Journal of the Egyptian Society of Parasitology, Vol. 47, No. 2, August 2017
J. Egypt. Soc. Parasitol. (JESP), 47(2), 2017: 323- 330

ASSOCIATION BETWEEN POST TREATMENT EXISTENCE OF HCV/ RNA POSITIVE STRAND AND/OR NEGATIVE STRAND IN THE PBMNCS AND HCV RELAPSE

By

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Abstract

Hepatitis C virus (HCV) has been found to infect peripheral blood mononuclear cells (PBMCs), using them as a reservoir, which might contribute to development of resistance to treatment. The study evaluated the predictive value of existence of HCV RNA in PBMCs of chronic hepatitis C patients and its association with HCV seroconversion following the completion of therapy with direct antiviral agents (DAAs). Methods: 58 Egyptian patients were included with chronic HCV infection who achieved negative serum HCV RNA after completion of therapy with DAAs. HCV RNA in PBMCs and serum was investigated at the end of treatment and 12 weeks later. At the end of treatment patients were subdivided into three groups. GA: included 23 patients with negative (positive & negative strands of HCV RNA) in PBMCs. GB: included 24 patients with positive (positive strand) of HCV RNA in PBMNCs. GC: included 11 patients with positive (positive & negative strands of HCV RNA) in PBMCs.

The results showed that 16 relapsers out of 58 patients (27.59 %). GA only one patient out of 23 (4.3 %) failed to achieve SVR, so the absence of HCV RNA in PBMNCs is associated with high SVR rate (95.7%).GB 10/24 patients failed to achieve SVR, so the presence of HCV RNA positive strand in PBMNCs is associated with high relapse rate (41.7 %), GC 5/11 patients failed to achieve SVR, where the presence of HCV RNA (negative replicative and positive genomic strands) in PBMNCs is associated with higher rate of relapse (45.45%).

Keywords: Patients, HCV, PBMNCs, Relapse, DAAs.

Introduction

Hepatitis C is considered to be hepatotropic virus (Gallegos-Orozco *et al*, 2008), extrahepatic HCV replication evidenced by using of highly sensitive assays has shown the viral RNA to exist in the material obtained from PBMCs, liver tissue, or other tissue sites such as the thyroid, salivary glands, pancreas, kidney, and even the brain (Castillo *et al*, 2010). The prevalence of OCI infection in apparently "healthy" population was 3.3% of individuals who were HCV-Ab negative and serum HCV-RNA negative and had detectable HCV-RNA in their PBMCs (de Marco *et al*, 2009). Immune cell subtypes isolated from individuals with chronic hepatitis C. HCV- RNA occurred at a similar frequency in all various cell subtypes; monocytes had the greatest viral load (Pham and Michalak, 2011). As these cells accessible, they can evaluate status of extra hepatic HCV replication during the course of treatment and after the apparent development of a putative SVR. The detection of HCV positive cells and in some cases the replicative negative viral strand in PBMCs confirms the role of these cells as a HCV reservoir both during ongoing antiviral treatment and most importantly after its completion (Chen *et al*, 2013). PBMCs PCR is a valid diagnostic test that diagnoses intracellular HCV in negative SRT-PC, eradication of intracellular strands was recommended to avoid RNA seroconversion (Abd-Alla and El Awady, 2017). This proved to be critically important. The use of the terms cure and total eradication of HCV following treatment occurring in association with a currently defined SVR (an undetectable serum HCV RNA level using a sensitive assay 12 weeks after completion of

therapy for hepatitis C) may not be appropriate (Pham *et al*, 2004). The presence of PBMC-associated HCV-RNA shown to be a strong predictor of a reduced likelihood of a SVR and was associated also with a reduced rate of rapid viral response (RVR) of 60% as compared to that occurring in individuals without detectable HCV-RNA in the PBMC (Angulo *et al*, 2013). The absence of HCV in the serum of patients by the end of treatment does not exclude future viremia. The patient might still be a source of infection to others. It was strongly encouraged to test for HCV in peripheral blood mononuclear cells to detect lack of response to treatment and persisting infection (Zaman *et al*, 2014).

