

Accepted Manuscript

Safety and anti-HCV Effect of Prolonged Intravenous Silibinin in HCV-Genotype 1 Subjects in the Immediate Liver Transplant Period

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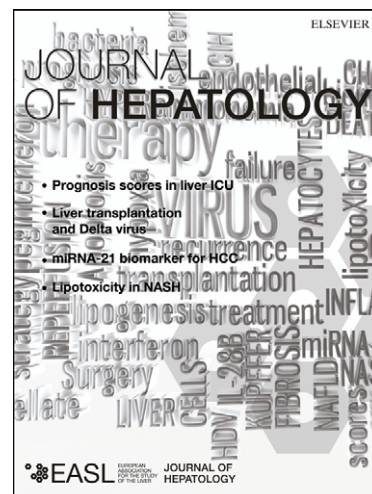
PII: S0168-8278(12)00777-5
DOI: <http://dx.doi.org/10.1016/j.jhep.2012.10.009>
Reference: JHEPAT 4444

To appear in: *Journal of Hepatology*

Received Date: 1 August 2012
Revised Date: 1 October 2012
Accepted Date: 8 October 2012

Please cite this article as: Bárcena, R., Moreno, A., Rodríguez-Gandía, M.A., Albillos, A., Arocena, C., Blesa, C., García-Hoz, F., Graus, J., Nuño, J., López-Hervás, P., Gajate, L., Martínez, A., Bermejo, T., Mateos, M.L., Campo, S.d., on behalf of the Hospital Ramón y Cajal Liver Transplant Group Safety and anti-HCV Effect of Prolonged Intravenous Silibinin in HCV-Genotype 1 Subjects in the Immediate Liver Transplant Period, *Journal of Hepatology* (2012), doi: <http://dx.doi.org/10.1016/j.jhep.2012.10.009>

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**Safety and anti-HCV Effect of Prolonged Intravenous Silibinin in
HCV-Genotype 1 Subjects in the Immediate Liver Transplant Period**

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Electronic word count: pending until definite manuscript

Number of Figures and Tables: 3 Figures and 2 Tables

List of Abbreviations in order of appearance

HCV: hepatitis C virus

LT: liver transplantation

SVR: sustained virological response

Peg-IFN: pegylated interferon

RBV: ribavirin

iv-SIL: intravenous silibinin

HIV: human immunodeficiency virus

HBV: hepatitis B virus

HBIg : anti hepatitis B immunoglobulin

iv-SIL-CR: intravenous silibinin complete responders

iv-SIL-PR: intravenous silibinin partial responders

MELD: model for end-stage liver disease

AST: aspartato aminotransferase

ALT: alanine aminotransferase

GGT: gamma glutamyl transpeptidase

AP: alkaline phosphatase

BR: bilirubin

ACR: acute cellular rejection

DAAs : direct-acting antivirals

PIs: HCV protease inhibitors

TPV: telaprevir

BOC: boceprevir

Conflict of interest: The authors of this manuscript have no conflicts of interest to disclose as described by the *Journal of Hepatology*

Finacial Support: this work was partially supported with a grant from the Fondo de Investigación Sanitaria (FIS PI11/02906)

Introduction

Hepatitis C virus (HCV) is one of the leading causes of chronic liver disease with life-threatening sequelae such as end-stage cirrhosis and liver cancer [1], and is the main indication for liver transplantation (LT) in Europe and USA [2,3]. HCV reinfection of the graft is the rule in patients with positive HCV-RNA at the time of LT, leading to impaired graft and patient survival [4]. Moreover, some patients develop early severe forms of HCV recurrence, such as fibrosing cholestatic hepatitis [5], and almost 35% of the patients evolve to cirrhosis within the first 5 years [6].

Several treatment strategies have been evaluated to avoid HCV recurrence. The first approach is antiviral therapy prior to LT [7], aimed to suppress viral replication at the time of surgery. However, currently most HCV-patients at the waiting list have already received unsuccessful antiviral therapy, and no more than 20-30% of patients with advanced cirrhosis tolerate therapy [8, 9], that it associated to significant toxicity and an increased risk of severe infections [8-10]. Another strategy has been the use of prophylactic antiviral therapy in the immediate post-transplant period, but it is also associated to poor tolerability, low efficacy and increased toxicity [11, 12]. In addition, treatment of acute hepatitis C after LT does not significantly reduce toxicity and yields low rates of sustained virological response (SVR) [13]. To date, treatment of established graft lesions with pegylated interferon (peg-IFN) plus ribavirin [RBV] beyond one or more years after LT seems to be the most appropriate scenario, but SVR ranges between 30-45% [14, 15].

