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Relationship between the hepatitis C viral load and the serum interferon concentration during the first week of peginterferon-alpha-2b-ribavirin combination therapy

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Figure 1A

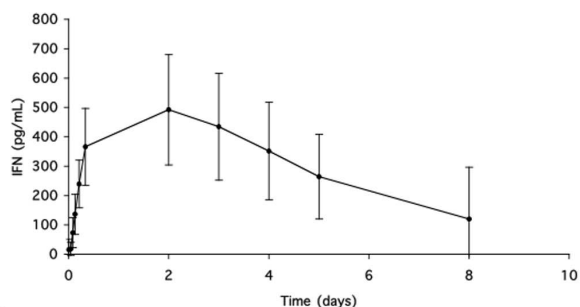
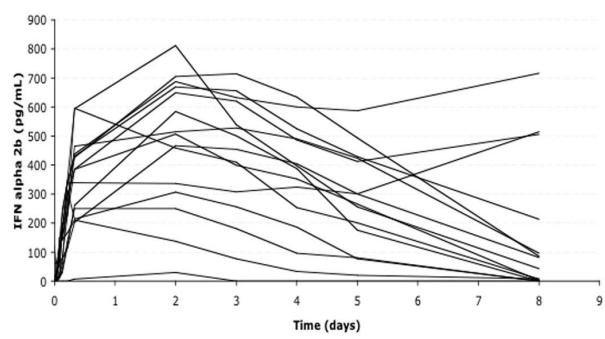


Figure 1B



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Figure 2A

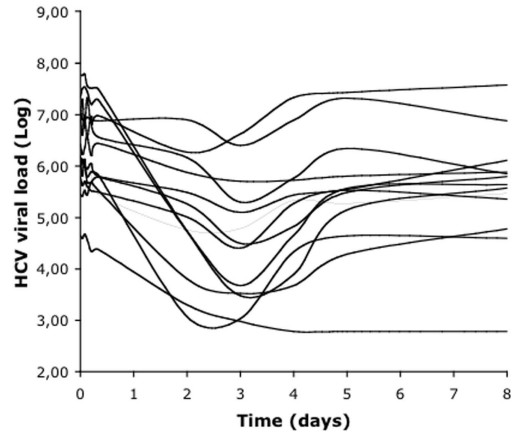


Figure 2B

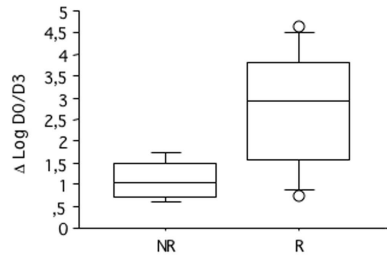
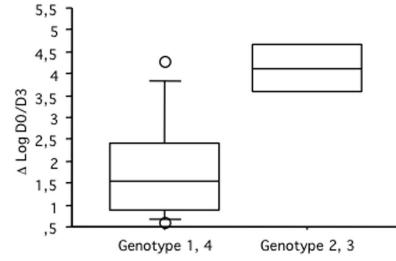