This study was designed to evaluate association between post treatment exis-tence of HCV/RNA positive strand and/ or negative strand in PBMNCs and HCV relapse and to answer a question is HCV SRT-PCR 12 weeks after treatment with DAAs considered as cure from HCV?

Subjects and Methods

A case control study was conducted on 58 Chronic Hepatitis C Egyptian patients Their age ranged from 29-70 ys and their sex distribution are 24 females and 34 males who achieved negative serum HCV RNA at the end of treatment with DDAs who subdivided into 3 groups according to presence or absence of positive and negative strands of HCV RNA in PBMNCs, GA: included 23 patients with negative (positive & negative strands of HCV RNA) in PBMCs, GB: included 24 patients with positive (positive strand) and negative (negative strand) of HCV RNA in PBMNCs, GC: included 11 patients with positive (positive & negative strands of HCV RNA) in PBMCs.

The patients who refused to be enrolled in the study, Non responders (HCV/RNA positive in serum after completion of treatment) co-infection with HBV, Previous treatment with DAAs and immunosuppressive agents and Chronic hepatitis C patients complicated with hepatocellular carcinoma were excluded from the study. The study was done on the out and in patients of Department of Tropical Medicine, Al-Azhar University Hospitals (Al-Hussein & Sayed Galal Hospitals). The study was approved by the local Ethics Committee, Faculty of Medicine, and Al-Azhar University.

All patients were subjected to thorough history taking and clinical examination, routine laboratory examinations including CBC, liver function tests. HBsAg, PCR for HCV RNA, Alpha feto-protein, renal function tests, abdominal U/S, The HCV RNA in the PBMCs and serum was investigated at the end treatment &12 weeks later (Goergen *et al*, 1994).

Extraction of RNA from PBMCs: Peripheral blood (200uL) was diluted with 10mL freshly prepared red blood cell alkaline buffer (38.8mmol/L NH4Cl, 2.5mmol/L $K2HCO_3$, 1mmol/L EDTA pH 8.0). After 10 minutes incubation at room temperature, nucleated cells were washed with the same buffer and lysed in 500mL antinuclease solution (4mol/L guanidinium isothiocyanate containing 25mmol/L sodium citrate, 0.5 sarcosyl and 0.1mol/L ß- mercaptoethanol). A single step method previously described and subsequently modified was followed for the RNA extraction.

Retrotranscription-PCR of genomic & antigenomic strands of HCV RNA: Detection of HCV RNA strands in PBMCs were carried out (Löhr *et al*, 1995). Briefly, the reaction mixture (50 mL) contained 400ng of cellular RNA, RT-PCR bead (HVD), 50 pmol from each of the primers (1CH, P2 & 2CH) for amplification of the genomic strand, or 50pmol from P2 and 75pmol from 2CH for antigenomic strand and 20 U of AMV reverse transcriptase.

The mixture was incubated at 42^{o}C for 1 hour and denatured for 15 minutes at 94^{o}C. Amplification of cDNA was performed in a thermal cycler for 30 cycles (94^{o}C for1 minute, 55^{o}C for 1 minute, & 72^{o}C for 1 minute). Nested PCR amplification was similar to the first-round PCR except for using 10mL from first PCR and two nested pri-

mers (P3 & P4) and 2U Taq DNA polymerase. To assure specificity the two controls were used: C1, ddw instead of RNA in cDNA synthesis reaction to exclude RNA contamination; C2, PCR step with only F1 or R1 to exclude mixed primer contamination. These two controls provided negative amplification; special attention was paid to heat inactivate the reverse transcriptase at 95^{o}C for 1 hour in order to reduce false detection of the antigenomic strand before adding the forward strand.

