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Pretreatment serum interleukin-12 levels in predicting sustained virological response among hepatitis C patients following Pegylated Interferon-α2β plus Ribavirin treatment

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Abstract

Background Dendritic cells activated by hepatitis C virus (HCV) produce high amounts of interleukin (IL)-12, considered to be associated with HCV clearance. The aim of this study was to investigate the IL-12 levels in HCV-infected patients, before and after the application of combination therapy with Pegylated Interferon-α2β plus Ribavirin.

Methods Laboratory data of IL-12 levels and other clinical characteristics were selected from 26 HCV-infected patients. Comparisons of IL-12 serum levels before and after treatment or between responders and non-responders (including relapsers) were performed using non-parametric tests. The study moreover investigated the probable relationship of IL-12 concentrations with viral load, HCV genotypes, liver function tests (LFTs), histological activity and the response to combination treatment.

Results The baseline IL-12 levels were found significantly higher in patients who achieved sustained virological response (SVR), compared to patients who did not respond to the combination treatment (P=0.029). The IL-12 levels at the end of treatment were not statistically different from the IL-12 baseline levels, in both responders and non-responders. Baseline serum levels of IL-12 higher than 3 pg/mL (cut-off) were found to positively predict patients who successfully responded to treatment. No statistical correlation was found between the baseline serum IL-12 levels and viral load, HCV genotypes, histological activity or LFTs among the HCV patients.

Conclusion Pretreatment IL-12 levels seem to predict which patients will achieve SVR to treatment. Patients with increased IL-12 serum levels were more likely to achieve SVR.

Keywords Cytokine, dendritic cells, hepatitis C, interleukin-12, Pegylated interferon α2β, T-helper cells

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Conflict of Interest: None

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Introduction

Hepatitis C virus (HCV) infection is characterized by a high propensity to chronicity, estimated to be over 85% and is the leading cause of developing chronic liver disease, decompensated cirrhosis and hepatocellular carcinoma (HCC). For this reason HCV infection has become a worldwide health problem [1-3]. HCV is non-cytopathic in most circumstances. Therefore, immunologically-mediated events in response to HCV, involving innate and adaptive immune response (IR), play an important role in the immunopathogenesis and clinical outcome of the infection [4].

The predominance of T-helper (Th) 1 response, provoked by cytokines interleukin (IL-) 12, intreferon (IFN)-α, IFN-γ, is considered to be associated with HCV clearance. On the contrary, the Th2 predominance, provoked by cytokines IL-4,

IL-10, IL-13, is related with chronicity and disease progression [5-7]. These findings indicate that an imbalance between Th1 and Th2 response plays a pivotal role in disease elimination or chronic progression [6,8,9]. On the other hand, apart from the shift of host's IR, it is suggested that HCV-encoded proteins, core and NS3, inhibit the allo-stimulatory function of dendritic cells (DCs), the most important component of the innate immune system, critical for the initiation of adaptive IR [10,11]. There is also evidence of an apparent delay between the presence of high levels of viral titers and the presence of adaptive IR [4,12]. The high viral replication rate, which also induces a high error rate of the RNA-dependent RNA-P combined with the lack of proof-reading, promotes the emergence of quasispecies formation and gives HCV the ability to evade human IR by these mutants [13,14]. This suggests excessive concentration of viral antigens that apparently contributes to one of the main causes of disturbance to CD4+ T-cells impairment, which results in the inhibition of the proliferative capacity and cytotoxicity of CD8+ T-cells and the possible over-stimulation of these cells and the impairment of the IR [15,16].

Based on the observations of our study, we speculated that DCs from HCV-infected patients are influenced in their quantity and their functional properties, and display an impaired ability to produce appropriate amounts of IFN-γ and IL-12. IL-12 is the most critical cytokine in promoting a Th1-cell response. Observations from previous studies have shown controversial findings requiring further investigation [17,18]. It is important to note that these low levels of IL-12 production by the impaired DCs of HCV patients, has been shown to be increased after successful Pegylated IFN-α plus Ribavirin (PEG-IFNα/RBV) treatment, which promotes the predominance of Th1 cells, making the possibility of viral elimination higher [19,20].

