Original article

Evidence of a significant role for Fas-mediated apoptosis in HCV clearance during pegylated interferon plus ribavirin combination therapy

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Background: The role of apoptosis in treatment-induced HCV clearance is controversial. We sought to assess the kinetics of serum apoptosis-related cytokines during pegylated interferon-α2a or -α2b plus weight-based ribavirin therapy for genotype 1 chronic HCV infection.
Methods: Serum levels of soluble Fas (sFas), soluble Fas ligand (sFasL) and soluble tumour necrosis factor receptor I (sTNF-RI) were measured at baseline, week 12 and 24 weeks after the end of therapy.
Results: Sustained virological response (SVR) was achieved in 46% of the 164 included patients, 29% had a non-response (NR) and 25% had relapse (RR). NR patients presented with higher levels of sFasL at baseline and lower levels of sTNF-RI at week 12 as compared to RR and SVR patients. Lower concentrations of sFas were observed in SVR patients 24 weeks after treatment as compared to RR and NR patients. An increase in sFas at week 12 followed by a significant drop 24 weeks after therapy was observed among SVR patients. An increase in sFasL during and after treatment was observed in RR and SVR patients. NR patients exhibited an earlier drop in sTNF-RI levels as compared to RR and SVR patients.
Conclusions: Virological response during HCV therapy was associated with an increase of sFas and sFasL, and maintenance of increased concentrations of sTNF-RI.

Introduction

Over the last few years, HCV infection has been recognized as a major health problem worldwide, with estimated prevalence ranging from 2.2% to 3.0% [1]. In addition, hepatitis C infection is associated with significant morbidity, as 25% of patients with chronic hepatitis C will ultimately evolve to liver cirrhosis [2], and a significant proportion of these will develop hepatocellular carcinoma [3,4]. Failure to generate sufficiently effective immune responses during acute infection is considered a key factor in developing chronic hepatitis C [5,6]. Recent evidence suggests that HCV clearance requires a coordinated action of both the innate immune system (type I interferon [IFN] secretion and natural killer cell activation) and the adaptive immune system ($CD4^+$ and $CD8^+$ T-cells and neutralizing antibodies) [7].

Although recognized as the current standard therapy for chronic HCV infection, the exact mechanism of action of IFN is not completely understood. Although a direct inhibition of HCV replication has been demonstrated [8], it is possible that IFN-α, through its immunomodulatory properties, accelerates the clearance of infected cells at the same time as it inhibits viral replication [9]. Likewise, the mechanisms of action of ribavirin in chronic hepatitis C are still a matter of debate. A direct effect on viral replication, by reacting with the HCV-RNA-dependent RNA polymerase (NS5B) [10] or inhibiting the enzyme inosine monophosphate dehydrogenase [11] has been proposed. In addition, evidence suggests that ribavirin may enhance the host-adaptive antiviral immune response by preserving or augmenting a Th1 response pattern that may eventually lead to increased activity of cytotoxic T lymphocytes and secretion of antiviral cytokines, such as IFN-γ and tumour necrosis factor (TNF)-α [12]. Although the exact pathways remain to be elucidated, there is growing evidence suggesting that the clearance of infected cells by apoptosis represents a critical event of both IFN-α and ribavirin modes of action [13,14]. An experimental study showed that the 2–5A system

contributes to the antiviral effect of IFNs through the RNase L-induced apoptosis [13]. Similarly, an *in vitro* study showed that ribavirin decreased synthesis of total DNA, RNA and protein, and led to a dose-dependent increase in apoptosis rate [14].

Recently, the roles of the soluble Fas (sFas), soluble Fas ligand (sFasL) and soluble TNF receptor I (sTNF-RI) have been evaluated in chronic HCV infection [15–20]. sFas is produced by the shedding of the extracellular portion of Fas or by alternative splicing of transcripts [21,22], and it is reported to reduce Fas/Fas ligand mediated apoptosis [21,23]. sFas level has been correlated with liver fibrosis [16] and necroinflammatory activity [17,18] in chronic HCV infection. sFasL can be generated after proteolytic cleavage of the native and functional forms of Fas ligand, and exhibits pro-apoptotic capacity [24]. Higher levels of sFasL were observed in HCV patients with more intense necroinflammatory activity [18]. The soluble forms of TNF receptors apparently arise as a result of shedding of the extracellular domains of the transmembrane receptors, acting as TNF inhibitors by competing with the transmembrane TNF receptors [25]. In HCV infection, sTNF-RI levels were demonstrated to be related to the severity of liver disease [26,27]. The aim of our study was to assess the kinetics of serum levels of sFas, sFasL and sTNF-RI during combination therapy with pegylated interferon (PEG-IFN) plus ribavirin for chronic hepatitis C.