In 2008, Ferenci P *et al* proved that a chemically hydrophilized formulation of intravenous silibinin (iv-SIL, LEGALON™), up to doses of 20mg/kg/d, has a potent dose-dependent antiviral effect when used as monotherapy in prior non-responders to conventional peg-IFN based therapy, with mean HCV-RNA decreases of 3.02 ± 1.01 log₁₀ IU/ml after 7 days [16]. Since then, there have been reports on the successful eradication of HCV in prior peg-IFN/RBV non-responders [17], even HIV-coinfected [18], and several cases of successful prevention of HCV reinfection of liver grafts using

iv-SIL monotherapy [19, 20], or treatment of established graft hepatitis with iv-SIL plus standard therapy [21]. In all cases iv-SIL monotherapy has shown to be safe, with transient hyperbilirubinemia and mild sensation of heat with infusion as the most relevant drug-associated effects [15-21].

SIL seems to have multiple effects on the HCV lifecycle: *in vitro* it can inhibit HCV NS5B polymerase activity, but it also appear to block virus entry and transmission, possibly by targeting the host cell [22, 23], and modelling HCV kinetics *in vivo* suggests that SIL may block both viral infection and viral production/release with its main dose-dependent effect being blocking viral production/release [24].

Taking into account current knowledge of the *in vivo* effects and safety of iv-SIL, the hypothesis of the present work was that in HCV-cirrhotic patients undergoing LT, early administration of iv-SIL monotherapy (immediately before and during surgery), followed by prolonged administration thereafter, might be able to avoid HCV reinfection of the graft, or at least could reduce HCV RNA replication, delaying and reducing the severity of viral recurrence.

Methods

Study design and organization

This was a prospective, “proof of concept” study on the safety and anti-HCV effect of iv-SIL (Legalon™, Madaus-Rottapharm, Germany) administered in the immediate transplant period followed by prolonged administration in patients with cirrhosis due to HCV genotype 1 undergoing LT at the Liver Transplant Unit in the Hospital Ramón y Cajal, a Reference Center for LT in Madrid, Spain. The main objective of the study was to evaluate the feasibility to avoid HCV recurrence in the liver graft, in comparison to a control, non-treated group of consecutive previously transplanted patients receiving the same immunosuppressive regimen. Before the study started, the protocol was reviewed and approved by the institutional review board. Informed consent was obtained from all patients in the iv-SIL group after sufficient explanation was given and before they participated in the study. As this was an off-label use of iv-SIL, in all subjects access to the product was obtained using a “compassionate use” procedure.

Patients

iv-SIL group: patients enrolled in the study were all consecutive subjects with HCV-cirrhosis undergoing deceased-donor primary orthotopic LT from September 2011 and with the following criteria: signed informed consent, HCV genotype 1 with positive serum HCV RNA, negative HBsAg or negative HBV recipient of a positive HBV anticore donor (to avoid the potential effect of HBIG), HIV-negative antibodies. Figure 1 represents the dosing scheme of iv-SIL for the first 24 hours. All patients started iv-SIL at a dose of 20mg/kg/day over a 3h infusion period immediately before LT, at the beginning of the anhepatic phase, and at entry in the intensive care unit. Thereafter, each patient maintained daily doses for at least 21 days. Therapy was prolonged up to 6 weeks in two patients reaching negative HCV-RNA between week 2 and 3, whereas patients with absence of HCV RNA negativization at day 21 stopped iv-SIL. In any case admission was prolonged related to iv-SIL therapy, that was completed in the day care unit of the hospital after discharge.

There were considered two types of response to iv-SIL: *complete response* (iv-SIL-CR), in patients reaching negative HCV-RNA during therapy, and *partial response* (iv-SIL-PR), in patients with persistence of detectable HCV-RNA levels at the end of the scheduled time of therapy of 21 days.

Control group: this group was retrospectively selected, and included all consecutive patients undergoing LT at our center between the incorporation of basiliximab-based immunosuppression and the initiation of the study with iv-SIL (from April 1 to September, 23, 2011).

For each patient we recorded demographic data, the presence of hepatocellular carcinoma, and HCV-related data (HCV-RNA at LT, prior antiviral therapy with peg-IFN plus RBV, model for end-stage liver disease (MELD) score at LT, and analytical data, specially those related to liver function (AST, ALT, GGT, Alkaline phosphatase, total bilirubin).

Immunosuppressive regimen

All patients received the same standard immunosuppression consisting of basiliximab induction (20 mg iv bolus intraoperatively after liver graft reperfusion on day 0, and on day 4), tacrolimus (0.10 mg/kg/day p.o or through a nasogastric tube in two divided doses, starting at day 3), micophenolate mofetil (1000 mg b.i.d p.o), and steroids (a bolus of methylprednisolone 5mg/kg intraoperatively in the anhepatic phase followed by 20 mg/day for the first two weeks, and progressive tapering until complete withdrawal between months 3 and 6).

