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Replication of hepatitis C virus in peripheral blood mononuclear cells in patients with chronic hepatitis C treated with pegylated interferon alpha and ribavirin

Replikacja wirusa zapalenia wątroby typu C (HCV) w jednojądrowych komórkach krwi obwodowej (PBMC) u pacjentów z przewlekłym zapaleniem wątroby typu C, leczonych pegylowanym interferonem alfa i rybawiryną

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Summary

Introduction:

Hepatitis C virus (HCV) is a primarily hepatotropic virus, but hepatocytes are not the only localization of its replication. It is still unclear if extrahepatic HCV replication, measured as the detection of HCV RNA negative strand in peripheral blood mononuclear cells (PBMCs) before initiation of treatment, has an influence on therapy response. Detection of HCV RNA in extrahepatic sites for assessment of therapy efficacy is not routinely used in clinical practice. The aim of the study was to evaluate whether the replication of HCV in PBMCs affects the rate of sustained virological response (SVR).

Materials and methods:

The study group comprised 55 patients with chronic hepatitis C, originally treatment naive. They were treated with pegylated interferon (PEG-IFN) alpha 2a and ribavirin, with the standard dosing schedule. Parallel serum samples for HCV RNA and PBMC samples for HCV RNA negative strand were obtained at baseline, at the end of treatment and 24 weeks after finishing therapy.

Results:

Undetectable HCV RNA in serum at the end of therapy was found in 48 patients (87.3%), while 33 patients (60.0%) achieved sustained virological response (SVR) (51% for HCV genotype 1 and 78% for genotype 3, respectively). Fifteen individuals (31.3%) were relapsers. Factors associated with significantly higher rate of SVR were young age, mild or no fibrosis and infection with HCV genotype 3. HCV RNA negative strand in PBMCs before treatment was found in 21.8% (12 out of 55 patients). HCV RNA negative strand was detected at baseline more frequently in patients who later achieved SVR. Relapse appeared significantly more often in patients with negative strand at the end of therapy: in 2 out of 15 individuals compared to 0 out of 33 patients ($p=0.03$).

Conclusions:

Presence of negative HCV RNA strand in PBMCs before treatment may be suggested as a potential marker of good treatment response. Detection of negative strand at the end of therapy is a predictor of relapse.

Key words:

HCV • extrahepatic replication • negative HCV RNA strand • PBMC • pegylated interferon alpha

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BACKGROUND

Infection with hepatitis C virus (HCV) causes chronic hepatitis, which may lead to liver cirrhosis and subsequently to hepatocellular carcinoma (HCC). HCV is a primarily hepatotropic virus, but hepatocytes are not the only localization of its replication.

Extrahepatic replication is well recognized and HCV sequences have been found, among other sites, in peripheral blood mononuclear cells (PBMCs): T-cell and B-cell lines and monocytes/macrophages [12,24]. Some studies also found replication of HCV in perihepatic lymph nodes, salivary glands, oral epithelial cells, pancreas, adrenal glands, thyroid and brain [11,18,19].

HCV is a positive strand RNA virus and negative strand acts as a replicative intermediate. Both positive and negative strands of HCV RNA are found in PBMCs. Whereas presence of HCV RNA positive strand may be the result of virions adsorption from the serum, detection of negative strand confirms the active replication of virus in these cells. Average amounts of HCV RNA positive strand are 10 to 100 times higher than negative strand [12,19].

Persistence of the replicating virus in PBMCs, even if HCV RNA is undetectable in serum, may cause infection reactivation in the future, particularly under conditions of immunosuppression (e.g. long-term steroid therapy, organ transplantations, HIV infection). Moreover, it may also play a role in pathogenesis of extrahepatic manifestations of HCV infection [5].

The current standard of care (SOC) includes combination therapy with pegylated interferon alpha 2 and ribavirin. The aim of treatment of chronic hepatitis C is the sustained suppression of viral replication. Sustained virological response (SVR) is assessed 24 weeks after finishing treatment and is defined as negative HCV RNA in the serum. Detection of HCV RNA in extrahepatic sites for assessment of therapy efficacy is not routinely used in clinical practice. Several well-known factors influence the SVR rate: age, sex, HCV genotype, baseline level of HCV viremia, liver fibrosis stage, interleukin (IL)28b polymorphism. The presence of HCV RNA negative strand in PBMCs at the end of treatment as a factor determining higher risk of viral relapse has been confirmed in some studies [3,13,21]. However,

it is not clear if extrahepatic replication in PBMCs before treatment affects the treatment efficacy.

