

## Peripheral Blood Monocytes in Prediction of Response to Direct Acting Antiviral Drugs in Chronic HCV Patients

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### ABSTRACT

**BACKGROUND:** Eradication rate of hepatitis C virus (HCV) infection had dramatically increased in era of direct acting antivirals (DAAs). However, predicting relapse post-treatment had to be more speculated.

**AIM:** To evaluate role of peripheral blood monocytes (PBMcs) HCV PCR as predictor of post-treatment relapse. **Methods:** For chronic HCV patients who achieved end of treatment response, HCV PCR at PBMcs was assessed in relation to SVR.

**RESULTS:** 112 out of 118 cases had SVR 24 with only six relapsers

(5.1%). Negativity of triple HCV RNA was significantly lower in relapsers than in patients who achieved SVR (16.7% versus 92.9%,  $p = 0.001$ ). Cases with two positive strands were relapsers ( $n = 2,33.3\%$ ). Single RNA positive strand was detected in 50% of relapsers ( $n = 3$ ) and in 8 out of 112 (7.1%) of patients who achieved SVR. Significant factors affecting treatment response were absence of esophageal varices, negativity of triple HCV RNA, albumin, platelets, INR, creatinine and MELD score. On multivariate analysis, negativity of triple HCV RNA was significant predictor of achieving SVR with 208.38 Odds Ratio and 95% CI (4.93-8809.97,  $p = 0.005$ ).

**CONCLUSION:** Relying on presence of HCV on PBMcs at end of treatment with DAAs might be a trustworthy independent predictor of relapse.

**Key words:** HCV; Direct acting antiviral drugs; Peripheral Blood Monocytes

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### INTRODUCTION

Despite evolutionary advances in hepatitis C virus infection (HCV), paradoxical challenges are still representing unmet needs for more effective management and viral eradication<sup>[1]</sup>. Viral, environmental and host factors, including immunologic and genetic susceptibilities, may contribute to differences in the disease expression and treatment response<sup>[2]</sup>.

Peripheral blood monocytes as an extrahepatic potential reservoir of HCV virus; had been involved in many studies reflecting the viral replicative cycle<sup>[3]</sup>. Studying predictors of treatment response in current era of direct acting antivirals (DAAs) with the highest success rates had become questionable<sup>[4]</sup>. However, aiming at HCV eradication, necessitates a better understanding to every single element might affect HCV treatment response. Detection of HCV positive cells in

peripheral blood mononuclear cells (PBMCs) confirms the role of these cells as HCV reservoir both during ongoing antiviral treatment and most importantly after its completion<sup>[5]</sup>. The discordance between serum and PBMCs HCV RNA was reported by Cavalheiro et al. 2007 as an early sign of HCV persistence or reinfection<sup>[6]</sup>. HCV relapse might be a consequence of continuous or recurrent intracellular PBMCs viral replication. Occult hepatitis C (negative viremia with positive HCV PCR RNA in PBMCs and/ or liver tissue) prevalence had been investigated among SVR achievers post DAAs and was assigned to be present in 25% chronic HCV patients treated with the combination of sofosbuvir and daclatasvir for 3 months<sup>[7-8]</sup>. HCV relapse might be a consequence of continuous or recurrent intracellular PBMCs viral replication<sup>[9]</sup>.

## PATIENTS AND METHODS

### Study population

This study was conducted on 118 chronically HCV infected patients (males represented 51.7%,  $n = 61$ ) who were eligible for treatment with DAAs and they were recruited from hepatology and gastroenterology department, National Liver Institute, Menoufia University and Al-Hussein University hospital in the period from March 2017 to June 2018. Establishment of diagnosis and evaluation were based on clinical examination, laboratory investigations and abdominal ultrasound examination (US). The study was approved by the local Ethics Committee, National Liver Institute, Menoufia University. Enrolment of the individuals to the study was conditioned by an obtained written informed consent. One hundred and eighteen patients were consecutively selected and submitted to either dual or triple therapy treatment protocols (sofosbuvir 400 mg, daclatasvir 60 mg with or without ribavirin) and achieved end of treatment response. At 24 weeks post treatment; 112 cases achieved sustained virological response (SVR24) (57 males / 55 females; with mean age  $46.9 \pm 10.2$  years old), with only six relapsers. Non-responders (HCV/RNA positive in serum after completion of treatment), patients co-infected with HBV, patients with previous treatment with DAAs and/or immunosuppressive agents and chronic hepatitis C patients complicated with hepatocellular carcinoma all were excluded from the study.