Figure 2C



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Figure 3A

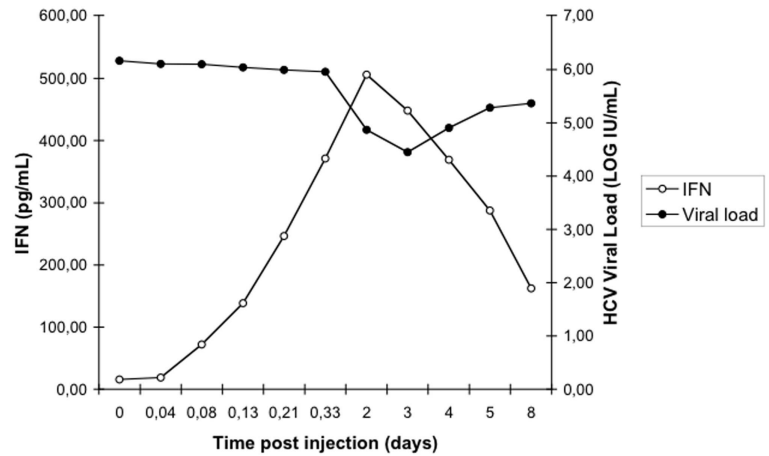
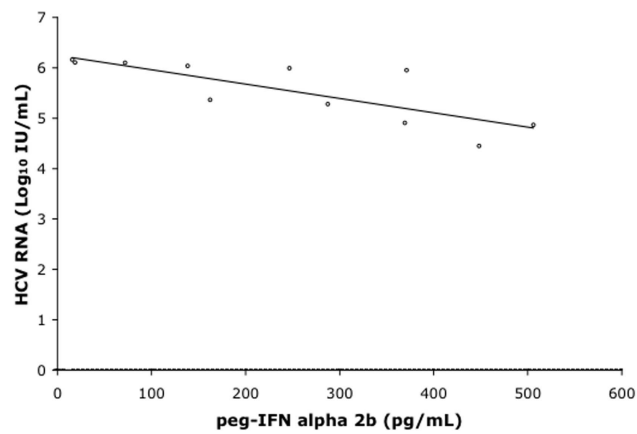


Figure 3B



846x1128mm (72 x 72 DPI)

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6 2 **Relationship between the hepatitis C viral load and the serum interferon concentration**
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8 **during the first week of peginterferon-alpha-2b-ribavirin combination therapy.**
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16 6 Catherine François¹, Véronique Descamps¹, Etienne Brochot¹, Isabelle Bernard¹, Valérie
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18 7 Canva², Philippe Mathurin^{2,3}, Sandrine Castelain¹ and Gilles Duverlie¹
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25 10 ¹ Unité de Virologie clinique et fondamentale EA4294, CHU d'Amiens, Université Picardie
26
27

28 11 Jules Verne, Amiens, France
29

30 12 ² Service d'Hépatogastroentérologie, Hôpital Huriez, Lille, France
31

32 13 ³ INSERM U795, Lille, F-59037, Université de Lille 2, Lille, France.
33
34
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36
37 15 **Corresponding author:**
38

39 16 Dr Catherine François
40

41 17 Faculté de Médecine et de Pharmacie, Laboratoire de Virologie
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43

44 18 3 rue des Louvels, 80000 Amiens, France
45

46 19 Phone: +33 322 827 917
47

48
49 20 Fax: +33 322 827 928
50

51 21 E-mail: catherine.francois@u-picardie.fr
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53 22 Running title: HCV load and IFN concentration
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Abstract

25 In chronic hepatitis C virus (HCV) infections, the current standard of care (combination
26 therapy with pegylated alpha interferon (PEG-IFN α) and ribavirin) is only effective in around
27 50% of cases. The aim of the present study was to analyze the relationship between the HCV
28 load and the PEG-IFN concentration during the first week of treatment.

29 Fifteen treatment-naive patients with chronic hepatitis C infection (genotypes 1, 2, 3 and 4)
30 underwent PEG-IFN α -2b/ribavirin combination therapy. Blood samples were collected before
31 the first injection (T₀) and then at different time points until the next injection a week later.
32 The PEG-IFN concentration and the HCV load were assayed.

33 The serum interferon concentration peaked two days after the first injection (mean value for
34 the study population; T_{max}=40.9 hours; C_{max}=490 pg/ml) and a trough in viral load was seen at
35 day three. The PEG-IFN α -2b concentration decreased from day 2 to day 7, enabling a viral
36 rebound in all patients. The change in viral load between day 0 and day 3 differed
37 significantly according to whether the patients were responders at week 12
38 ($\Delta\log_{10}D0/D3=2.729\pm 1.419$ Log₁₀ IU/mL) or not ($\Delta\log_{10}D0/D3=1.102\pm 0.472$ Log₁₀ IU/mL).