The aim of the study was to evaluate whether the extrahepatic replication of HCV affects therapy response.

MATERIALS AND METHODS

The study group comprised 55 patients with chronic hepatitis C, treatment naive, 28 females and 27 males, aged 24-68 years (mean 47.3). All the patients were anti-HCV positive, HCV RNA positive. HCV genotype 1 was detected in 35 patients (63.6%), genotype 3 in 18 individuals (32.7%) and genotype 4 in 2 patients (3.6%). All the individuals were treated with pegylated interferon alpha and ribavirin, with the standard dosing schedule regarding HCV genotype and body weight. All patients were receiving peginterferon alfa-2a 180 µg once weekly (Pegasys; Hoffmann-La Roche, Basel, Switzerland) combined with ribavirin (Copegus; Hoffmann-La Roche, Basel, Switzerland) in the dose of 1000 mg/day if their body weight was <75 kg or 1200 mg/day if their body weight was ≥75 kg. Duration of treatment was 48 weeks for genotypes 1 and 4, and 24 weeks for genotype 3.

Baseline HCV RNA in serum ranged from 1.4×10^3 to 11.1×10^7 IU/ml. Inflammatory activity in liver histological examination was 0-3 (mean 1.97), and staging was in the range 0-4 (mean 2), according to the METAVIR score.

Parallel serum samples for HCV RNA and PBMC samples for HCV RNA negative strand were obtained at baseline, at the end of treatment and 24 weeks after finishing therapy.

Anti-HCV antibodies in serum were analyzed using a 2nd generation anti-HCV kit (Organon Teknika, Dublin, Ireland), qualitative and quantitative HCV RNA in serum by RT-PCR (Abbott RealTime HCV (Abbott Molecular Inc.), lower limit of detection 30 IU/ml). HCV genotype was determined by INNO-LiPA test (Innogenetics).

Peripheral blood mononuclear cells (PBMC) (3×10^5 - 10^6 cells) were isolated from blood by centrifugation over a density gradient and RNA was extracted by means of a modified guanidinium thiocyanate-phenol/chloroform technique using a commercially available kit (TRIZOL LS, Gibco/BRL). The specificity of our real-time RT-PCR

assay for the detection of negative-strand HCV RNA was ascertained by conducting cDNA synthesis at a high temperature with the thermostable enzyme *Tth* (Applied Biosystems). Descriptions of the *Tth*-based assay and its real-time modification have been published elsewhere [10]. The strand-specific assay is capable of detecting ~100 viral genomic eq/mL of the correct negative strand. Although the assay is quantitative over a wide range of template, the results were commonly close to the level of detection and, thus, were reported only as being either positive or negative.

Data were compared using the parametric Student's t test and nonparametric Mann-Whitney U test and Chi-square test. Spearman's coefficient was used to calculate the correlations. Values of $p \leq 0.05$ were considered to be statistically significant. Calculations were performed using Statistica 64 version 10 for Windows (StatSoft Inc., Tulsa OK USA).

RESULTS

Negative HCV RNA in serum at the end of treatment, meaning end of treatment response (ETR), was found in 48 patients (87.3%) and sustained virological response (SVR) at 24 weeks after therapy in 33 patients (60%); 51% for HCV genotype 1 and 78% for genotype 3, respectively. Relapse of replication at follow-up 24 weeks after the end of therapy was detected in 15 individuals (31.3%).

Comparison of 33 patients with SVR with 22 non-SVR patients, including age, sex, body weight, HCV genotype, quantitative HCV RNA, grading and staging, is presented in Table 1. Rates of HCV RNA in serum and HCV RNA negative strand in PBMCs at certain time points of observation are shown in Fig. 1.