HCV-RNA assessment

HCV-RNA was measured using a fully automatized COBAS TaqMan HCV assay (Roche Diagnostics, Pleasanton, USA) with a lower detection limit of 15 IU/ml.

iv-SIL group: HCV-RNA was measured prospectively with the following schedule: on the day of surgery, both before and immediately after each iv-SIL dose (Figure 1), at days 1, 3, 5, 7, 10, 14 and 21 post-LT (always before iv-SIL administration), and at least once weekly thereafter.

Control group: HCV-RNA was assessed in available stored samples belonging to the same period, (at least at baseline of LT and day 7), and once weekly, if feasible.

IL28B assessment

IL28B genotype analyses (rs 12979860 SNP) were performed prospectively (iv-SIL group), or using stored samples (control group). In all cases the patients gave specific informed consent.

Safety

Any clinical adverse event during infusion in the iv-SIL group was recorded. In addition, haematological and biochemical parameters were assessed in both groups, with the same schedule used for HCV-RNA assessment, and/or if deemed medically necessary.

Histology

At our Center there is not a protocol for scheduled liver biopsies after transplantation, and its performance is restricted to patients with abnormal liver function tests, suspected acute cellular rejection, or in case of suspected liver injury related to HCV recurrence.

Statistical analysis

Mean and standard deviation or median and interquartile range were used for the description of continuous variables. Absolute and relative frequencies were used for categorical variables. For the comparison of baseline data between iv-SIL and control group, chi-square or Fisher's exact test for categorical variables and Mann-Whitney U-test for continuous variables were used.

The comparison of HCV-RNA decays during follow-up, and variations in liver function tests were performed by non-parametric tests (Mann-Whitney U test), with significance for p values <0.05. All statistical analyses were carried out using the SPSS package (version 15.0).

Results

The overall cohort included 16 subjects (9 in the iv-SIL group and 7 in the control group, respectively). Table 1 shows the lack of significant differences in baseline features between the groups, including demographic and analytical data, and HCV-RNA levels.

Figure 2 shows the evolution of HCV-RNA levels in the control (Fig 2A) and iv-SIL groups (Fig 2B). Patients in the control group showed an initial decrease in HCV-RNA, but none of them reached undetectable levels, and viral rebound was the rule after day 7. By contrast, the slope of the observed HCV-RNA drop during the first week in the iv-SIL group was much more greater, all subjects showed a maintained and progressive decrease during the period of iv-SIL administration, and HCV-RNA reached undetectable levels in four patients (44%): P1 (day +21), P3 (day+14), P7 (day+21) and P8 (day+21). During iv-SIL monotherapy there were no cases of HCV-RNA breakthrough; however, following iv-SIL withdrawal HCV-RNA rebounded in all subjects, with progressively higher HCV-RNA levels. In patients with negative HCV-RNA the median time to the first positive HCV-RNA was 21 days (range, 16-28 days). Table 2 shows the comparative mean HCV-RNA decreases between controls and iv-SIL, and between controls and iv-SIL-PR, respectively.

Among iv-SIL patients, there was a trend to lower baseline HCV-RNA levels in those reaching negative HCV-RNA when compared with those that did not (5.1 ± 0.8 vs 5.7 ± 0.5 \log_{10} IU/ml, $p=0.1$). Partial responders to iv-SIL showed marked and higher decreases in HCV-RNA when compared to controls (Table 2), but lower than complete responders to iv-SIL at week 1 (-2.9 ± 0.69 vs -3.13 ± 1.04 , $p=0.7$), week 2 (-3.42 ± 0.7 vs -4.25 ± 0.9 , $p=0.18$), and specially at week 3 (-3.15 ± 0.8 vs -5.15 ± 0.85 , $p=0.01$).

The observed mean HCV-RNA decreases during iv-SIL were higher among naïve patients when compared to prior nonresponders to peg-IFN/RBV: week 1 (-3.53 ± 0.87

vs $-2.59 \pm 0.50 \log_{10}$ IU/ml, $p=0.08$), week 2 (-4.45 ± 0.67 vs $-3.26 \pm 0.66 \log_{10}$ IU/ml, $p=0.03$), week 3 (-4.38 ± 1.66 vs $-3.91 \pm 1.05 \log_{10}$ IU/ml, $p=0.65$)

IL28B genotype did not affect response to iv-SIL; moreover, although non-significant, the observed mean HCV-RNA decreases during iv-SIL were higher among non-CC subjects than in CC-subjects at week 1 (-3.30 ± 0.77 vs $-2.64 \pm 0.80 \log_{10}$ IU/ml, $p=0.24$), week 2 (-4.18 ± 0.80 vs $-3.29 \pm 0.79 \log_{10}$ IU/ml, $p=0.14$) and week 3 (-5.00 ± 1.05 vs $-3.30 \pm 0.99 \log_{10}$ IU/ml, $p=0.057$).