39 Our results emphasize the potential clinical importance of achieving viral decay immediately
40 after initiation of interferon-ribavirin combination therapy.

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42 **Key words:** interferon, hepatitis C, HCV, IFN, pharmacokinetics

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Introduction

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8 45 The hepatitis C virus (HCV; family: *Flaviviridae*; genus: *Hepacivirus*) is a major causative
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10 46 agent of chronic hepatitis - a condition that can progress to cirrhosis and hepatocellular
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12 47 carcinoma [Sangiovanni et al., 2006]. The first treatment for chronic hepatitis C was
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15 48 monotherapy with interferon alpha (IFN α); this produced a sustained virologic response
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18 49 (SVR) in only 10-15% of cases. The combination of ribavirin with IFN α (from 1998
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20 50 onwards) increased the response rate to 40% [McHutchison et al., 1998; Nyberg et al., 1999;
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22 51 Poynard et al., 1998]. Pegylation of IFN (the covalent attachment of polyethylene glycol
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24 52 (PEG) chains) increases the protein's size and molecular weight, slows renal clearance,
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27 53 maintains a more stable serum concentration and increases the effective dose at certain body
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29 54 sites [Harris et al., 2001]. Indeed, the use of pegylated IFN raises the sustained response rate
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31 55 to 54-56% [Fried et al., 2002; Manns et al., 2001]. PEG-IFN α -2b is formed by linking a linear
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34 56 12-kDa PEG moiety to a histidine residue in IFN α -2b via an unstable urethane bond.
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37 57 Hydrolysis of this bond releases native IFN α -2b into the circulation [Grace et al., 2001; Wang
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39 58 et al., 2002]. The serum half-life of PEG-IFN α -2b is approximately 40h (10 times longer than
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41 59 for standard IFN α -2b); this yields a sustained, high serum concentration for 48-72h post-
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43 60 injection and makes a regimen with weekly injections possible [Glue et al., 2000a]. The
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45 61 combination of ribavirin with PEG-IFN α -2b does not modify the latter's pharmacokinetic
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47 62 parameters [Glue et al., 2000b].

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52 63 Although the reasons for non-response to interferon are not completely understood, both
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54 64 virologic factors (such as load and genotype) [Fried et al., 2002; Manns et al., 2001] and host
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56 65 factors (such as age, histology and prior induction of an IFN system) [Sarasin-Filipowicz et
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58 66 al., 2008] are thought to be involved.

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3 67 Given that novel drugs (such as protease and polymerase inhibitors) will not become available
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5 68 in the very near future, efforts are being made to optimize current therapies and thus increase
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8 69 the response rate. The first parameter to have been modified was the treatment duration.
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10 70 Whereas a shorter course of treatment will be sufficient for some patients, longer treatment is
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12 71 advisable for others [Francois et al., 2009; Mangia et al., 2008]. These new therapeutic
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15 72 strategies are based on evaluating the early-stage viral kinetics in general and the HCV load
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17 73 one month after treatment initiation in particular. Indeed, the rapid virologic response (RVR,
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19 74 defined as an undetectable viral load after 4 weeks of treatment) is highly predictive of the
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21 75 sustained response rate [Martinot-Peignoux et al., 2009]. Hence, analysis of the change in
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23 76 viral load over the first month may be helpful for modifying the treatment as soon as possible,
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26 77 as a function of the observed antiviral efficacy.
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29 78 In order to establish whether or not the early treatment response is a useful parameter, we
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31 79 studied the pharmacokinetics of PEG-IFN α -2b and HCV levels during the first week of
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34 80 treatment in 15 patients and evaluated the correlation between these two parameters.
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Patients and Methods

Samples:

Serum samples were obtained from 15 patients chronically infected with HCV (genotype 1: n=10; genotype 2: n=1; genotype 3: n=1; genotype 4: n=3). All patients were interferon-naïve and eligible for combination therapy (Table 1). HCV RNA had been detectable for at least the previous year. The treatment was once weekly 1.5 µg/kg PEG-IFN α 2b, combined with daily, oral ribavirin given according to bodyweight recommendations and regardless of the genotype (1000 mg/day for patients under 75 kg and 1200 mg/day for those over 75 kg). Enrollment began in 2003.