Primer sequences used included below: a- 1CH 5-ggtgcacggtctacgagacctc-3′, b- 2CH 5-aactcatgtcttcacgcagaa-3′, c- P2 5-tgctca tggtgcacggtcta-3′, d- P3 5-ctttcgcgacccaacactac-3′, and e- P4 5-agagccatagtggtctgcgg-3′

Medicine administration: The antiviral therapeutic regimens included: 1- Sofosbuvir at 400mg + Daclatasvir at 60mg for 12 weeks, 24 weeks. 2- Sofosbuvir at 400mg + Daclatasvir at 60mg + weight-based Ribavirin daily (1000mg for those who weigh 75kg and 1200mg for those who weigh >75kg) for 12 weeks. 3- Sofosbuvir at 400mg + Ledipasvir at 90mg (Harvoni) once daily for 12 weeks, 24 weeks. 4- Sofosbuvir at 400mg + Ladepasvir at 90mg once daily weight-based ribavirin daily for 12 week. 5- Sofosbuvir at 400mg + weight-based ribavirin daily for 24 weeks.

Statistical analysis: Data were tabulated, computerized and shown in the form of rate (%) and the standard deviation (SD). Chi-Square (χ^2) test and Fischer's exact test were used where appropriate. The P-value less than 0.05 were considered as statistically significant one.

Results

The results are shown in tables (1, 2, 3, 4 & 5) and figures (1 & 2).

Table 1: Baseline characteristics of treated patients in relation to HCV RELAPSE

		Negative serum	Positive serum	Independent t-test	
		No. = 44	No. = 16	T	p-value
Age	M±SD	54.91 ± 8.85	59.00 ± 9.04	-1.534	0.131
	Range	29 – 69	37 – 70		
Sex	Male	26 (61.9%)	8 (50.0%)	0.677	0.410
	Female	16 (38.1%)	8 (50.0%)		
Hb g/dL	M±SD	13.09 ± 1.38	13.08 ± 1.74	0.024	0.981
	Range	9.4 – 16	11 – 16		
WBCs 10^9/L	M±SD	6.96 ± 1.70	6.86 ± 1.67	0.198	0.844
	Range	3.6 – 11	4.2 – 11		
Platelets 10^9/L	M±SD	188.86 ± 78.51	190.67 ± 97.34	-0.072	0.943
	Range	52 – 371	78 – 380		
Creatinine mg/dL	M±SD	0.91 ± 0.23	0.96 ± 0.16	0.777	0.441
	Range	0.3 – 1.6	0.9 – 1.5		
Albumin g/dL	M±SD	3.74 ± 0.56	3.25 ± 0.64	2.831	0.006
	Range	2.6 – 4.6	2.4 – 4.3		
T.Bilirubin Mg/dL	M±SD	1.19 ± 0.95	1.90 ± 1.04	-2.447	0.018
	Range	0.3 – 4.7	0.4 – 3.4		
INR	M±SD	1.16 ± 0.26	1.39 ± 0.40	-2.484	0.016
	Range	0.9 – 2.1	1 – 2.1		

All patients showed viral response at the end of treatment. Only 16/58 patients (27.59 %) failed to achieve SVR 12 weeks after the end of treatment. In GA only one/23 (4.3 %) failed to achieve SVR. So, absence of HCV RNA in PBMNCs was associated with high SVR rate (95.7%). In GB 10/24 patients failed to achieve SVR. So, presence of HCV RNA positive strand in PBMNCs were associated with high relapse rate (41.7%) and low SVR rate (58.3%), In GC 5/11 patients failed to achieve SVR.

This means that the presence of HCV RNA (negative replicative and positive genomic strands) in PBMNCs was associated with higher rate of relapse (45.45%) and lower SVR rate (54.5%)

Table 2: Prevalence of relapse between studied groups:

		GA		GB		GC		Chi-square test	
		No.	%	No.	%	No.	%	X^2	P-value
Serum	Negative	22	95.7%	14	58.3%	6	54.5%	10.358	0.006
	Positive	1	4.3%	10	41.7%	5	45.5%		
Positive strand	Negative	22	95.7%	14	58.3%	3	27.3%	17.270	0.000
	Positive	1	4.3%	10	41.7%	8	72.7%		
Negative strand	Negative	23	100.0%	24	100.0%	7	63.6%	18.357	0.000
	Positive	0	0.0%	0	0.0%	4	36.4%		

There was high significant difference between the HCV relapse and the presence of positive & negative strands in PBMNCs (P < 0.001).