Neither HCV subtype (1a vs 1b) nor gender mismatch affected HCV-RNA kinetics or the rates of negative HCV-RNA in iv-SIL treated recipients: patients with HCV genotype 1a (N=4) showed similar baseline HCV-RNA levels (5.90 ± 0.80 vs $5.30 \pm 0.80 \log_{10}$ IU/ml, $p=0.29$), and week 1 (-3.20 ± 1.10 vs $-2.90 \pm 0.60 \log_{10}$ IU/ml, $p=0.64$), week 2 (-3.70 ± 1.20 vs $-3.80 \pm 0.70 \log_{10}$ IU/ml, $p=0.80$) and week 3 (-4.06 ± 1.90 vs $-4.20 \pm 0.50 \log_{10}$ IU/ml, $p=0.86$) HCV-RNA decays than subjects with HCV genotype 1b (N=5), with similar rates of negative HCV-RNA: 50% vs 40% ($p=1$). Regarding gender mismatch (N=4), values were as follows: baseline (5.43 ± 0.83 vs $5.70 \pm 0.81 \log_{10}$ IU/ml, $p=0.63$), week 1 (-2.71 ± 0.49 vs $-3.24 \pm 0.99 \log_{10}$ IU/ml, $p=0.36$), week 2 (-3.57 ± 0.42 vs $-3.96 \pm 1.16 \log_{10}$ IU/ml, $p=0.56$) and week 3 (-4.02 ± 0.32 vs $-4.22 \pm 1.72 \log_{10}$ IU/ml, $p=0.81$), and 25% vs 60% rates of negative HCV-RNA ($p=0.52$).

There were not adverse events during iv-SIL monotherapy; none of the patients reported sensation of heat during infusion, and all subjects completed the scheduled administration. iv-SIL patients showed lower but non-significant lower median ALT levels at each time point: week 1 (129 vs 197 U/L, $p=0.08$), week 2 (55 vs 131 U/L, $p=0.31$), week 3 (26 vs 43 U/L, $p=0.22$), and a trend to higher GGT levels: week 1 (567 vs 346 U/L, $p=0.10$), week 2 (536 vs 261 U/L, $p=0.19$), week 3 (247 vs 193 U/L, $p=0.75$). As reported previously, iv-SIL led to significantly higher total bilirubin levels when compared to controls (Figure 3): week 1 (5.16 vs 1.64 mg/dL, $p=0.01$), week 2 (3.99 vs 1.1 mg/dL, $p=0.02$), week 3 (2.85 vs 0.94 mg/dL, $p=0.03$). In all cases this

effect was transient and asymptomatic. In addition, iv-SIL was not associated to a higher rate of biopsy proven acute cellular rejection (ACR): 1/9 (11%) vs 2/7 (29%), $p=0.58$. Only one patient in the control group received steroid boluses; in the remaining cases rejection was managed adjusting oral immunosuppressive drugs.

ACCEPTED MANUSCRIPT

Discussion

In the present study, administration of iv-SIL (20mg/kg/d) monotherapy up to six weeks in patients with HCV genotype 1 undergoing orthotopic LT was safe and showed a potent and sustained antiviral effect, with 44% of subjects reaching undetectable HCV-RNA levels during therapy. This is a relevant finding, for to date, any available anti-HCV drug used as monotherapy has been able to achieve similar results, safely and without significant toxicity or interactions with immunosuppressive drugs.

As reported previously in studies on viral dynamics during and immediately after LT, [25-27], among non-treated patients there was also a rapid decline in HCV-RNA levels, supporting that the liver is the principal site of HCV replication. However, this effect was observed only during the first week, the slope of HCV-RNA decay was much less pronounced, and none of the control patients reached undetectable HCV-RNA levels.

By contrast, iv-SIL monotherapy was associated to significant, progressive and maintained decreases in HCV-RNA ($-4.15 \pm 1.31 \log_{10}$ IU/ml at week 3), ranges that are at least similar or even greater than those reported during monotherapy with currently marketed direct-acting antivirals (DAAs), such as the HCV-NS3/4A protease inhibitors (PI), telaprevir (TPV) or boceprevir (BOC). In the first, phase 1 trial of TPV in patients with chronic hepatitis C, TPV reduced HCV-RNA levels by $2 \log_{10}$ or greater after 14 days of monotherapy [28]. A recently published, open-label study in naïve Japanese patients with HCV genotype 1b using TPV alone for 12 weeks [29], showed a median decrease of $5.175 \log_{10}$ IU/ml on day 14, with viral breakthrough and emergence of resistant variants in 80% of the subjects; moreover, in one subject that achieved undetectable HCV-RNA levels, relapse was observed as soon as 1 week after completion of therapy. On the other hand, there is little information on the efficacy of BOC monotherapy, with available data only after one week and at doses below those approved for triple therapy: in a multicenter, open-label, 2-dose level, 3-way crossover, randomized study, in patients with HCV genotypes 1a or 1b, prior nonresponders to peg-IFN/RBV [30], mean maximum \log_{10} changes in HCV-RNA were -1.08 ± 0.22 and