All cases were subjected to thorough history taking, clinical examination, and routine laboratory examinations including complete blood counts, liver tests [aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum total and direct bilirubin, albumin, prothrombin concentration and international normalized ratio (INR)]. HBsAg was assessed by third generation ELISA, detection of HCV RNA level by real time PCR with lower detection limit (15 IU/mL), serum creatinine, HCV RNA in the PBMCs and serum were investigated at the end treatment & 12 weeks later. Model of End-Stage Liver Disease (MELD) was calculated<sup>[10]</sup>. Cirrhosis was diagnosed based on clinical findings, imaging studies (abdominal ultrasound) and laboratory results.

### Laboratory investigations

Ten ml venous blood samples collected from all patients were divided into three aliquots. Two aliquots used for routine laboratory investigations using fully automated auto analyzer SYNCHRON CX9ALX (Beckman Coulter Inc., CA, USA) for liver and renal tests, Sysmex K-21, (Sysmex Corporation, Kobe, Japan) for CBC and immunoassay (Abbott Laboratories, Abbott Park, IL, USA) for serum HBsAg and Anti-HCV. The third one was collected in EDTA containing tube for molecular and Triple PCR.

### Molecular testing

**HCV Real time PCR:** Whole blood samples on EDTA have been collected to separate plasma by centrifugation. Then, used for extraction of viral RNA by using QIAamp® Viral RNA: Qiagen (Qiagen, Germany). Real Time PCR for HCV performed by using ArtusHCV RG RT-PCR Kit Handbook- September 2013.

### Triple PCR

**(1) PBMC isolation:** PBMC were isolated by using Ficoll Technique. Washing steps have been skipped to keep part of plasma with the buffy coat for ability of detection of positive, negative strands in cells and positive strands in plasma.

**(2) RNA extraction:** Cellular and plasma viral RNA were extracted from 200  $\mu$ l of PBMCs & plasma sample using SV Total RNA Isolation System spin column-based technique to final 60  $\mu$ l Elution volume. Protocol described originally by Promega in "SV Total RNA Isolation System manual 07/18 was followed (Promega Corporation, USA).

**(3) cDNA synthesis:** RNA converted to cDNA by using GoScript™ Reverse Transcription System: Promega as per manufacturer instructions (Promega Corporation, USA). Up to 5  $\mu$ g RNA extract + random primer (0.5  $\mu$ g) completed the volume to 5  $\mu$ l with nuclease free water were heated in a 70°C heat block for 5 minutes. Immediately, chilled in ice water for at least 5 minutes and then, centrifuged for 10 seconds in a micro centrifuge. The mix components were GoScript™ 5X reaction buffer = 4.0  $\mu$ l, MgCl<sub>2</sub> (final concentration 1.5-5.0mM) = 1.2  $\mu$ l, PCR nucleotide mix (final concentration 0.5mM each dNTP) = 1.0  $\mu$ l, GoScript™ reverse transcriptase = 1.0  $\mu$ l and nuclease-free water = 7.8  $\mu$ l to a final total volume of 15  $\mu$ l. RNA samples (5  $\mu$ l) were added and mixed with the previous prepared components then inserted in the thermal cycler: Veriti96: (Applied Biosystem, USA). Thermal profile was 25°C for 5 minutes (annealing), 42°C for up to one hour (extension), then, 70°C for 15 minutes (reverse transcriptase inactivation).

**(4) qPCR detection:** First targets primer has been picked and blasted for the triple PCR HCV Target to target positive and negative HCV RNA strands. Primers were designed by the Oligo Primer D bioinformatics tool primer sequence (Gene Bank Accession number: D10749). And primers used were synthesized by (Metabion, Germany) as the following:

Forward: 5' GAGTGTCTGTCAGCCT 3'

Reverse: 5' CACTCGCAAGCACCCTATCA 3'

Detection was done by using 5x HOT FIREPol® EvaGreen® PCR Supermix from (Solis BioDyne, Estonia) on Rotor Gene Q-plex Real Time PCR Machine (Qiagen, Germany). Melting curve have been performed to eliminate nonspecific reaction (Figure 1). Reaction components were as the following: 5x HOT FIREPol®EvaGreen® qPCR Super mix = 4  $\mu$ l, primer forward (10 pmol/ $\mu$ l) = 0.3  $\mu$ l, primer reverse (10 pmol/ $\mu$ l) = 0.3  $\mu$ l, DNA template (0.1 ng/ $\mu$ l) = 2  $\mu$ l and H<sub>2</sub>O PCR grade = 13.5  $\mu$ l with total volume 20  $\mu$ l. Thermal profile and cycling conditions were 95°C for 12 min as initial activation for one cycle, 95°C for 15 seconds as denaturation step, 57°C for 20 seconds as annealing step, 72°C for 30 seconds as elongation step. Denaturation, annealing and elongation steps were repeated for 45 cycles. Then, final step was 70°C- 95°C continuous as melting step (Table 1).