Methods

Patients

This retrospective cross-sectional study included consecutive adult patients with genotype 1 HCV infection treated with PEG-IFN-α2a or -α2b plus ribavirin at our institution, between January 2001 and December 2007, after giving their written informed consent. Antiviral therapy was considered in patients with positive HCV RNA by PCR (>50 IU/ml) and liver biopsy showing fibrosis stage ≥1 and/or portal/periportal necroinflammatory activity ≥2 by using Scheuer's classification [28]. Contraindications to antiviral therapy included haemoglobinopathies, evidence of hepatic decompensation (ascites, encephalopathy or gastrointestinal bleeding secondary to portal hypertension), uncontrolled cardiovascular diseases, severe pre-existing psychiatric disorders, alcohol abuse (ethanol consumption >50 g/day) or immunosuppressive therapy over the last 6 months, coexisting systemic autoimmune diseases or malignancy, and platelet count $<75\times10^9$/l or neutrophil count $<1.5\times10^9$/l. In addition, patients with the following conditions were excluded: HBV and/or HIV coinfection, end-stage renal disease, prior IFN treatment and absence of stored sera (at baseline and at week 12 of therapy). The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration and was approved by our institutional review board.

Procedure

Demographics, laboratory and other clinical variables were reviewed and extracted from medical records. Patients with ethanol consumption >50 g/day (before 6 months prior to the start of antiviral therapy) were considered as alcohol abusers. The laboratory results were expressed as absolute values. Only laboratory results performed within 2 weeks from the start of treatment were used for this study.

Serum cytokines

Serum levels of sFas, sFasL and sTNF-RI were measured by ELISA in samples, stored at -20°C, at baseline, at week 12 and 24 weeks after the end of therapy using commercially available assays (R&D Systems, Inc., Minneapolis, MN, USA). All tests were performed strictly according to the manufacturer's instructions. The results are expressed as pg/ml.

Viral assays

Qualitative HCV RNA was tested before treatment, at week 48 and at week 72 with a PCR-based assay (Amplicor 2.0; Roche Diagnostic Systems, Indianapolis, IN, USA), with a lower detection limit of 50 IU/ml. Quantification of HCV RNA was performed at week 12 by the Cobas Amplicor HCV Monitor 2.0 assay (Roche Diagnostic Systems), with lower limit of detection of 600 IU/ml. HCV genotyping was performed by Inno Lipa (Innogenetics, Ghent, Belgium) according to the manufacturer's protocol.

Treatment regimens

Patients were treated with PEG-IFN-α2a (180 μg/week) or -α2b (1.5 μg/kg/week) in combination with ribavirin at a dose of 1,000 mg/day (for patients with a body weight <75 kg) or 1,250 mg/day (for those with a body weight ≥75 kg). Early virological response was defined as either an undetectable HCV RNA (<50 IU/ml) or a minimum 2-$\log_{10}$ decrease from baseline in HCV RNA at week 12. Treatment was interrupted at week 12 in those who fail to achieve early virological response; otherwise, therapy was continued until week 48. Relapse (RR) was assessed between week 48 and week 72. Sustained virological response (SVR) was defined as undetectable HCV RNA at 6 months after the end of treatment. According to the pattern of virological response, patients were classified as having non-response (NR), RR or SVR.