Table 3: Ultrasonoghraphic findings in relation to relapse

		Total patients		Negative serum		Positive serum		Chi-square test	
		no.	%	No.	%	No.	%	X^2	p-value
Liver	Hepatomegaly	21	36.2%	19	90.48%	2	9.52%	34.696	<0.001
	Coarse liver	18	31.0%	12	66.67%	6	33.33%	18.000	<0.001
	Cirrhotic liver	19	32.8%	11	57.89%	8	42.11%	15.481	<0.001
Splenomegaly PV dilated		26	44.8%	11	42.31%	15	57.69%	13.951	<0.001
Ascites		18	31.0%	6	33.33%	12	66.67%	7.200	0.007

There was high significant difference between ultrasonoghraphy (liver echo-pattern, splenic span, PV diameter & presence of ascites) and HCV relapse (P= <0.001).

Table 4: Impact of Treatment regimns on HCV relapse:

Treatment	Total patients		Negative serum		Positive serum		Chi-square test	
	no.	%	No.	%	No.	%	X2	p-value
Sofo/dacla	12	20.7%	8	66.67%	4	33.33%	2.667	0.102
Sofo/dacla/riba	19	32.8%	16	84.21%	3	15.79%	17.789	<0.001
Sofo/ledipasvir	9	15.5%	6	66.67%	3	33.33%	2.000	0.157
Sofo/ledipa /riba	15	25.9%	11	73.33%	4	26.67%	6.533	0.010
Sofo/riba	3	5.2%	1	33.33%	2	66.67%	0.667	0.414

There was high significant difference between the triple therapy regimens (Sofo/ Dakla/Riba, Harvoni/Riba), and SVR (P=<0.001).

Table 5: Relation between duration of treatment and HCV relapse.

Serum	Duration				Chi-square test	
	12 weeks		24 weeks		X^2	P-value
	No.	%	No.	%		
Negative	9	40.90%	29	80.6%	9.501	0.002
Positive	13	59.10%	7	19.4%		

There was high significant difference between prolongation of treatment and SVR (P= <0.01).

Discussion

HCV is not a strictly a hepatotropic viral pathogen, clinical and experimental evidence strongly indicates that the virus also invades and replicates in cells of other organs, particularly PBMNCs. The present results demonstrated that the 16/58 patients (27.59%) failed to achieve SVR, and absence of HCV RNA in PBMNCs was associated with high SVR rate (95.7%), Presence of HCV RNA positive strand in PBMNCs at the end of treatment is associated with high relapse rate (41.7%) and the low SVR rate (58.3%), while the presence of HCV RNA (negative replicative & positive genomic strands) in PBMNCs was associated with higher rate of relapse (45.45%) and lower SVR rate (54.5%). This agreed with Abd-Alla and El Awady (2017) who reported that PBMCs PCR was a valid diagnostic test that can diagnose intracellular HCV when the SRT-PCR was negative. The antisense and sense strands were respectively recognized more often in naïve and experienced patients. The expected overall relapsing rate in our cohort was 18.02%. Intra-PBMC infections associated with liver cirrhosis in naïve non-viremic patients. Eradication of intracel-

lular strands was recommended to avoid the RNA seroconversion.

The present results agreed with Zayed *et al.* (2010) who reported the presence of detectable HCV RNA in the PBMC of 27% of patients despite the clearance of serum HCV RNA. During follow-up, 80% of the patients who became serum HCV positive 6 months after the end of treatment had a detectable level of HCV RNA in PBMC at the end of treatment. They concluded that the absence of HCV in the serum of patients by the end of treatment does not exclude future viremia. The patient might still be a source of infection to others and they were recommended to testing for HCV in PBMC to detect lack of response to treatment and persisting infection.