-1.61±0.21 for BOC 200mg and 400mg, respectively. A more interesting finding in our study, however, was the lack of HCV-RNA breakthrough and “viral resistance” during iv-SIL monotherapy, in contrast to what has been reported with PIs; attributable to the high genetic variability of HCV, variants with reduce susceptibility to PIs can occur naturally even before treatment begins, precluding their use in monotherapy [31]. Therefore, the observed differences in the *in vivo* activity of iv-SIL compared to DAAs might support that the main antiviral effect of iv-SIL could be the blockage of viral infection and viral release rather than a direct inhibition of HCV replication impairing the activity of the HCV NS5B RNA-dependent RNA polymerase [22-24]. In fact, SIL may inhibit HCV replication in part by blocking binding of the RNA polymerase to its template, but direct inhibition of the RNA polymerase activity is unlikely to be a major contributor to its antiviral effect [32].

Despite its progressive effect on HCV-RNA and the lack of viral rebound while on therapy, the effect of iv-SIL was transient, and progressive increases in HCV-RNA levels were observed in all subjects following iv-SIL withdrawal. In contrast to the early rebound observed after TPV monotherapy [29], HCV-RNA rebound was significantly delayed (median 21 days) in patients reaching undetectable HCV-RNA levels during iv-SIL.

Our results with iv-SIL therapy were also better than those reported with the use of intravenous HCV-Ab^{XTL}68, a fully human monoclonal antibody that binds to the virion E2 envelope glycoprotein [33, 34]. A randomized, double-blind, dose escalation study in the immediate transplant period showed a greatest median HCV-RNA decrease at day 1 of 2.5 log₁₀ IU/ml for the 240-mg dose that was not sustained. Moreover, no patient reached undetectable HCV-RNA levels at any time during the study, and 21% of patients discontinued infusion as a result of an adverse event, fatal in one subject [35].

In our patients IL28B-CC genotype was not associated to a better response, but taking into account the proposed mechanisms of action of iv-SIL and the lack of peg-IFN coadministration, it seems not surprising. Moreover, IL28B may have limited predictive

value in patients with prior failure to peg-IFN/RBV receiving triple therapy with HCV-PIs, and there was no significant difference in SVR rates across the different IL28B genotypes with TPV triple therapy in genotype 1 experienced patients [36].

To our knowledge, our study represents to date the most prolonged use of iv-SIL, especially in the setting of LT. iv-SIL showed an excellent safety profile, that makes its use an attractive therapeutic alternative in this population. Although the new standard-of-care treatment for HCV genotype 1 infection is the combination of peg-IFN, RBV and TPV or BOC, both drugs have a synergistic and/or additive effect with peg-IFN/RBV increasing the rates of anemia and rash [37, 38], and lead to higher levels of cyclosporine and tacrolimus, with potentially harmful effects [39]. To enhance the effect of iv-SIL, its combination with RBV seems an interesting approach. Although the main mechanism of action of RBV in patients with chronic hepatitis C remains undetermined, RBV increases the rates of SVR, is critical to avoid HCV-RNA recurrence in peg-IFN responders, has a direct suppressive effect of viral polymerase activity, and also shows some immunomodulatory activity [40, 41]. A case report published in 2009 [42] showed a rapid suppression of HCV-RNA in a peg-IFN/RBV non-responder cirrhotic patient receiving 14 days of iv-SIL after a 4-week course of RBV monotherapy (800mg/day). Another approach could be the addition of standard peg-IFN/RBV during iv-SIL monotherapy or immediately after its withdrawal, when HCV-RNA levels still remain negative or at very low levels. This “early iv-SIL” strategy, from our point of view, might be more efficient than delaying iv-SIL plus peg-IFN/RBV to the phase of established graft damage, as reported previously [21].

The main limitations of our study were the sample size –although it was planned as a pilot study–, the inability to determine the optimal duration of iv-SIL, at least if used as monotherapy, *and the absence of data concerning liver graft histology during iv-SIL administration that could have confirmed if iv-SIL was really able to delay HCV reinfection of the graft.*

In conclusion, taking into account the unique profile of iv-SIL (lack of toxicity during prolonged administration, no pharmacokinetic interactions with immunosuppressants or other anti-HCV drugs, potent dose- and time-dependent antiviral effect, and specially the absence of HCV-RNA rebound and/or resistance during administration), the results of the present study support further investigation on its use in combination with currently or in the nearest future available anti-HCV combinations in patients with viral recurrence following LT.