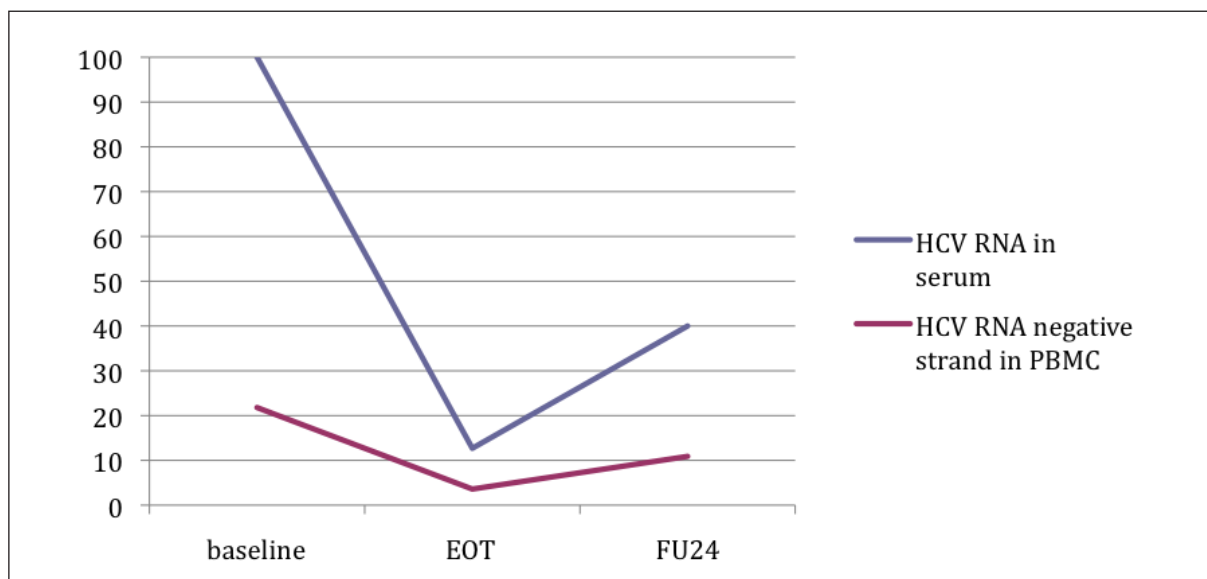


Fig. 1. Rates of detectable HCV RNA in serum and HCV RNA negative strand in PBMCs during observation [% of patients]

Table 1. Comparison of groups with and without sustained virological response.

	SVR n=33	non-SVR n=22	Statistical tests
Age; mean (range)	44.27 (24-66)	51.72 (28-68)	t=2.57; p=0.013
Male : female	16:17	12:10	Chi-square=0.19; p=0.66
Body weight	80.33	80.81	t=0.1; p=0.92
HCV genotype (%)			
1	18 (55)	17 (77)	Chi-Square=39; p<0.001
3	14 (42)	4 (18)	
4	1 (3)	1 (5)	
HCV RNA, quantitative [IU/ml]	14.2 x 10 ⁶	9.47 x 10 ⁶	t=-0.81; p=0.52
ALT; mean (range) [IU/l]	96 (24-317)	81 (20-221)	t=-0.9; p=0.37
Grading; mean (range)	1.9 (0-3)	2.11 (1-3)	t=1.1; p=0.3
Staging; mean (range)	1.82 (0-3.5)	2.28 (1-3.5)	t=2.1; p=0.039

SVR, sustained virological response; ALT, alanine transaminase

A parallel significant decline of detectable HCV RNA in serum and HCV RNA negative strand in PBMCs was observed at the end of treatment, compared to baseline. Also relapse of HCV replication in serum 24 weeks after therapy was parallel to re-appearance of HCV RNA negative strand in PBMCs.

Among 33 individuals who achieved SVR, HCV RNA negative strand before start of treatment was detected in 10 patients, compared to only 2 patients among 15 without SVR (30.3% vs 9.1%). Twenty-four weeks after the end of therapy HCV RNA negative strand was detected 3-fold more frequently in the non-SVR group versus the SVR group (18.2% vs 6.1%). Comparison of HCV RNA negative strand rates in PBMCs in SVR and non-SVR groups at particular time points of observation is shown in Table 2.

Comparison of ETR and SVR rates in groups with and without HCV RNA negative strand before treatment is shown in Table 3.