Zaman *et al.* (2010) reported that total of 62 patients who achieved SVR treated with interferon and ribavirin for 24 weeks were included in the study. Viral RNA in PBMCs and HCV NS5A protein in were detected. Seven (11.3%) patients were relapsers. A positive association between viral RNA in the PBMCs and relapse was observed. Relapse significantly occurred more often in patients with HCV RNA in their PBMCs at SVR stage than the patients who did not have the RNA (25% vs. 4.8%). HCV protein expression in PBMCs showed the significant association with relapse (31.6% vs. 2.3%). The study concluded that patients having HCV RNA and NS5A protein in PBMCs after achieving the SVR are more likely to go in relapse as compared to those negative for HCV/RNA and protein in the PBMCs (Pawełczyk *et al*, 2013).

Hanno *et al.* (2014) conducted a similar study in 25 Egyptian patients infected with HCV. HCV/RNA was found in 32% of the PBMCs of infected patients. They also found that the patients with HCV/RNA in PBMCs after therapy had significantly higher relapse rate (50%) when compared with patients negative for the HCV/RNA in both PBMCs and serum after finishing therapy (6%). These results are in complete agreement with our study as we also found that the patients having HCV RNA in PBMCs have significantly higher rate of viral relapse Also, Gong *et al.* (2003) reported that HCV is capable of infecting and replicating in PBMCs. HCV plus strand RNA as well as minus strand RNA and HCVNS5 protein were found in PBMCs of 62.9%, 40.0% & 85.0% of chronic HCV patients respectively. Patients with minus strand HCV/RNA in PBMCs showed a significantly lower 6-month sustained response to IFN, suggesting a minus-strand (aviral replicative form) HCV/RNA in PBMCs may be one of the factors influencing response to IFN therapy. Cavalheiro *et al.* (2007) reported that the HCV/RNA was detected in both serum and PBMC in 35 (64%) patients without RNA in 16(29.6%). Disagreement between the serum and PBMC results was observed for three patients (5.6%), with HCV/RNA being detected in PBMC but not in serum. Four months later, new serum and PBMC samples were collected from one of the patients and HCV/RNA was detected in both samples, showing that PBMC can reveal signs of a lack of response to treatment.

El-Awady *et al.* (2003) used a triple assay comprised of RT-PCR tests for the detection of HCV-RNA plus strand in serum and peripheral blood mononuclear cells (PBMC), together with testing for the minus strand in PBMC for prediction of relapse after interferon + ribavirin combination therapy in 45 patients with chronic HCV. The only four patients with a negative triple assay had no relapse one year after the end of therapy. In contrast, two-thirds of the 12 patients who tested negative for viral RNA in serum at the end of therapy relapsed 1 year later so, they concluded that the absence of both minus and plus strands in patients who tested negative for serum PCR may indicate the total eradication of HCV.

In the present study, low albumin, elevated bilrubin and elevated INR which represent parameters of decompensated cirrhosis are predictor for low SVR rate that agreed with

Charlton *et al.* (2015) who reported that advanced cirrhosis lower SVR rate which has persisted to a lesser extent in the DAA era, with differences that are more pronounced in patients with greater liver disease severity. The regimens containing the triple therapy (Sofo/dacla/riba, Sofo/ledipasvir/riba) 28/37 (75.68%) gave high SVR rate (Sofo/Dacla/ Riba) 16/19 (84.21%)> (Sofo/Ledipasvir/ Riba) 11/15 (73.33%). Combining DAA with ribavirin also suggested a positive effect of ribavirin in such treatment strategies. The first study reporting a potential effect of ribavirin involved the nucleoside inhibitor and showed a synergic effect of ribavirin (Pradat *et al*, 2015), where the patients that did not receive ribavirin in PROVE-studies and those with a low ribavirin dosage in SPRINT-1 study had a higher probability of viral breakthrough or relapse and a lower SVR rate. These studies show that standard ribavirin dosage is necessary to get the best response to a first generation PI-based treatment. In the present study, lengthening duration of therapy was associated with high SVR rate 29 (80.6%) patients received DAAs for 24 weeks, in comparison to 9(40.9%) patients received DAAs for 12 weeks and this agreed with Hanno *et al.* (2014) who reported that the addition of ribavirin to the regimen and/ or lengthening the duration of therapy was associated with high SVR rate may be a recommended strategy, a hypothesis that is in need for further verification.