The aim of this study was to determine the serum IL-12 levels from HCV-infected patients and correlate these with the possibility of achieving sustained virological response (SVR); to discover the possible influence of PEG-IFNα2β/RBV treatment on IL-12 levels, so that modifications of this marker might be used to predict a disease prognosis; and to investigate any correlation between the IL-12 levels, before treatment and the known parameters of HCV patients, such as viral load, HCV genotypes, histological activity, and liver function tests (LFTs).

Patients and methods

Twenty-six patients with chronic HCV infection (12 men; mean age 43.1±12.9 years) were enrolled in the present study after their written informed consent had been obtained. This study was approved by the Human Ethics Committee of "Hygeia" Hospital. Patients had been followed at Hygeia Diagnostic and Therapeutic Center of Athens, "Hygeia" Hospital of Athens and "Sismanoglio" General Hospital of Athens, from May 2005 to May 2008.

Initial diagnosis was made by positive third generation ELISA for antibodies to HCV and was confirmed by qualitative reverse transcriptase polymerase chain reaction (RT-PCR) for HCV-RNA in serum (Roche Amplicor Assay, USA). HCV-RNA viral load in IU/mL was performed in all patients by Cobas Amplicor HCV monitor Test version 2.0 Roche, with sensitivity level 600 IU/mL, as well as HCV genotype, obtained by a Versant HCV genotype 2.0 assay (INNO-LiPA HCV 2.0). A percutaneous liver biopsy was performed in all patients who consented, as part of the diagnostic evaluation. All patients had shown persistent or fluctuating alanine aminotransferase (ALT) abnormalities at baseline (mean 92±71 IU/mL; median 108, range 73-201 IU/mL).

Main exclusion criteria included: evidence of co-infection with HBV or HIV, acute infection with Epstein-Barr virus, Cytomegalovirus, Herpes Simplex virus, alcoholic liver disease, metabolic disorders, autoimmune liver disorders, or previous treatment with IFN or RBV. The source of HCV infection was assumed to be known in 16 of 26 patients. HCV infection was considered to be due to blood transfusions (before 1991) in 11 patients; IV drug addiction in 4 patients; and probably sexual transmission by his wife in 1 patient (similar genotype). The demographics, biochemistry, molecular biology and histological data are summarized in Table 1.

Treatment protocol

All patients received standardized doses of PEG-IFNα2β (1.5 mg/kg/week) and RBV at a dose of 800-1200 mg daily, according to body weight, for either 24 or 48 weeks, depending on patient's genotype (24 weeks for patients with genotype 3 and 48 weeks for genotypes 1 or 4). Among the 31 patients initially enrolled in the study, 5 were excluded. Specifically, 2 intravenous drug users with genotype 3a refused to participate; 2 with genotypes 1 and 4 respectively discontinued the treatment because of noncompliant behavior; and 1 patient with genotype 1 could not participate due to financial difficulties (no health insurance).

HCV-RNA quantity/quality, HCV genotypes, definition of responses

After the initial diagnosis of HCV infection was made, HCV-RNA viral load in IU/mL was measured in all patients (Cobas Amplicor HCV monitor test version 2.0 Roche) as well as HCV genotypes, determined by a Versant HCV genotype 2.0 assay (INNO-LiPA HCV 2.0), prior to the onset of combination treatment.