Blood samples were collected before (T₀) the first injection and then 1, 2, 3, 5 and 8 hours, 2, 3, 4, 5, 8, 15 and 29 days and 8 and 12 weeks afterwards. Samples were processed within four hours of collection and stored at -70°C. The treatment outcome was defined as the response at week 12, which is considered by the French consensus group as a good time point for predicting non-response to combination treatment [Dhumeaux et al., 2003]. The study design and protocol were approved by the local independent ethics committee. Prior to enrolment in the study, all patients gave their written informed consent to participation.

Cell line:

MDBK-t2 cells (containing the human interferon-inducible MxA promoter linked to a chloramphenicol acetyl transferase (CAT) reporter gene) were a kind gift from Dr Martin D. Fray [Fray et al., 2001]. The cells were maintained in Glutamax I-Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 10 µg/mL blasticidin (Invitrogen, Cergy Pontoise, France).

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Bioassay for the quantitative determination of serum IFN activity:

For each assay, MDBK-t2 cells were plated at a density of 1×10^6 per well in six-well plates. After 18H of incubation at 37°C in 5% CO₂, the culture medium was discarded and replaced with 1 mL of DMEM with 10% fetal bovine serum and 100 µL of either a sample or known amounts of PEG-IFN α -2b (Schering-Plough, NJ). The cells were cultured for an additional 24H at 37°C in 5% CO₂. The standard curve was constructed by serial 1:2 dilutions (from 625 pg/mL down to 19.5 pg/mL) of PEG-IFN α -2b. Chloramphenicol acetyl transferase expression was determined as previously described [Francois et al., 2005]. The intra-assay and inter-assay coefficients of variation were 8.6% and 14.4%, respectively. The assay's limit of detection (LOD) was 19.5 pg/mL [Francois et al., 2005].

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Determination of HCV RNA levels:

To prepare total RNA from sera, we used the QIAamp RNA kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Next, HCV RNA was quantitated specifically by a real-time RT-PCR assay, as described previously [Castelain et al., 2004].

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Statistical analysis:

All statistical analyses were performed using Statview® (Frankfurt, Germany) software. Inter-group comparisons were made using a non-parametric Mann-Whitney test or a correlation test. The threshold for statistical significant was set to $p < 0.05$.

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Results

The concentration of peginterferon- α 2b

The serum concentration of PEG-IFN α -2b was measured as described in the Materials and Methods section. PEG-IFN α -2b activity was generally detected between two and three hours after the injection. One patient was excluded because the PEG-IFN concentration was below the LOD throughout the first week (Figure 1B). In the remaining 14 patients, the peak serum concentration (C_{\max}) of PEG-IFN α -2b (490 ± 219.8 pg/ml) was observed 2 days after the injection (T_{\max} : 40.9 ± 21.9 hours) (Figure 1A). This peak was followed by a linear decline in the serum PEG-IFN α -2b concentration over the next few days. On day 8 (i.e. just before the scheduled injection of the next dose), the PEG-IFN α -2b concentration had fallen to below 100 pg/ml for ten of the 14 patients and was even below the LOD in seven cases (50%). In three cases, the second injection had been performed before collection of the day 8 blood sample (Figure 1B). The same decline was observed over the next three weeks for the five patients for whom a blood sample was collected before the subsequent injections (data not shown). Only two patients had a detectable IFN concentration in the serum before the treatment.

HCV load.