### Statistical analysis

Data were tabulated, computerized and shown in the form of rate (%) and the standard deviation (SD). Chi-square ( $\chi^2$ ) test and Fischer's exact test were used where appropriate. Predictors of SVR and

relapse were analyzed first by univariate analysis. We then performed multivariate analysis, including those variables with  $p < 0.05$ . The p-value less than 0.05 were considered as statistically significant one.

## RESULTS

By evaluation of the studied parameters, as predictors for SVR, both who achieved SVR and relapsers showed no significant difference regarding age, sex, diabetes mellitus, presence of liver cirrhosis, direct acting antiviral regimens, bilirubin, albumin, AST, ALT, hemoglobin levels and white blood cells count (WBCs) count as shown in Tables 2, 3 and Figure 2. In the 6 relapsers, 5 patients had detectable triple HCV RNA (83.3%) while in patients who achieved SVR; detectable triple HCV RNA was found in 8 patients (7.1%) and p value was 0.001. Cases with two positive strands were relapsers ( $n = 2$ , 33.3%). Single RNA positive strand was detected in 50% of relapsers ( $n = 3$ ) and in 8 out of 112 (7.1%) of patients who achieved SVR (Table 3). Univariate analysis revealed low platelet counts, higher INR levels, higher serum creatinine, higher basal HCV RNA viral load, higher MELD score, presence of esophageal varices, and triple HCV RNA negativity to be the offending factors affecting SVR achievement post DAAs. Patients who had negative triple HCV RNA were liable to achieve SVR 208.3 times more than patients who did not have triple HCV RNA negativity. Factors affecting treatment response were; absence of esophageal varices ( $p = 0.019$ ), triple HCV RNA negativity ( $p = 0.001$ ), albumin ( $p = 0.018$ ), platelets ( $p = 0.049$ ), INR ( $p = 0.003$ ), creatinine ( $p = 0.030$ ) and MELD score ( $p = 0.007$ ) (Table 4). On multivariate regression analysis of the significant parameters for SVR, negativity of triple HCV RNA was the only significant independent predictor for achieving SVR after treatment with different regimens of directly acting antiviral drugs.

## DISCUSSION

HCV infection is a global health burden particularly in Egypt<sup>[11-12]</sup>. Prevalence is the highest in the world with about 10% of the population having chronic HCV-related liver diseases<sup>[13-14]</sup>. Several host and viral factors affect natural history of HCV infection<sup>[15-16]</sup>. Combination of peginterferon and ribavirin regimen was considered as standard of care of treating chronic HCV for years<sup>[17-18]</sup>. New direct-acting antiviral drugs, generic or brand, showed an improvement in liver tests and health-related quality of life in patients with chronic hepatitis C related decompensated liver cirrhosis<sup>[19-20]</sup> with higher efficacy and safety in elderly patients<sup>[21]</sup>. Accordingly,

**Table 1** cDNA synthesis protocol.

(up to 5µg RNA Extract + Random Primer (0.5µg) complete the volume to 5µl with nuclease free water) then. Heat in a 70°C heat block for 5 minutes. Immediately chill in ice water for at least 5 minutes. Centrifuge 10 seconds in a microcentrifuge.		
Mix Component Volume		
GoScript™ 5X Reaction Buffer	4.0 µl	
MgCl2 (final concentration 1.5–5.0mM)	1.2 µl	
PCR Nucleotide Mix (final concentration 0.5mM each dNTP)	1.0 µl	
GoScript™ Reverse Transcriptase	1.0 µl	
Nuclease-Free Water (to a final volume of 15µl)	7.8 µl	
Final volume	15 µl	
+ 5µl of RNA Mix - Then the mix inserted in the Thermal cycler - Veriti96 - Applied Biosystem- for the below profile		
Anneal	Extend	Inactivate Reverse Transcriptase
25°C for 5 minutes.	42°C for up to one hour	70°C for 15 minutes