Among 55 individuals there were 48 patients (87.3%) with negative HCV RNA in serum at the end of therapy. Relapse of HCV replication after the end of treatment was observed in 15 of them (31.3%). Negative strand in PBMCs at the end of therapy was detected in 2 among 15 of these patients (13.3%). Negative strand at the end of therapy was observed significantly more often in patients with relapse than with SVR (2/15 vs 0/33; $p=0.03$).

In 2 patients, who achieved SVR, both infected with HCV genotype 3, negative strand in PBMCs was detected 6 months after the end of treatment. Repeated assessment was performed 36 months after therapy and HCV RNA in serum was still negative.

DISCUSSION

Treatment with pegylated interferon alpha and ribavirin caused the elimination of HCV RNA negative strand from PBMCs in parallel with elimination of HCV RNA from serum.

Based on former studies there was evidence of persisting HCV RNA negative strand in PBMCs despite sustained elimination of HCV RNA from serum. The majority of these studies assessing HCV replication in PBMCs and its influence on therapy outcome were conducted in the era of recombined interferon alpha [13,16,18,23]. Our finding may be proof of the stronger action of pegylated interferon compared to recombinant IFN. PEG-IFN might be able to eliminate the extrahepatic reservoir, as no negative strand was detected at the end of therapy in the group with SVR. Pugnale et al. analyzed the dynamics of decline of HCV RNA in PBMCs on treatment with pegylated interferon alpha and ribavirin. Fast viral decay in PBMCs was associated with sustained virological response, but viral kinetics in PBMCs added only minor predictive information compared with kinetics in plasma [17].

In our analysis, well-known predictive factors – younger age, less advanced fibrosis and infection with HCV genotype 3 – were associated with a significantly higher rate of SVR ($p<0.05$ for each parameter), as in many other studies [1,6,14].

As previously reported, the presence of negative HCV RNA strand in PBMCs at the end of treatment is a predictor of replication relapse [8,15,18]. Our observation was consistent with other authors.

In some studies, also presence of HCV RNA negative strand before treatment was suggested to be a negati-

Table 2. Rates of negative HCV RNA strand in PBMCs in SVR and non-SVR groups

	SVR (%)	non-SVR (%)	Chi-square, p
Baseline	10/33 (30.3)	2/22 (9.1)	2.33, $p=0.13$
EOT	0/33 (0.0)	2/22 (9.1)	2.85, $p=0.09$
FU24	2/33 (6.1)	4/22 (18.2)	1.57, $p=0.21$

EOT, end-of-treatment; FU24, follow-up 24 weeks

Table 3. Rates of ETR and SVR in groups with and without HCV RNA negative strand at baseline

	Negative strand at baseline (n=12; 21.8%)	Without negative strand at baseline (n=43; 78.2%)	Statistical tests
ETR (yes/no)	12 (100%)/0 (0%)	36 (83.7%)/7 (16.3%)	Chi-square=2.24; $p=0.13$
SVR (yes/no)	10 (83.3%)/2 (16.7%)	23 (53.5%)/20 (46.5%)	Chi-square=3.48; $p=0.062$

ETR, end of treatment response; SVR, sustained virological response

ve prognostic factor [2]. Surprisingly, in our study patients who had detectable HCV RNA negative strand in PBMCs before treatment had a higher rate of SVR ($p=0.062$). This potentially beneficial influence of negative strand presence on SVR rate is unclear. Different hypotheses of mechanisms concerning the biology of HCV infection, host immune response and IFN alpha-mediated response have been proposed over the last few years. PBMCs are important target cells for IFN; they may express IFN receptors at a level comparable with liver cells [22]. It was demonstrated that pegylated interferon alpha modulates the expression of approximately 1000 genes in PBMC in vitro and in vivo [20]. There are observations suggesting presence of distinct variants of HCV in different compartments, such as plasma, PBMCs, and brain. PBMC-specific variants might attenuate the cell-mediated immune response. PEG-IFN therapy, suppressing HCV replication in PBMCs, might however antagonize this negative effect on the immune response. Moreover, negative strand, as a replicative intermediate for HCV, may induce viral protein expression and subsequently stimulate an anti-HCV immune response [4,24].

Possibly, PBMC infection by HCV genotypes 2 and 3, highly sensitive to interferon alpha, was more predictive of the treatment response than was the presence of these genotypes in plasma. However, we did not determine the HCV genotype of HCV extracted from PBMCs.