A qualitative HCV-RNA test was performed at the end of weeks 24 and 48 for patients with genotypes 1 and 4 and at the end of week 24 for patients with genotype 3, to define the patients' virological response at this time frame (ETR, End-of-Treatment Response). All patients were tested again for the qualitative HCV-RNA, 24 weeks after the cessation of the combination treatment, so that their SVR could be

Table 1 Demographics and clinical characteristics of hepatitis C virus patients

Characteristics	Patients (n=26)	
Demographics		
Mean age (min-max) (years)	43.1	(24-69)
Male, n (%)	12	(46%)
Clinical characteristics		
Continuous variables	median	(range)
Glucose (mg/dL)	98.0	(89-100)
Cholesterol (mg/dL)	200.0	(191-210)
Triglycerides (mg/dL)	148.0	(130-155)
Alanine aminotransferase (ALT, IU/mL)	108.0	(73-201)
Pre-treatment viral load ($x10^5$ IU/mL)	3.5	(0.016-27.0)
Categorical variables	n	(%)
Source of infection		
Transfusion	11	(42)
IV drug addiction	4	(15)
Sexual transmission	1	(4)
Unknown	10	(39)
Genotype		
1	14	(54)
3	9	(35)
4	3	(11)
Pre-treatment histology (stage) based on HAI index*		
No fibrosis (0)	1	(7)
Portal fibrosis (1-2)	9	(65)
Fibrous septa (3-4)	1	(7)
Transition to cirrhosis (5-6)	3	(21)
Pre-treatment histology (grade) based on HAI index (necroinflammatory)*		
Minimal (1-4)	2	(14)
Mild (5-8)	8	(57)
Moderate (9-12)	4	(29)
High (13-18)	0	(0)
Interleukin-12 serum levels (pg/mL)	median	(range)
before treatment	3.6	(1.9-7.6)
after treatment	4.0	(1.6-7.6)
6-months after treatment	4.2	(1.4-8.8)

**14 of 26 patients accepted to undergo biopsy*

defined, according to standard criteria [21].

Patients, infected by genotypes 1 or 4, were assessed for virological response after 12 weeks and treatment was continued only if a ≥2 log reduction in viremia level had occurred. Patients with genotypes 1 or 4 who had detectable HCV-RNA by week 24 were considered as non-responders and the treatment was terminated at week 24.

Histology

Liver biopsy was obtained in 14 of 26 patients as the rest refused to undergo biopsy. For these 14 patients, biopsy was performed within 6 months or less, prior to treatment initiation and 1 to 3 months post-treatment. Biopsies were scored according to the Ishak modified histological activity index (HAI) scoring system [22]. The necroinflammatory grading was scored from 0 (absence of necroinflammation), to a maximum of 18. The fibrosis staging was based on scores from 0 (absence of fibrosis) to 6 (cirrhosis, probable or definite).

Analysis of immunological parameter (IL-12) levels

A quantitative sandwich enzyme immunoassay technique (Quantikine HS High sensitivity, R&D systems) was used for the quantitative measurement of the IL-12 in patients' sera. The optical density (average of duplicate readings) of the standards versus their concentration was plotted and the best curve after linearization by log transformation was drawn. IL-12 concentration of each sample was determined according to the standard curve. Reference value was 0-7.9 pg/mL.

Samples

Blood samples from all patients were drawn prior to treatment initiation, at week 24 after the treatment initiation and at 24 weeks after the cessation of treatment. Another sample was obtained at week 48 for genotypes 1 and 4. The serum samples were collected in tubes mixed with sodium citrate and were then transported to the lab to be spun. The time allowed until centrifugation was 1 h to avoid RNA breakdown. The serum from each sample was aliquoted in 3 to 4 separate tubes stored at -70°C, until use.

Statistical analysis

Due to the low number of cases, descriptive results are presented both as median and range or mean and corresponding standard deviation for the continuous variables and as frequencies and percentages for the categorical variables. The Wilcoxon signed ranks test was used for paired samples' comparisons of IL-12 serum levels before and after treatment, while an independent samples t-test and non-parametric Mann-Whitney test were performed for mean IL-12 differences between responders and non-responders (including relapsers) at baseline or at the end of treatment. The influence of immunological parameters on the virological response to the treatment among the patients was assessed

by univariate logistic regression. Multivariate analyses were not fitted due to the very small sample size. Both Pearson and Spearman rank correlation coefficients were estimated to assess potential relationships of IL-12 levels with variables of interest. A P value of ≤0.05 was considered for statistical significance.