Acknowledgments

The authors wish to thank Alfonso Muriel and Javier Zamora, from the Clinical Biostatistics Department, for their valuable help in the performance of statistical analyses.

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Figure legends

Fig. 1. Administration schedule of intravenous silibinin in the immediate period of transplantation.

Fig. 2. Evolution of HCV-RNA following liver transplantation. (2A) control group, (2B) intravenous silibinin group.

Fig. 3. Evolution of total bilirubin (mg/dL, median) up to week 3 following liver transplantation. Black line: intravenous silibinin (iv-SIL) group. Dotted line: control group.

ALL FIGURES SHOULD BE IN BLACK AND WHITE

Table 1. Baseline features

	Iv-SIL (N=9)	Controls (N=7)	<i>P</i>
Age (years; mean \pm SD)	52 \pm 6	56 \pm 4	0.24
Male sex (% , N)	78% (N=7)	57% (N=4)	0.37
MELD at LT (mean \pm SD)	15 \pm 4,5	14,6 \pm 6	0.87
Peg-IFN/RBV prior to LT (% , N)	56% (N=5)	43% (N=3)	0.61
HCC (% , N)	33% (N=3)	57% (N=4)	0.34
Non-CC IL28B genotype (%)	56%	57%	0.95
HCV RNA (log ₁₀ IU/ml; mean \pm SD)	5.6 \pm 0.78	5.5 \pm 0.39	0.88
AST (U/L; median)	85	130	0,49
ALT (U/L; median)	58	73	0,71
GGT (U/L; median)	71	40	0,48
AP (U/L; median)	178	143	0,72
Total BR (mg/dL; median)	3.26	2.86	0,71

SD: standard deviation; **iv-SIL:** intravenous silibinin; **MELD:** model for end-stage liver disease; **LT:** liver transplantation; **peg-IFN:** pegylated interferon; **RBV:** ribavirin; **IL28B:** rs12979860 SNP; **HCC:** hepatocellular carcinoma; **AST:** asparatate aminotranseferase; **ALT:** alanine aminotransferase; **GGT:** gammaglutamil transpeptidase; **AP:** alkaline phosphatase; **BR:** bilirubin

Table 2. Comparative mean HCV RNA log₁₀ IU/ml decline in control vs iv-SIL groups

	Controls (N=7)	iv-SIL (N=9)	<i>p vs control</i>	iv-SIL-PR (N=5)	<i>p vs control</i>
Week 1	-1.8±1.0	-3.0±0.8	0.019*	-2.9±0.7	0.063
Week 2	0.2±1.25	-3.8±0.9	<0.001*	-3.4±0.7	0.005*
Week 3	-0.4±0.6	-4.1±1.3	<0.001*	-3.1±0.8	0.002*

iv-SIL: intravenous silibinin; **iv-SIL-PR:** intravenous silibinin partial responders (no HCV RNA negativization)