Statistical analyses

Continuous variables were compared using the Student's *t*-test or the Mann–Whitney U test. Categorical variables

Table 1. Baseline characteristics of included patients and comparison of clinical, virological and histological variables according to the type of PEG-IFN

Patient characteristic	All (*n*=164)	PEG-IFN-α2a (*n*=66)	PEG-IFN-α2b (*n*=98)	*P*-value
Mean age at treatment, years (±SD)	46.7 (11.3)	46.2 (10.8)	47.1 (11.7)	0.649
Male gender, *n* (%)	83 (51)	39 (59)	44 (45)	0.075
Caucasian, *n* (%)	116 (71)	47 (71)	69 (70)	0.912
Alcohol consumption ≥50 g/day, *n* (%)[a]	28 (17)	15 (23)	13 (13)	0.114
HCV RNA≥800,000 UI/ml, *n* (%)	75 (54)	32 (54)	43 (54)	0.982
Mean sFas level, pg/ml (±SD)	1,203.5 (494.0)	1,326.8 (487.9)	1,106.9 (462.1)	0.010
Median sFasL level, pg/ml	87.0	100.0	84.0	0.088
Mean sTNF-RI level, pg/ml (±SD)	145.8 (47.6)	153.2 (50.3)	141.1 (45.6)	0.128
Fibrosis stage				0.259
0, *n* (%)	6 (4)	1 (1)	5 (5)	_
1, *n* (%)	54 (33)	22 (34)	32 (33)	_
2, *n* (%)	56 (35)	22 (34)	34 (36)	_
3, *n* (%)	22 (14)	7 (11)	15 (16)	_
4, *n* (%)	22 (14)	13 (20)	9 (10)	_
Portal-periportal activity				0.817
0, *n* (%)	4 (3)	1 (2)	3 (3)	_
1, *n* (%)	3 (2)	1 (2)	2 (2)	_
2, *n* (%)	88 (55)	38 (59)	50 (53)	_
3, *n* (%)	56 (35)	20 (31)	36 (38)	_
4, *n* (%)	8 (5)	4 (6)	4 (4)	_
Non-response, *n* (%)	48 (29)	17 (26)	31 (32)	0.428
Relapse, *n* (%)	41 (25)	17 (26)	24 (25)	0.883
Sustained virological response, *n* (%)	75 (46)	32 (49)	43 (44)	0.502

[a]Before 6 months prior to the start of antiviral therapy. PEG-IFN, pegylated interferon; sFas, soluble Fas; sFasL, soluble Fas ligand; sTNF-RI, soluble tumour necrosis factor receptor I.

were compared using the χ^2 test. ANOVA or Kruskal–Wallis analyses were used to compare the serum levels of sFas, sFasL and sTNF-RI according to the pattern of response. Repeated measures ANOVA or Friedman's test were used for comparisons of the levels of cytokines in specific time points during treatment. Test results with *P*-values <0.05 were considered as statistically significant. Statistical analysis was performed by using SPSS software version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

From January 2001 to December 2007, 181 patients with genotype 1 HCV infection were treated with PEG-IFN plus ribavirin in our institution. Among these, 17 patients had insufficient stored sera at baseline or at week 12 and were excluded. These patients were not significantly different from the included individuals regarding demographic, laboratorial and histological variables (*P*>0.05; LLS *et al.*, data not shown). Among the 164 included patients (Table 1), the mean age ±SD was 46.7 ±11.3 years, 71% were Caucasian and 51% were male. Previous alcohol abuse was observed in 17% of the patients and 54% exhibited baseline HCV RNA levels >800,000 UI/ml. Histological findings and serum levels of sFas, sFasL and sTNF-RI at baseline are depicted in Table 1. A total of 66 (40%) patients received PEG-IFN-α2a and 98 (60%) patients received PEG-IFN-α2b. As shown in Table 1, patients treated with PEG-IFN-α2a exhibited higher levels of sFas at baseline as compared to those treated with PEG-IFN-α2b. No other significant differences were observed among the two PEG-IFN groups. When evaluating the pattern of virological response, 48 (29%) patients were considered as having NR, 41 (25%) had RR and 75 (46%) individuals achieved SVR.

Previous alcohol consumption had no influence on cytokine levels. Mean ±SD sFas and sTNF-RI levels were, respectively, 1,184.10 ±473.66 pg/ml and 145.19 ±49.92 pg/ml in abstainers, and 1,293.30 ±580.39 pg/ml and 148.71 ±35.39 pg/ml in patients who were considered ethanol abusers (*P*=0.290 and =0.723, respectively). Similarly, median sFasL levels were 88.00 pg/ml in patients who consumed ≥50 g/day of alcohol and 83.00 pg/ml in those who consumed <50 g/day (*P*=0.205).