HCV RNA was quantitated in a real-time RT-PCR assay, as described in the Materials and Methods section. All 14 patients responded to the first dose of PEG-IFN α -2b, as evidenced by a decrease in the viral load. During the first week, the lowest viral load was obtained on day three for all patients (Fig. 2A). In order to characterize the interferon's antiviral activity, we calculated the difference in viral load between day 0 and day 3 (expressed in \log_{10} units ($\Delta\log$)). As shown in Figure 2B, the mean $\Delta\log$ d0/d3 was 1.820 ± 1.203 for genotype 1 or 4

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3 152 HCV patients and 4.115 ± 0.757 for genotype 2 or 3 patients ($p=0.0679$). The same
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6 153 comparison was made for responders vs. non-responders at week 12 (i.e. no detectable HCV
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8 154 RNA at week 12). The mean $\Delta\log d0/d3$ values were 1.102 ± 0.472 and 2.729 ± 1.419 for the
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10 155 non-responder and responder patients, respectively ($p=0.0388$) (Fig. 2C). The same trend was
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12 156 observed when considering only genotype 1 patients, although the $\Delta\log d0/d3$ values did not
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14 157 differ significantly (2.57 ± 1.19 and 1.69 ± 0.96 for responders and non-responders,
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16 158 respectively; $n=10$; $p=0.28$). As shown in Table 2, all the patients with a $\Delta\log d0/d3 > 2$ were
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18 159 responders at week 12 and 80% of these individuals showed a sustained virologic response.
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25 161 **The relationship between HCV load and IFN concentration during the first week of**
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27 162 **treatment.**
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30 163 The treatment's antiviral activity was visualized by plotting the HCV load and the IFN
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32 164 concentration over the first week of treatment on the same graph and for all patients (Figure
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34 165 3A). The two parameters were inversely correlated, with a correlation coefficient of -0.794
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36 166 ($p<0.01$) (Figure 3B). There was a decrease in the HCV load when the PEG-IFN
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38 167 concentration was at its highest; conversely, the fall in IFN concentration was accompanied
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Discussion

171 One of today's main goals in monitoring the treatment for chronic HCV infection is to predict
172 the SVR as soon as possible after initiation of the therapy. This knowledge may usefully
173 enable therapy to be discontinued in patients who will ultimately fail to benefit or, on the
174 contrary, may help patients to maintain good compliance if the treatment appears to be
175 effective. Hence, understanding the early pharmacokinetics and pharmacodynamics of IFN
176 could be useful for predicting the treatment response.

177 Here, we first looked at pharmacokinetic parameters. Our results confirm previous reports on
178 the pharmacokinetics of PEG-IFN α -2b [Zeuzem et al., 2003]. The serum PEG-IFN
179 concentration peaked at day 2 post-injection and then fell slowly over the course of the week.
180 Hence, in contrast to what had been expected [Wang et al., 2002], the PEG-IFN α -2b
181 concentration did not remain stable over the week as a whole. At the end of the week (i.e.
182 before the next scheduled injection) serum IFN could not be detected in most patients. These
183 findings confirmed a number of other reports [Bruno et al., 2004; Formann et al., 2003].
184 When IFN was no longer detectable in the serum, the viral load increased until the next IFN
185 injection. This observation suggests that the antiviral effect of IFN is not optimal during the
186 first week of treatment and that the pharmacokinetic parameters could be improved. Indeed,
187 this is why twice-weekly injection of PEG-IFN α -2b has been suggested by Formann and
188 collaborators [Formann et al., 2003]. In the latter report, the blood PEG-IFN α -2b
189 concentration was maintained throughout the week and the HCV rebound was avoided. These
190 results were confirmed by Bruno and collaborators, who compared the change in HCV RNA
191 load with the pharmacokinetics of PEG-IFN α -2a and PEG-IFN α -2b [Bruno et al., 2004]. At
192 week 12, the HCV load was significantly lower in the PEG-IFN α -2a-treated group than in the
193 PEG-IFN α -2b-treated group - suggesting that the difference in HCV RNA values may be

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3 194 related to the different absorption and distribution profiles of the two drugs. No differences in
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6 195 gene induction were observed when comparing the pharmacodynamics of PEG-IFN α -2a and
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9 196 PEG-IFN α -2b. However, the latter study was performed by testing the patients' serum during
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11 197 the first week of treatment and did not necessarily reflect pharmacodynamics of interferon in
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13 198 the liver [Bruno et al., 2007].