Fig. 1

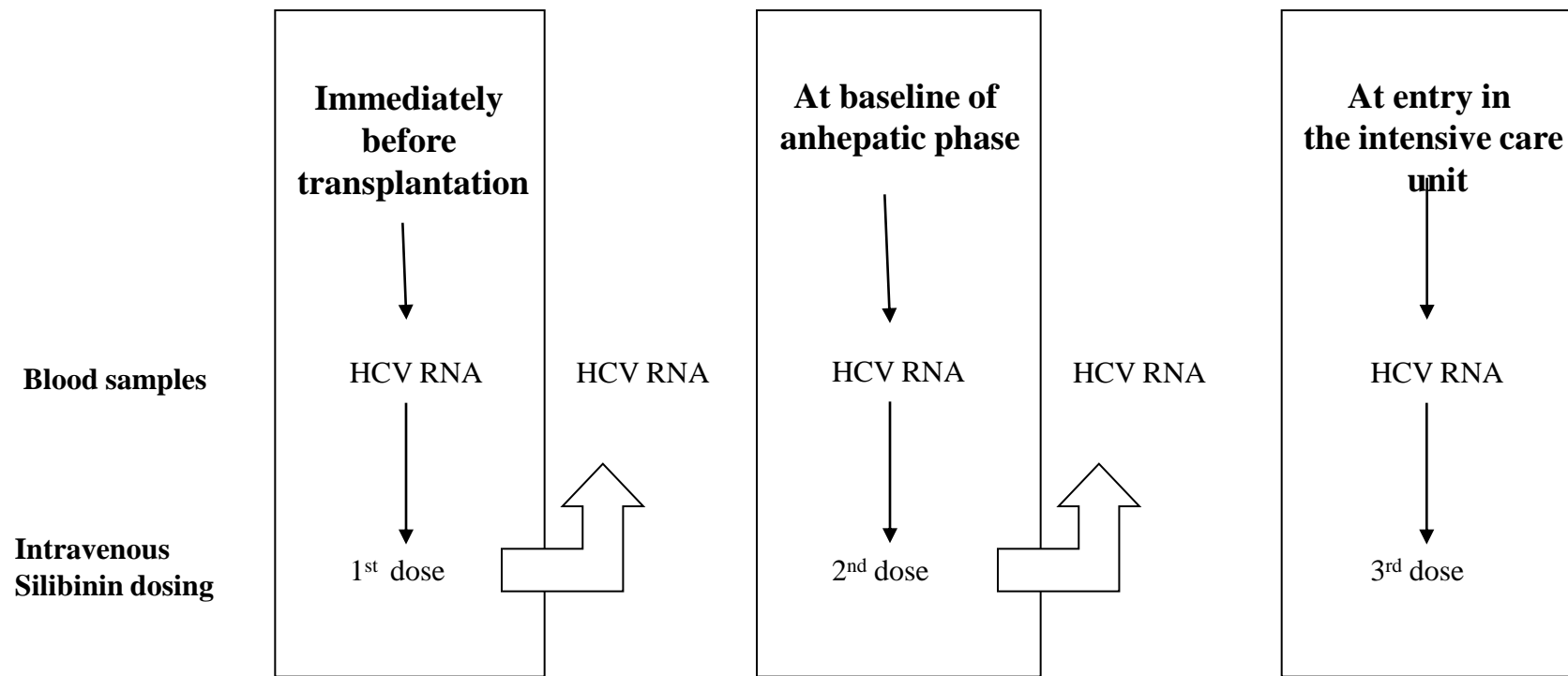


Fig. (2A)