Generally speaking, Guerra *et al.* (2012) reported that with 14.7% of 15-59-year-olds testing anti-HCV positive, and added that Egypt had the highest HCV prevalence in the world. Kenyon and Colebunders (2014) identified the risk factors for the HCV infection within married couples and gave additional evidence of the importance of intrafamilial transmission of HCV in Egypt. They added that husbands whose wives had experienced female genital cutting had a higher prevalence of HCV and this relationship was driven by a strong association in urban areas. Esmat *et al.* (2016) stated that HCV showed highly prevalent and that the overall prevalence rate of HCV antibody among Cairo University's students was 4.6%. PCR for HCV/ RNA was detected in 31.4% of HCV antibody positive subjects (43/ 137) with statistical significant difference between males (29/51) and females (14/86).

Conclusion

The outcome results showed that the existence of HCV RNA positive and /or negative strand in PBMNCs was strongly associated with HCV relapse 12 weeks post-treatment with DAAs. Also, the prolongation of treatment course and /or addition of ribavirin to DDAs were associated with better SVR and lower relapse rate 12 weeks post-treatment.

No doubt, HCV prevention in Egypt must become a national priority. Public health authorities must introduce and implement more prevention measures targeting the routes of HCV transmission. Moreover, the massive HCV epidemic at the national level must have occurred with substantial transmission still ongoing today