An additional finding of our study was the low rate of HCV RNA negative strand in PBMCs before treatment (18%) compared to other studies: 92% in children [8], and 40% in adults with chronic hepatitis C [7]. An influence of method sensitivity cannot be excluded [9].

Persistence of negative strand in PBMCs more than 24 weeks after the end of therapy may not mean the risk of late relapse of HCV replication in serum, as in 2 patients after 36 months of post-treatment observation HCV RNA in serum is still negative.

In summary, our findings suggest that examination of negative HCV RNA strand in PBMCs may have some impact for prediction of treatment success. With respect to the role of well-established response predictive factors, our observation suggests that HCV replication in PBMCs may have a positive influence on immune mechanisms enhanced by antiviral therapy. Extrahepatic replication of HCV is an intriguing phenomenon and its evaluation brings essential information for knowledge about the pathogenesis and natural history of HCV infection.

The most significant limitations of our study include the small study group, particularly with detectable negative HCV RNA strand and no data concerning the polymorphism of IL28b, as in the period of conducting the study it was not yet an established prognostic factor in clinical practice.

REFERENCES

- [1] Berg T., Sarrazin C., Herrmann E., Hinrichsen H., Gerlach T., Zachoval R., Wiedenmann B., Hopf U., Zeuzem S.: Prediction of treatment outcome in patients with chronic hepatitis C: significance of baseline parameters and viral dynamics during therapy. *Hepatology*, 2003; 37: 600-609
- [2] Cribrier B., Uhl G., Schmitt C., Doffoël M., Vetter D., Kirn A., Stoll-Keller F.: Follow-up of hepatitis C virus RNA in peripheral blood mononuclear cells during interferon therapy. *Arch. Virol.*, 1999; 144: 355-364
- [3] de Felipe B., Leal M., Soriano-Sarabia N., Gutiérrez A., López-Cortés L., Molina-Pinelo S., Vallejo A.: HCV RNA in peripheral blood cell subsets in HCV-HIV coinfecting patients at the end of PegIFN/RBV treatment is associated with virologic relapse. *J. Viral Hepat.*, 2009; 16: 21-27
- [4] Di Liberto G., Roque-Afonso A.M., Kara R., Ducoulombier D., Falot G., Samuel D., Feray C.: Clinical and therapeutic implications of hepatitis C virus compartmentalization. *Gastroenterology*, 2006; 131: 76-84
- [5] Ferri C., Monti M., La Civita L., Longombardo G., Greco F., Pasero G., Gentilini P., Bombardieri S., Zignego A.L.: Infection of peripheral blood mononuclear cells by hepatitis C virus in mixed cryoglobulinemia. *Blood*, 1993; 82: 3701-3704
- [6] Fried M.W., Shiffman M.L., Reddy K.R., Smith C., Marinos G., Goncalves F.L.Jr., Häussinger D., Diago M., Carosi G., Dhumeaux D., Craxi A., Lin A., Hoffman J., Yu J.: Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med.*, 2002; 347: 975-982
- [7] Gong G.Z., Lai L.Y., Jiang Y.F., He Y., Su X.S.: HCV replication in PBMC and its influence on interferon therapy. *World J. Gastroenterol.*, 2003; 9: 291-294
- [8] Januszkiewicz-Lewandowska D., Wysocki J., Pernak M., Nowicka K., Zawada M., Rembowska J., Lewandowski K., Mańkowski P., Nowak J.: Presence of hepatitis C virus (HCV)-RNA in peripheral blood mononuclear cells in HCV serum negative patients during interferon and ribavirin therapy. *Jpn. J. Infect. Dis.*, 2007; 60: 29-32
- [9] Lanford R.E., Chavez D., Chisari F.V., Sureau C.: Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. *J. Virol.*, 1995; 69: 8079-8083
- [10] Laskus T., Operskalski E.A., Radkowski M., Wilkinson J., Mack W.J., deGiacomo M., Al-Harhi L., Chen Z., Xu J., Kovacs A.: Negative-strand hepatitis C virus (HCV) RNA in peripheral blood mononuclear cells from anti-HCV-positive/HIV-infected women. *J. Infect. Dis.*, 2007; 195: 124-133
- [11] Laskus T., Radkowski M., Wang L.F., Vargas H., Rakela J.: Search for hepatitis C virus extrahepatic replication sites in patients with acquired immunodeficiency syndrome: specific detection of negative-strand viral RNA in various tissues. *Hepatology*, 1998; 28: 1398-1401
- [12] Lerat H., Berby F., Trabaud M.A., Vidalin O., Major M., Trépo C., Inchauspé G.: Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. *J. Clin. Invest.*, 1996; 97: 845-851
- [13] Majda-Stanisławska E., Bednarek M., Józwiak B., Sidorkiewicz M., Piekarska A., Kuydowicz J.: Effect of interferon alfa and ribavirin treatment on hepatitis C virus RNA in serum and peripheral blood mononuclear cells in children with chronic hepatitis C. *Acta Gastroenterol. Belg.*, 2006; 69: 187-190
- [14] Manns M.P., McHutchison J.G., Gordon S.C., Rustgi V.K., Shiffman M., Reindollar R., Goodman Z.D., Koury K., Ling M., Albrecht