Results

Descriptive results

Table 1 presents the descriptive data for the HCV-infected patients. The pretreatment viral load with a median of 3.5 x 10^5 IU/mL was grouped with a cut-off of 4 x 10^5 IU/mL, as "high" (38%) and "low" (62%). The predominant genotype was 1 in more than half of the patients (54%), while genotypes 3 and 4 were found in the remaining 9 and 3 patients respectively. Mild necroinflammatory activity, as a descriptive grade of inflammation, was dominant in histopathological findings of liver biopsies. Portal fibrosis with or without short fibrous septa was present in the majority of the liver specimens (9 patients, 65%).

IL-12 serum levels and corresponding associations

The median (and corresponding range) baseline serum IL-12 levels in the studied group of 26 patients with HCV infection before the combination treatment were 3.6 (1.9-7.6) pg/mL (mean 3.8±1.5 pg/mL), at the end of treatment they were 4.0 (1.6-7.6) pg/mL (mean 4.0±1.52 pg/mL), and 6 months after the end of treatment they were 4.2 (1.4- 8.8) pg/mL (mean 4.1±1.9 pg/mL) (Table 1).

Seventeen of 26 patients 65% showed SVR. The median IL-12 serum levels of responders, was 3.9 pg/mL before treatment and 4.0 pg/mL at the end of treatment. The median IL-12 serum levels of non-responders (including relapsers) before treatment was 2.5 pg/mL, and at the end of treatment 3.7 pg/mL. The combination treatment effect (pre- versus post-treatment) on IL-12 serum levels did not differ among patients (P=0.756). The corresponding effect moreover was statistically significant neither in sustained responders, nor in non-responders or relapsers (Fig. 1). The baseline IL-12 serum levels were significantly (P=0.029) higher in the group of the sustained responders, compared with non-responders or relapsers (Fig. 2), while the corresponding difference for IL-12 serum levels after treatment was slightly higher but not statistically significant (P=0.454).

Using logistic regression analysis it was found that for an increase in IL-12 serum levels per 1 pg/mL, patients were more likely to respond by 163% (OR 2.63, 95%CI: 0.89-7.78, P=0.081), although the result was marginally non-significant. When a cut-off of 3 pg/mL was used, as obtained through a receiver operating curve (ROC) analysis (data not shown),