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15 199 The significance of the fact that the trough in HCV load was seen 24 hours after the peak
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18 200 serum IFN and that a HCV rebound occurred rapidly after the IFN concentration had
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20 201 decreased is subject to debate. However, this finding could mean that IFN-induced host
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22 202 proteins with a short half-life could be involved in the early antiviral response.

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25 203 Both viral and host factors have been implicated in the response to IFN therapy. In terms of
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27 204 the viral factors, genotype seems to be an important predictive factor. In particular, genotypes
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31 206 more sensitive to IFN therapy [Fried et al., 2002; Manns et al., 2001]. The present study
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33 207 confirmed that the early decline in viral load ($\Delta\log d0/d3$) is lower for genotypes 1 and 4 than
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35 208 for genotypes 2 and 3. However, this difference was not statistically significant - no doubt due
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37 209 to the low number of patients in general and genotype 2 or 3 patients in particular. Several
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39 210 lines of evidence now suggest that early viral clearance in patients infected with HCV is a
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41 211 sign of more effective IFN-ribavirin combination therapy [Bekkering et al., 2001; Galeras JA,
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43 212 2006; Jessner et al., 2003; Rosen et al., 2002]. The analysis of the $\Delta\log d0/d3$ appears to
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45 213 further corroborate this hypothesis. Just as the rapid virologic response is predictive of the
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47 214 sustained response rate [Jensen et al., 2006; Martinot-Peignoux et al., 2009; Yu et al., 2007]
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49 215 [Deltenre et al., 2009], the $\Delta\log d0/d3$ might also be an early-stage parameter with predictive
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51 216 value. However, the present results must be verified with a large number of patients.
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3 327 **Figure legends**

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5 328 **Figure 1**

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8 329 The serum interferon concentration in patients treated with a combination of peginterferon
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10 330 α 2b (1.5 μ g/kg/week) and ribavirin. Figure 1A shows the mean interferon α 2b concentration
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12 331 in 14 patients during the first week of treatment. The Figure 1B represents the change over
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14 332 time in the interferon α 2b concentration for each of the 14 patients.
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20 334 **Figure 2**

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22 335 The HCV load (Log_{10} IU/mL) in the 14 patients during 12 weeks of treatment with
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24 336 peginterferon α 2b. (2A). The box-plots represent the $\Delta \log d_0/d_3$ as a function of the HCV
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26 337 genotype (2B) and the treatment response at week 12 for all genotypes (2C). R and NR
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28 338 respective indicate patients in whom HCV RNA was undetectable or detectable at week 12.
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36 341 **Figure 3**

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38 342 A: The change over time in the mean serum HCV RNA load (black dots) and the mean serum
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40 343 IFN concentration (white dots) for all the patients during the first week of treatment. The
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42 344 HCV load was expressed in log IU/mL and the IFN concentration was expressed in pg/mL, by
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44 345 comparison with a standard curve of peginterferon alpha 2b.
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48 347 B: Correlation between the mean HCV load and the mean IFN concentrations measured at the
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50 348 same time point, for all patients (regardless of the genotype). Each circle represents a time
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350**Table 1: Patients**

Patients: n=15	
Male	3
Female	12
Mean (range) viral load in log ₁₀ IU/mL	6.16 (4.65-7.78)
Genotype	
1	10
2	1
3	1
4	3*
Responders at week 12	9
Non-responders at week 12	6

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* One patient was excluded from further analysis because the peginterferon concentration was below the assay's LOD throughout the first week.

Table 2 : $\Delta\log d0/d3$ correlates with the drop in HCV RNA at week 12

All genotypes		Undetectable HCV RNA			
$\Delta\log d0/d3$	n	Week 12 (n)	%	SVR after 6 months of follow-up (n)	%
< 2	9	4	44%	4	44%
> 2	5	5	100%	4	80%
All	14	9	64%	8	57%