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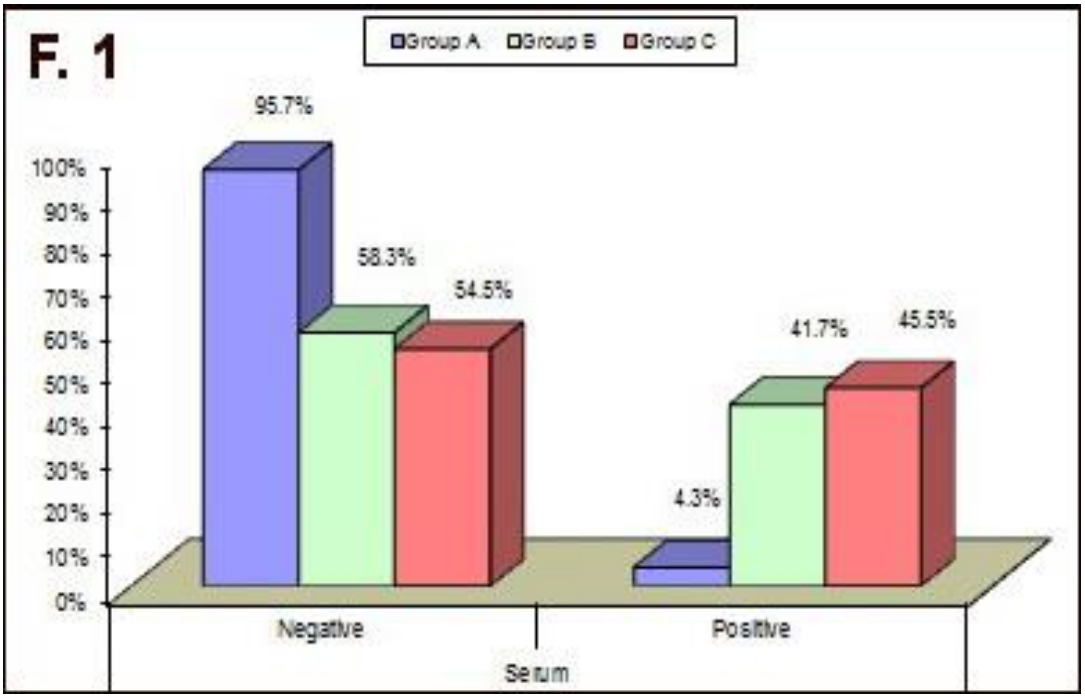
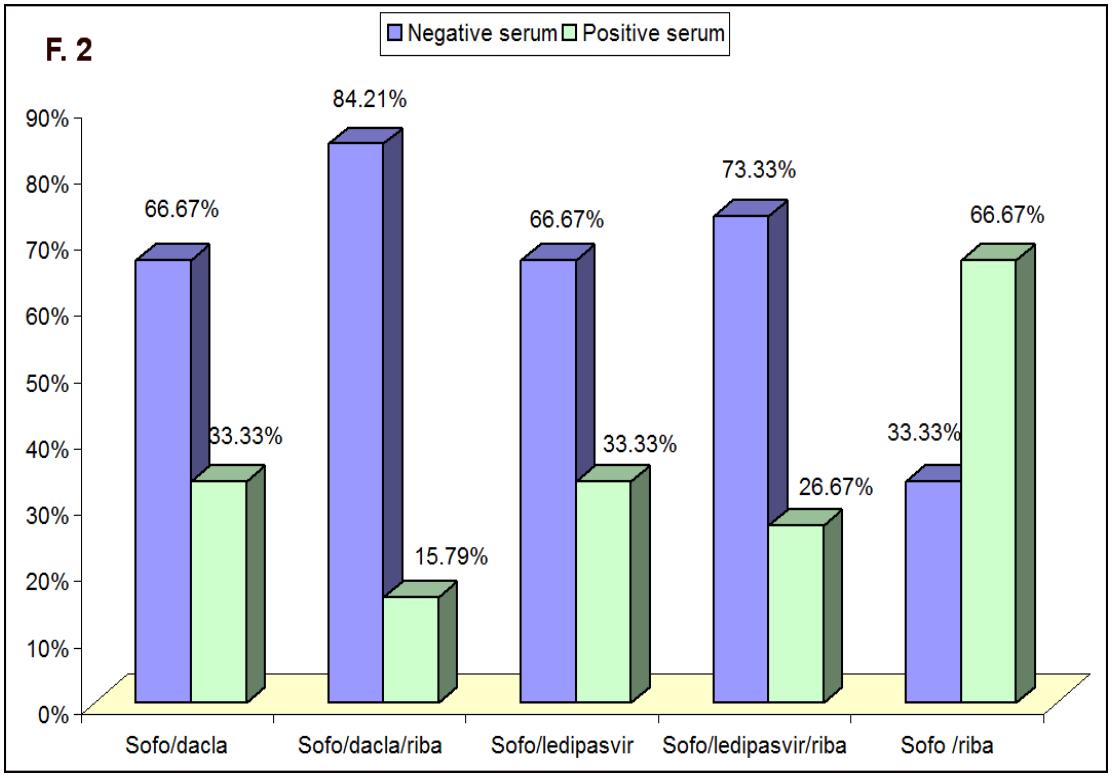
Explanation of figures

Fig. 1: Prevalence of serum relapse between studied groups.
Fig. 2: Treatment regimens and relation to HCV relapse.


F. 1
Group A
Group B
Group C
95.7%
58.3%
54.5%
4.3%
41.7%
45.5%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Negative
Positive
Serum


F. 2
Negative serum
Positive serum
84.21%
66.67%
66.67%
73.33%
66.67%
33.33%
33.33%
15.79%
26.67%
33.33%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Sofo/dacla
Sofo/dacla/riba
Sofo/ledipasvir
Sofo/ledipasvir/riba
Sofo /riba