Cytokine levels according to the pattern of response

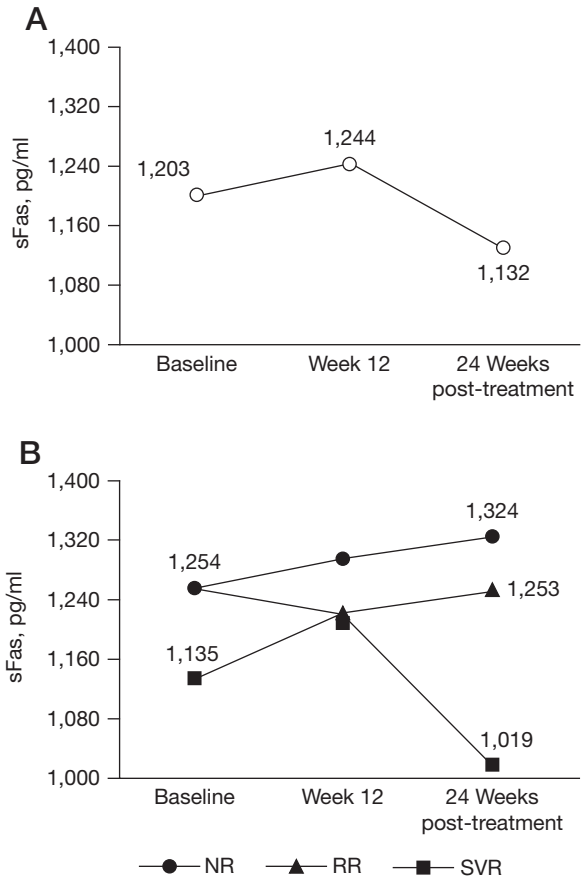
Among 164 included patients, 98 patients had stored sera at 24 weeks after the end of therapy. Table 2 shows the serum levels of the apoptosis markers

Table 2. Serum levels of sFas, sFasL and sTNF-RI according to the pattern of response to antiviral therapy

Parameter	Non-response (*n*=48)	Relapse (*n*=41)	Sustained virological response (*n*=75)	*P*-value
sFas				
Baseline	1,254.20 ±535.74	1,254.07 ±464.93	1,135.29 ±482.05	0.332
Week 12	1,294.43 ±471.36	1,221.03 ±431.98	1,218.88 ±506.15	0.703
24 Weeks post-treatment	1,324.66 ±527.88	1,253.22 ±353.77	1,019.24 ±400.88	0.010[a]
sFasL				
Baseline	97.50	79.50	82.00	0.051
Week 12	101.00	98.00	86.00	0.263
24 Weeks post-treatment	123.50	108.00	109.00	0.778
sTNF-RI				
Baseline	145.44 ±55.82	147.36 ±50.35	144.73 ±41.39	0.963
Week 12	111.34 ±55.63	142.55 ±59.49	135.08 ±57.40	0.041[b]
24 Weeks post-treatment	102.81 ±71.74	88.25 ±68.54	69.84 ±52.09	0.101

Data are mean ±SD for soluble Fas (sFas) and soluble tumour necrosis factor receptor I (sTNF-RI), and median for soluble Fas ligand (sFasL). Units of measures are pg/ml for all parameters. [a]Statistical significance for comparison between non-responders and sustained virological responders. [b]Statistical significance for comparison between patients with non-response and relapse.

Figure 1. Serum sFas levels according to the period of pegylated interferon plus ribavirin therapy



(A) Considering all patients, no differences were observed (*P*=0.254). **(B)** Sustained virological response (SVR) patients showed an increase of soluble Fas (sFas) at week 12 (not statistically significant; *P*>0.05) followed by a significant decrease in its levels at 24 weeks post-treatment (*P*=0.007). NR, non-response; RR, relapse.