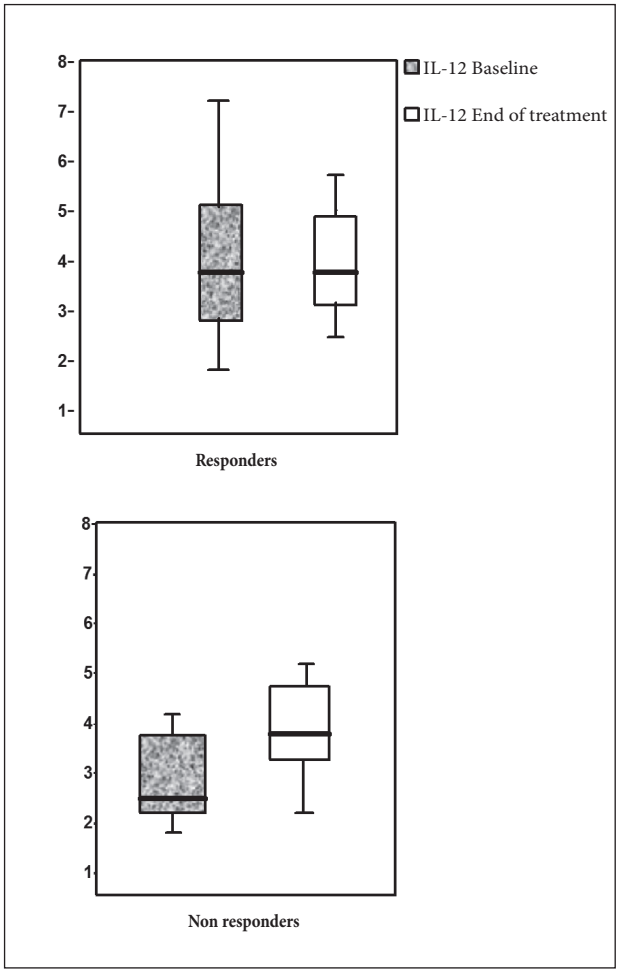

Figure 1 Serum interleukin (IL)-12 at baseline and the end of treatment in patients who achieved a sustained virological response (Responders) and in those who did not (Non-Responders)

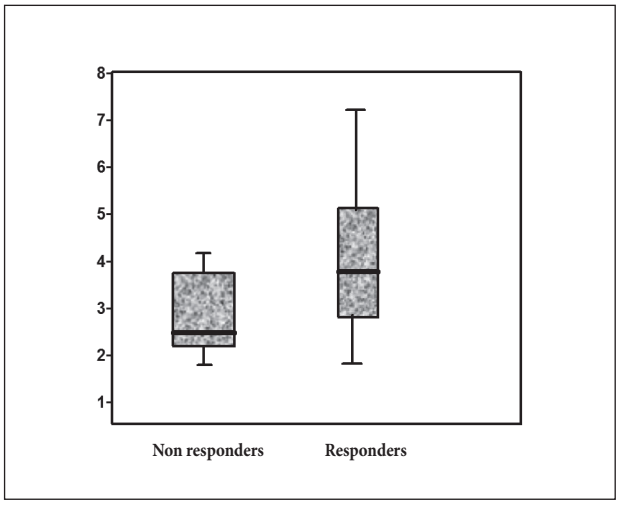

Figure 2 Serum interleukin (IL)-12 at baseline in patients who achieved a sustained virological response (Responders) and in those who did not (Non-Responders)

it was found that pretreatment IL-12 serum levels higher than this cut-off positively predicted the sustained response.

Correlations with IL-12 serum levels

No statistically significant correlation was found between the serum IL-12 levels, the age of patients and the probable duration of HCV infection (for 16 of the 26 patients in whom the source and the time of infection could be assumed). Neither viral load nor genotype was found to correlate significantly with IL-12 serum levels. Finally no statistically significant correlations were found between the baseline or after-the-treatment serum IL-12 levels and the severity of necroinflammatory changes (r=0.10 P=0.768 and r=-0.29, P=0.367 respectively) or the extent of fibrosis in liver tissue specimens (r=-0.26 P=0.435 and r=-0.39, P=0.207 respectively).

Discussion

There is evidence that HCV actively participates in the chronicity of HCV infection, though the actual mechanisms involved are poorly understood [3,5]. Several previously known hypotheses have been proposed to explain how an inefficient cellular IR allows HCV to establish chronic infection. Taking into consideration the crucial role of antigen presenting cells (APCs) in antigen processing and presentation to T cells, the recent approach has been drawn to the most potent APCs, the DCs. Within the context of major histocompatibility complex (MHC) class I and II molecules on the surface they thereby trigger the activation of innate and adaptive IR. The strength of this IR is largely dependent on the stimulatory function of DCs. Myeloid DCs (mDCs), the most typical APCs, mainly produce large amounts of IL-12, while plasmatocytoid DCs (pDCs) produce IFNs when stimulated by viral infections [23].