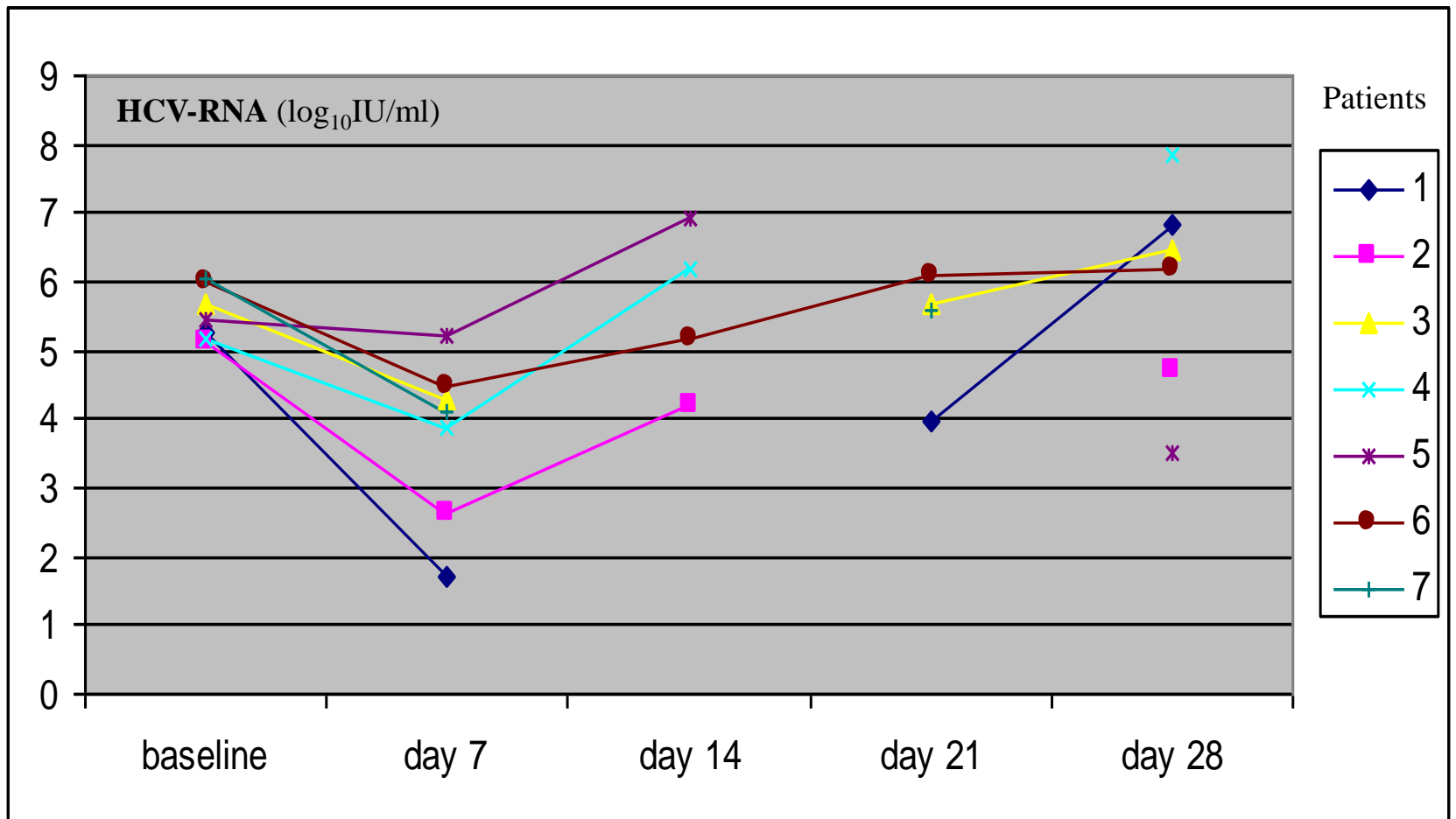


Fig. (2B)

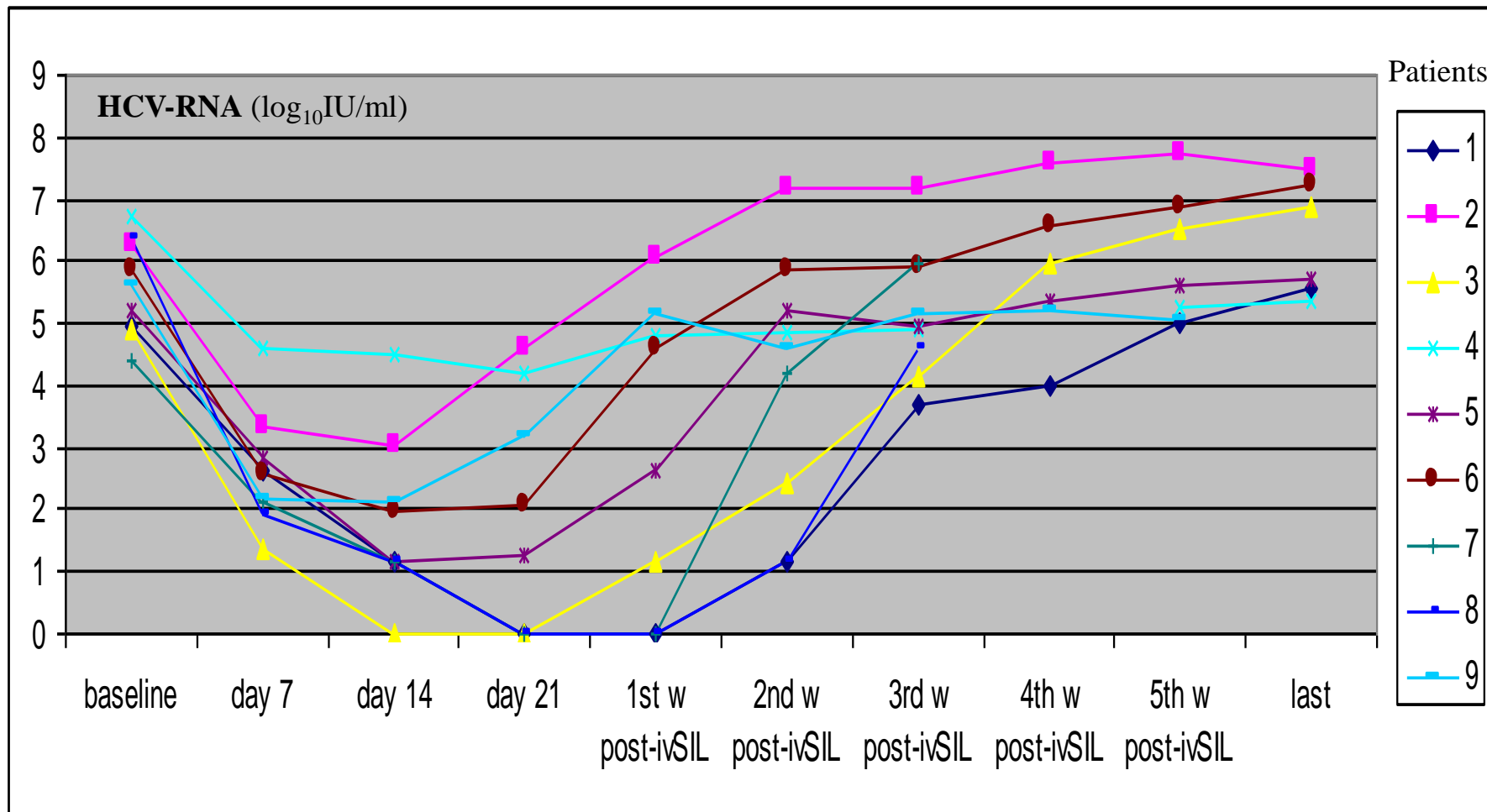


Fig. 3