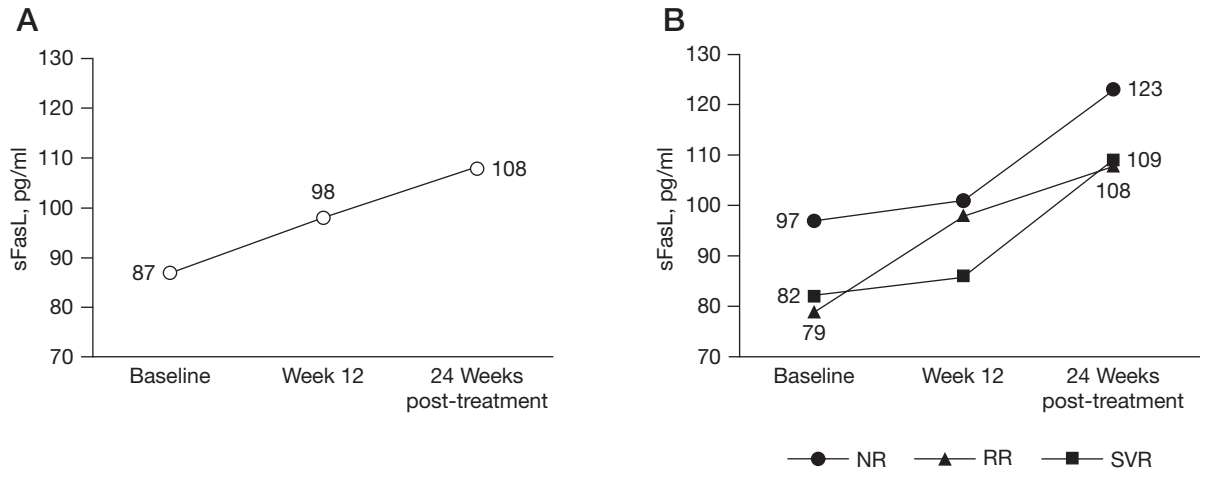
at baseline, at week 12 of treatment and 24 weeks after the end of therapy according to the pattern of response. No differences were observed in sFas levels at baseline and at week 12 among the three groups. SVR patients exhibited post-treatment sFas levels that were significantly lower than NR patients (*P*=0.010). Median sFasL levels at baseline were significantly higher in NR patients as compared to RR and SVR patients (*P*=0.051). Conversely, sFasL levels at week 12 and 24 weeks after the end of therapy were similar among the three groups. When sTNF-RI levels were evaluated according to the pattern of response, no differences were found at baseline and at 24 weeks post-treatment. NR patients exhibited sTNF-RI levels at week 12 that were significantly lower as compared to RR and SVR patients (*P*=0.041).

Behaviour of serum cytokines during treatment

Considering all patients, no significant differences were observed when the serum levels of sFas were compared in different time points during treatment (Figure 1A). Similarly, no differences were noted for the comparison of sFas at baseline, at week 12 and 24 weeks after the end of therapy in NR and RR patients. However, SVR patients showed an increase of sFas at week 12 (not statistically significant), followed by a significant decrease in its levels at 24 weeks post-treatment (*P*=0.007).

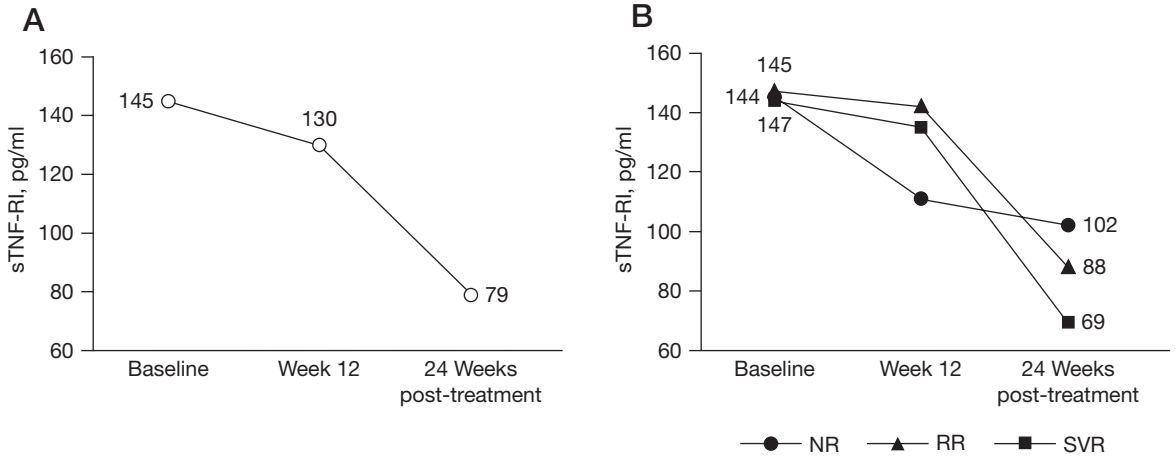
A significant increase of sFasL during and after therapy was noted (*P*<0.001) when all patients were analysed as a group (Figure 2A). However, when evaluated according to the pattern of response, this difference was restricted to RR (*P*=0.038) and SVR patients (*P*<0.001; Figure 2B). No significant differences were observed when the serum levels of sFasL were compared in distinct periods of treatment for NR patients.