IL-12 is a crucial mediator between innate and adaptive IR and promotes the development of Th1 in response to HCV infection, correlating with viral clearance [18]. Previous studies suggested that HCV has a direct effect on DCs by disturbing their function, thereby reducing their allo-stimulatory capability and secretion of cytokines, especially those of type I IFNs and IL-12 [12,24]. The reduced production of these cytokines may explain the shift from Th1 to Th2 cells found in chronic HCV patients [9].

In the present study we evaluated serum IL-12 concentration levels among 26 HCV patients, before and after the application of PEG-IFNα2β/RBV combination treatment. We found statistically significant higher baseline IL-12 serum concentration levels among HCV patients who achieved SVR when compared to non-responders, which indicates the potential role of the IL-12 in promoting Th1 cells differentiation and suppressing the Th2 function. In addition, the chances to achieve SVR were higher for patients in whompretreatment IL-12 levels were higher than 3 pg/mL. Of course, there are other well-known factors in the literature that also contribute to SVR in HCV-infected patients, such as patients with genotype 2 or 3; pretreatment low viral load; young age (<40); female sex; minimal liver fibrosis stage; and the recently identified genetic polymorphisms of IL28B CC genotype [25,26].

Our study also rendered that the IL-12 serum levels were not statistically significantly raised in responders or in non-responders, after successful combination treatment with PEG-IFN/RBV. In contrast, the subgroup of patients who achieved an increase in IL-12 levels by 1 pg/mL at the end of treatment appeared to be more than 2.5 times more likely to achieve SVR. Both findings support the significant value of IL-12 as SVR predictor marker.

Taking into account that both IL-12 and type I IFNs activate the signal transducers and activators of transcription (STAT)-4, the essential transcription factor for Th1 differentiation [27], the previously mentioned findings indicate that sustained IL-12 signaling, together with exposure from the beginning to appropriate levels of IL-12, are essential for successful Th1 cell development provided there is HCV clearance, which cannot be induced only by a transient IL-12 pulse, possibly due to IFN/RBV activity. The conclusions of the study are in agreement with findings from previous studies which suggested that sustained exposure to appropriate concentrations of IL-12 levels was crucial for successful Th1 cell differentiation [28,29]. In the present study no correlation was found between the levels and patients' viral load, HCV genotype, LFTs or histological activity. Quiroga and colleagues (1998) found that HCV-infected patients with more advanced necro-inflammatory activity of the liver showed increased IL-12 production by peripheral blood mononuclear cells, suggesting that IL-12 may play an important role in promoting inflammatory reactions [30]. They also found that the increase in serum IL-12 levels of HCV-infected patients was confirmed with ALT flares [30].

A major limitation of the present study was the relatively small number of patients that were used in each group. While significant results were obtained, further studies with larger groups of patients are suggested to confirm these findings. Additional limitations were the inevitable exclusion of patients for various reasons, as well as the restriction of the sample (to only 14 of 26 patients) for the analyses of liver biopsy because they refused to undergo biopsy.

In conclusion, the present study suggests that IL-12 plays a significant role in the immunopathogenesis and outcome of HCV infection by affecting the Th1/Th2 balance. Patients with increased baseline levels of IL-12 were more likely to achieve SVR, when compared to patients with low baseline levels of IL-12. Maybe this can be achieved if the PEG-IFN/RBV combination treatment could succeed in increasing the IL-12 levels by 1 pg/mL at the end of treatment. It was found that pretreatment IL-12 serum levels seem to predict which patients will achieve SVR to treatment. No statistical correlation was found between baseline serum IL-12 levels and patients' viral load, HCV genotypes, histological activity or LFTs.

Summary Box

What is already known:

- Successful treatment in patients with hepatitis C correlates with:
  - Genotype other than 1, 4
  - Pre-treatment low viral load
  - Young age (<40)
  - Female sex

What the new findings are:

- Pre-treatment high interleukin-12 levels seem to predict which hepatitis C-infected patients will achieve sustained virological response to treatment

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