 representa los resultados de la carga viral en función de la respuesta al tratamiento.

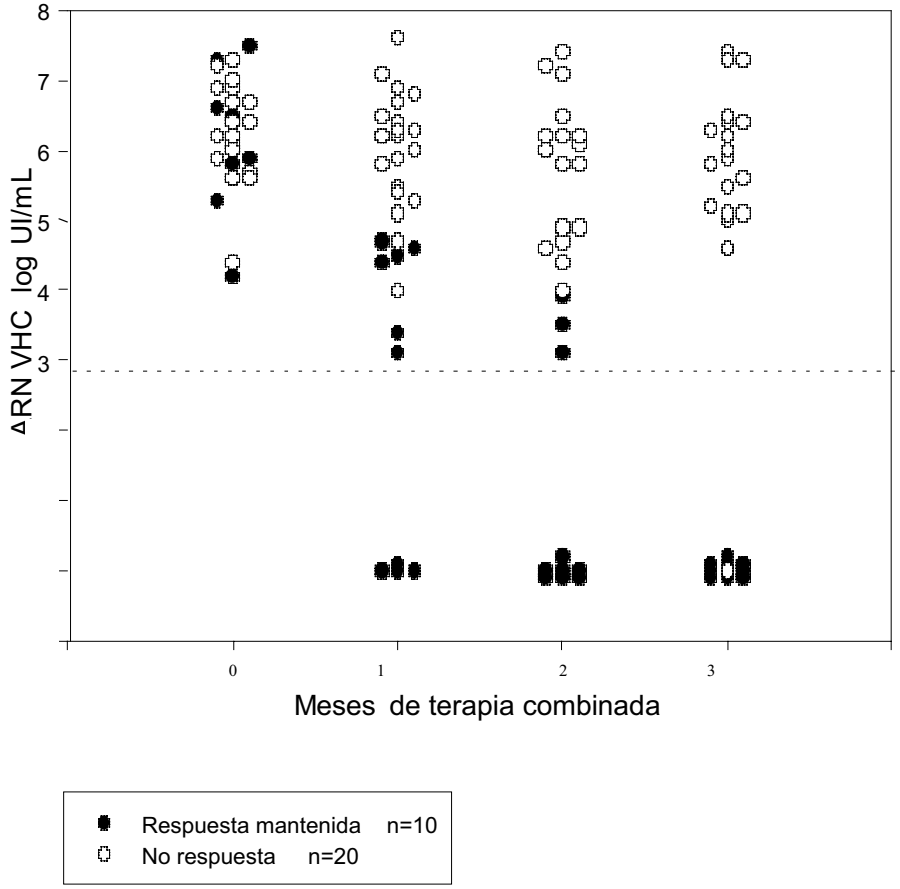


Figura 18. Valor de la carga viral de los 30 pacientes durante los primeros tres meses de tratamiento combinado, agrupados en función de la respuesta al tratamiento. El límite inferior de detección de Amplicor HCV Monitor v2.0 es de 2.8 log (600) UI/mL.

Como se puede observar, la carga viral no mostró variaciones significativas en los pacientes no respondedores durante los primeros meses de terapia combinada (6.3±0.7 versus 6±0.9, 5,7±1 and 5,9±0.9 log UI/mL al inicio y tras uno, dos y tres meses, respectivamente). Sin embargo la carga viral de los pacientes que presentaron respuesta mantenida disminuyó progresivamente en cada muestra mensual, llegando

a ser indetectable al tercer mes en todos los pacientes. La tabla 7 resume las cargas virales medias y el número de pacientes con ARN del VHC indetectable en cada muestra.

Tabla 7. Dinámica viral durante los primeros meses de terapia combinada en función de la respuesta.

	Mes 1			Mes 2			Mes 3		
	RM	NR	P	RM	NR	P	RM	NR	P
ARN-VHC indetectable*	4/10	0/20	0.02	7/10	0/20	0.002	10/10	1/20	0.004
Carga viral media	3.9+1	6+1	0.001	3.4+0.6	5.7+1	0.002	<3	5.9+1	<0.001
❒ARN VHC	3.5±2	0.2±1	<0.001	5.1±2	0.5±1	<0.001	6±1	0.8±1	<0.001

*El límite inferior de detección de Amplicor HCV Monitor v2.0 es de 600 IU/mL. RM: respuesta mantenida. NR: no respuesta. ❒ARN VHC: descenso en carga viral con respecto a la basal. La carga viral es expresada en log UI/mL

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Se construyeron curvas COR referentes a la carga viral en los meses 1, 2 y 3 para predicción de respuesta mantenida. Los valores del área bajo la curva son los siguientes: en el mes 1 de 0.977 (IC95%, 0.932-1.023), en el mes 2 de 1.0, y en el mes 3 de 0.969 (IC95%, 0.897-1.041). Fueron detectados los siguientes picos de sensibilidad: 5 log/mL en el mes 1, 4 log/mL en el mes 2 y 3 log/mL en el mes 3.