Figure 2. Serum sFasL levels according to the period of pegylated interferon plus ribavirin therapy



(A) A significant increase of soluble Fas ligand (sFasL) was noted ($P<0.001$) for the patients as a whole. **(B)** When evaluated according to the pattern of response, this difference was observed in patients with relapse (RR; $P=0.038$) and with sustained virological response (SVR; $P<0.001$), but not in patients with non-response (NR; $P>0.05$).

Figure 3. Serum sTNF-RI levels according to the period of pegylated interferon plus ribavirin therapy



(A) For all patients, post-treatment soluble tumour necrosis factor receptor I (sTNF-RI) levels were significantly lower than those observed at baseline and at week 12 ($P<0.001$). **(B)** When evaluated according to the pattern of response, the difference between sTNF-RI levels at week 12 and 24 weeks post-treatment was restricted to patients with relapse (RR; $P=0.004$) and with sustained virological response (SVR; $P<0.001$). NR, non-response.

As shown in Figure 3A, post-treatment sTNF-RI levels were significantly lower than those observed at baseline and at week 12 ($P<0.001$). Nevertheless, no differences were noted for the comparison of sTNF-RI levels at baseline and at week 12. When evaluated according to the pattern of response, RR and SVR patients exhibited post-treatment sTNF-RI levels significantly lower than those observed at baseline and at week 12. Similarly, NR patients showed lower concentrations of sTNF-RI after the treatment when compared to the baseline. However, no differences were noted for comparisons of sTNF-RI levels at week 12 and at 24 weeks post-treatment in NR patients, suggesting an early decrease in sTNF-RI concentrations in this group.

Discussion

Recent evidence has demonstrated a significant role for apoptosis in the pathogenesis of hepatitis-C-induced liver damage [29]. Although the importance of studying the cellular events related to the apoptotic signalling is unquestionable, from a practical point of view, serum markers may theoretically provide non-invasive tools that might be useful in evaluating HCV patients, especially during antiviral therapy.

In the present study, when we evaluated the serum levels of apoptosis-related cytokines according to the pattern of response, patients who achieved SVR exhibited significantly lower post-treatment concentrations of sFas as compared to NR and RR patients. Although there are no other studies evaluating serum levels of sFas after HCV therapy, a previous report showed that the hepatic Fas expression was significantly reduced after IFN treatment in those with no HCV RNA detected in liver samples [30]. Thus, it is possible that the lower serum levels of this marker observed in SVR patients after the end of PEG-IFN plus ribavirin treatment reflect a less intense Fas expression in liver tissue in those who successfully cleared the virus. By constrast, sFas at baseline and at week 12 of therapy was similar among the three groups. Although these findings are in agreement with those established by Kakiuchi *et al.* [31], two other studies reported higher baseline sFas levels among NR patients to conventional IFN monotherapy [17,32].