- J.K.: Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. *Lancet*, 2001; 358: 958-965
- [15] Mohamad H.I., El-Bab H.K., Kamal A.M.: HCV RNA in serum and peripheral blood mononuclear cells after successful interferon therapy. *Hepatology*, 2011; 58: 932-936
- [16] Moonka D.K., Henzel B.S., Gutekunst K., O'Brien C.B.: Quantitative assessment of hepatitis C virus RNA in peripheral blood mononuclear cells during therapy with interferon- α 2a. *J. Viral Hepat.*, 1998; 5: 27-33
- [17] Pugnale P., Herrmann E., Neumann A.U., Pawlotsky J.M., Schalm S.W., Ferrari C., Homburger Y., Zeuzem S., Negro F., DITTO-HCV Study Group: Hepatitis C viral kinetics in plasma and peripheral blood mononuclear cells during pegylated interferon- α 2a/ribavirin therapy. *J. Hepatol.*, 2008; 48: 932-938
- [18] Radkowski M., Gallegos-Orozco J.F., Jabłońska J., Colby T.V., Walewska-Zielecka B., Kubicka J., Wilkinson J., Adair D., Rakela J., Laskus T.: Persistence of hepatitis C virus in patients successfully treated for chronic hepatitis C. *Hepatology*, 2005; 41: 106-114
- [19] Revie D., Salahuddin S.Z.: Human cell types important for hepatitis C virus replication in vivo and in vitro. Old assertions and current evidence. *Virology J.*, 2011; 8: 346
- [20] Taylor M.W., Tsukahara T., Brodsky L., Schaley J., Sanda C., Stephens M.J., McClintick J.N., Edenberg H.J., Li L., Tavis J.E., Howell C., Belle S.H.: Changes in gene expression during pegylated interferon and ribavirin therapy of chronic hepatitis C virus distinguish responders from nonresponders to antiviral therapy. *J. Virol.*, 2007; 81: 3391-3401
- [21] Xu D.Z., Xie Y., Li Z.Q.: Clearance of HCV RNA in peripheral blood mononuclear cell as a predictor of response to antiviral therapy in patients with chronic hepatitis C. *Hepatobiliary Pancreat. Dis. Int.*, 2005; 4: 550-553
- [22] Yamaguchi Y., Hino K., Fujiwara D., Ren F., Katoh Y., Satoh Y., Okita K.: Expression of type I interferon receptor in liver and peripheral blood mononuclear cells in chronic hepatitis C patients. *Dig. Dis. Sci.*, 2002; 47: 1611-1617
- [23] Zayed R.A., Rushdy E., Saleh D.A.: Detection of HCV RNA in the peripheral blood mononuclear cells of serum HCV RNA-negative Egyptian patients under interferon treatment. *Am. J. Med. Sci.*, 2010; 340: 435-438
- [24] Zignego A.L., De Carli M., Monti M., Careccia G., La Villa G., Giannini C., D'Elios M.M., Del Prete G., Gentilini P.: Hepatitis C virus infection of mononuclear cells from peripheral blood and liver infiltrates in chronically infected patients. *J. Med. Virol.*, 1995; 47: 58-64

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