También fueron construidas curvas COR referentes al descenso de la carga viral con respecto a la basal en los meses 1, 2 y 3 para predicción de respuesta mantenida. Las áreas bajo la curva fueron similares a los de la carga viral absoluta y se identificaron

picos de sensibilidad en todas ellas. La tabla 8 resume los valores predictivos de los picos de sensibilidad.

El análisis multivariado mediante regresión logística identificó a la carga viral absoluta tras tres meses de tratamiento combinado como el predictor más potente de respuesta mantenida.

Tabla 4.Sensibilidad (Se), especificidad (Es), valor predictivo positivo (VPP) y valor predictivo negativo (VPN) para respuesta mantenida de la carga viral durante los tres primeros meses de terapia combinada

		Se	Es	VPP	VPN
Mes 1	Carga viral<5 log	100%	90%	83%	100%
	r ARN-VHC>1 log	100%	90%	83%	100%
Mes 2	Carga viral<4 log	100%	100%	100%	10 0%
	r ARN-VHC≥2 log	100%	90%	83%	100%
Mes 3	Carga viral<3 log	100%	95%	91%	100%
	r ARN-VHC>3 log	100%	95%	91%	100%

❒ARN VHC: descenso de la carga viral con respecto a la basal.

Estos resultados sugieren que la carga viral durante los tres primeros meses de tratamiento con interferón y ribavirina es el predictor de respuesta más potente de respuesta a largo plazo. El uso de este predictor de respuesta podría mejorar el manejo clínico de los pacientes con hepatitis C, al permitir la adaptación de la pauta

terapéutica de forma individualizada en función de la evolución de la carga viral durante los primero meses de tratamiento combinado.

El uso de un método cuantitativo para monitorizar la respuesta virológica tiene varias ventajas: en primer lugar, si el test cuantitativo está bien estandardizado, este tendrá con menor frecuencia limitaciones de especificidad (contaminación de la muestra por ARN proveniente de otra muestra) en comparación con los tests cualitativos; en segundo lugar, los tests cuantitativos parecen ser más sensibles en la detección precoz de no respondedores, especialmente en pacientes que siguen terapia combinada; y por último, la magnitud del descenso de la carga viral tiene un valor predictivo para respuesta mantenida superior a los resultados cualitativos.

Las características técnicas del método de RT/PCR cuantitativo utilizado en este estudio han sido analizadas previamente, mostrando una reproducibilidad e independencia de genotipo interesantes. Una versión semiautomatizada de Amplicor HCV Monitor v2.0 calibrada en unidades internacionales (Cobas v2.0; Roche Diagnostics Systems, Meylan, France) ha sido evaluada recientemente[123]. La correlación de sus resultados con los de SuperQuant fue excelente ($r=0.932$; $P<0.001$) y el análisis de regresión linear obtuvo una ecuación de conversión entre copias/mL (SuperQuant) y unidades internacionales/mL (Cobas v2.0):

$$\text{Log (Superquant)} = 1.344 + 0.789 \log (\text{UI,Cobas})$$

Aplicando esta fórmula, el pico de sensibilidad en la 4ª semana de 5.6 log copias/mL descrito por Poynard[122] equivale a 5.3 log UI/mL, cifra similar al punto de corte descrito en este estudio de 5 log UI/mL a la 4ª semana.

Si nuestros resultados fueran confirmados, la terapia antiviral combinada podría ser retirada durante los primeros tres meses en los pacientes cuya carga viral fuera superior a los puntos de corte establecidos. Esto permitiría una importante reducción de costes económicos y efectos secundarios, sin disminuir la tasa de respuesta mantenida.

Esta estrategia podría ser más atrayente para los pacientes afectos de hepatitis crónica C que una pauta de 12 meses de interferón y ribavirina sin monitorizar la carga viral. Los pacientes aceptarían con mayor probabilidad un tratamiento con múltiples efectos adversos y una tasa de respuesta mantenida inferior al 50%, si fueran informados de la posibilidad de identificar precozmente a los no respondedores para que la terapia les fuera retirada.

CONCLUSIONES

1) Las técnicas de RT/PCR cualitativa y cuantitativa de segunda generación evaluadas son más sensibles en 1 logaritmo y son menos genotipo-dependientes que las de primera generación.

2) La determinación del ARN del VHC mediante RT/PCR cualitativa tras 4 semanas de tratamiento con interferón es el predictor de respuesta más potente descrito.

3) La respuesta virológica a la terapia combinada (interferón más ribavirina) no es siempre tan precoz como durante el tratamiento con interferón en monoterapia, por lo que la RT/PCR cualitativa (Amplicor v2.0) no es un predictor útil durante los primeros meses de la terapia. (Recomendación actual de testar tras 6 meses de terapia combinada).

4) El análisis del descenso de la carga viral durante los primeros 3 meses del tratamiento combinado (interferón más ribavirina) mediante RT/PCR cuantitativa (Monitor v2.0) parece ser un predictor de respuesta muy potente, capaz de identificar a los pacientes no respondedores de forma precoz.

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