We have also evaluated serum levels of sFas in the distinct periods of PEG-IFN plus ribavirin therapy. SVR patients showed an increase of sFas at week 12 (not statistically significant) followed by a significant decrease in its levels at 24 weeks after the end of therapy. These findings are in agreement with Zekri *et al.* [33] who reported a significant increase in sFas levels during PEG-IFN plus ribavirin treatment in those who achieved SVR. Interestingly, these results were not observed in patients treated with conventional IFN. It is possible that this increment of sFas levels observed during PEG-IFN plus ribavirin therapy actually reflects an enhancement of the hepatic Fas expression, contributing to the IFN-induced HCV clearance process.

In the current study, higher baseline sFasL levels were observed among NR patients as compared to RR and SVR patients. This finding is contrary to that previously reported by Toyoda *et al.* [17], who found no differences in baseline sFasL levels among patients with SVR and NR. However, this difference could probably be explained by the use of conventional IFN monotherapy in the Japanese series. Similarly, Zekri *et al.* [33] described no differences in baseline sFasL according to response to PEG-IFN plus ribavirin therapy. Nevertheless, the small number of patients treated with PEG-IFN plus ribavirin therapy in this study limits the interpretation of its results. When we evaluated serum levels of sFasL at week 12 and at 24 weeks after therapy, no differences were observed according to the pattern of virological response. Similar findings regarding comparisons of sFasL levels during HCV treatment were previously described [17,33].

When we evaluated the serum levels of the apoptosis-related cytokines in specific time points of antiviral therapy, a significant increase in sFasL levels could be observed when the patients were analysed as a group. However, when evaluated according to the pattern of response, this difference was restricted to RR and SVR patients. Our results are in agreement with previous studies that demonstrated a significant increase in sFasL concentrations during HCV treatment, particularly among individuals with SVR [17,33]. These findings suggest that sFasL may have a role in the IFN-induced HCV clearance process. The persistently higher levels of sFasL observed after the end of HCV therapy among RR and SVR patients may indicate a residual effect of PEG-IFN or a stronger sFasL induction as a characteristic of those patients more prone to virological response.

Lower serum levels of sTNF-RI at week 12 of treatment were observed in NR patients as compared to RR and SVR patients. These findings were not observed by Zekri *et al.* [33] when sTNF-RI levels were measured at week 4 of therapy. Such differences may be justified by the smaller number of patients included and by the shorter interval between the beginning of treatment and sTNF-RI measurement in the cohort studied by Zekri *et al.* [33]. In the present study, no differences were observed in sTNF-RI levels at baseline and at 24 weeks after therapy. These results are in accordance with previous studies evaluating HCV patients treated with conventional IFN [26,27]. It is possible that the lower sTNF-RI levels observed after the end of combination therapy, irrespective of virological response, reflect a decrease in hepatic necroinflammatory activity as a residual effect of PEG-IFN treatment [34,35].

When serum levels of sTNF-RI were evaluated in distinct periods of therapy, post-treatment sTNF-RI concentrations were significantly lower than those observed at baseline and at week 12 for the patients as a whole. However, when evaluated according to the pattern of response, an early decrease in sTNF-RI levels was found in NR patients. These findings were not observed in RR and SVR patients, suggesting that the maintenance of higher sTNF-RI levels during PEG-IFN plus ribavirin reflects a stronger immune response that might be associated with virological response. Our results are contrary to previous studies that failed to reveal differences in sTNF-RI before and after HCV treatment [26,36,37]. However, all these studies

included small numbers of participants treated with conventional IFN.

We acknowledge some limitations to our analysis. First, the use of retrospectively collected data might have led to selection bias. However, this was unlikely to have occurred in this study because only a small proportion of the patients treated with PEG-IFN in our institution were excluded. In addition, excluded patients showed no significant differences as compared to those who were included. Secondly, the absence of established cutoff values for the apoptosis-related cytokines may limit the interpretation of the results. Although we were not able to assert whether the cytokine levels were outside the normal range or not, the behaviour of these tests during HCV therapy seems to be the most important finding of this study and these results were not affected by the absence of established cutoff values.

In conclusion, virological response during PEG-IFN plus ribavirin therapy for HCV infection was associated with an increase in serum levels of sFas and sFasL, and maintenance of increased concentrations of sTNF-RI. These findings suggest that apoptosis may have a significant role in HCV clearance induced by PEG-IFN plus ribavirin.

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Disclosure statement

The authors declare no competing interests.

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