**Table 2** Comparison between patients who achieved sustained virological response and those who did not achieve SVR.

	Relapsers (M±SD)		p
	N=6	N=112	
Age (years old)	43.00 ±8.83	46.89 ±10.16	0.332
Bilirubin (mg/dL)	0.94 ±0.11	0.91 ±0.24	0.688
Albumin (g/dL)	3.40 ±0.60	4.15 ±3.06	0.072
AST (U/L)	64.83 ±17.89	56.86 ±23.44	0.237
ALT (U/L)	57.17 ±21.00	66.66 ±26.87	0.401
Hemoglobin (g/dL)	13.32 ±1.52	13.20 ±1.64	0.75
WBCs ×10 <sup>9</sup> /L	5.28 ±1.33	5.96 ±1.53	0.345
Platelets ×10 <sup>9</sup> /L	136.50 ±72.29	203.44 ±74.39	0.043
INR	1.16 ±0.11	1.04 ±0.08	0.001
Creatinine mg/dL	1.13 ±0.10	0.96 ±0.17	0.016
HCV RNA level (IU/ml)	3732.50 ±1918.97	2083.29 ±7983.54	0.011
MELD	9.17 ±1.47	6.65 ±1.96	0.002

M= Mean; SD= Standard deviation; AST= Aspartate aminotransferase; ALT= Alanine aminotransferase; WBCs= White blood cells; INR= International normalized ratio; MELD= Model for end stage liver disease.

**Table 3** Comparison of the categorical data between patients who achieved sustained virological response and those who did not achieve SVR.

		Relapsers Number (%)	SVR Number (%)	Total	p
Sex	Female	2 (33.3%)	55 (49.1%)	57 (48.3%)	0.68
	Males	4 (66.7%)	57 (50.9%)	61 (51.7%)	
Drug protocol	Dual	6 (100%)	72 (64.3%)	78 (66.1%)	0.095
	Triple	0 (0%)	40 (35.7%)	40 (33.9%)	
Diabetes Mellitus	No	5 (83.3%)	98 (87.5%)	103 (87.3%)	0.566
	Yes	1 (16.7%)	14 (12.5%)	15 (12.7%)	
Cirrhosis	Non cirrhotic	2 (33.3%)	80 (71.4%)	82 (69.5%)	0.069
	Cirrhotic	4 (66.7%)	32 (28.6%)	36 (30.5%)	
Varices	No	3 (50%)	99 (88.4%)	102 (86.4%)	0.032
	Yes	3 (50%)	13 (11.6%)	16 (13.6%)	
Triple HCV RNA	Negative	1 (16.7%)	104 (92.9%)	105 (89%)	0.001
	Positive	5 (83.3%)	8 (7.1%)	13 (11%)	
Triple HCV RNA strands	Negative	1 (16.7%)	104 (92.9%)	105 (89%)	0.001
	Positive 1 strand	3 (50%)	8 (7.1%)	11 (9.3%)	
	Positive 2 strand	2 (33.3%)	0 (0%)	2 (1.7%)	

INR: International normalized ratio; MELD: Model for end stage liver disease; CI: Confidence interval.

**Table 4** Univariate and multivariate analysis of predictors of SVR.

	Univariate			Multivariate		
	p	Odds	95% CI	p	Odds	95% CI
Drug protocol	0.998	0.01	0			
Sex	0.458	1.93	0.34-10.97			
Diabetes mellitus	0.766	1.4	0.15-12.88			
Cirrhosis	0.071	5	0.87-28.66			
No Varices	0.019	7.62	1.39-41.75	0.94	0.86	0.02-48.35
Negative Triple HCV RNA	0.001	65	6.75-625.56	0.005	208.38	4.93-8809.97
Age	0.361	1.04	0.96-1.12			
Bilirubin (mg/dl)	0.732	0.55	0.02-17.31			
Albumin (g/dl)	0.018	8.65	1.44-52.08	0.983	1.01	0.47-2.16
Creatinine (mg/dl)	0.03	0.01	0.00-0.48	0.32	0	0.00-2706.57
Hemoglobin (g/dl)	0.863	0.96	0.58-1.58			
Platelets×10 <sup>9</sup> /L	0.049	1.01	1.00-1.03	0.505	0.99	0.96-1.02
INR	0.003	0.01	0.00-0.02	0.143	0	0.00-6507.61
HCV RNA level (IU/ml)	0.634	1	1.00-1.00			
MELD score	0.007	0.58	0.39-0.864	0.734	1.39	0.21-9.25

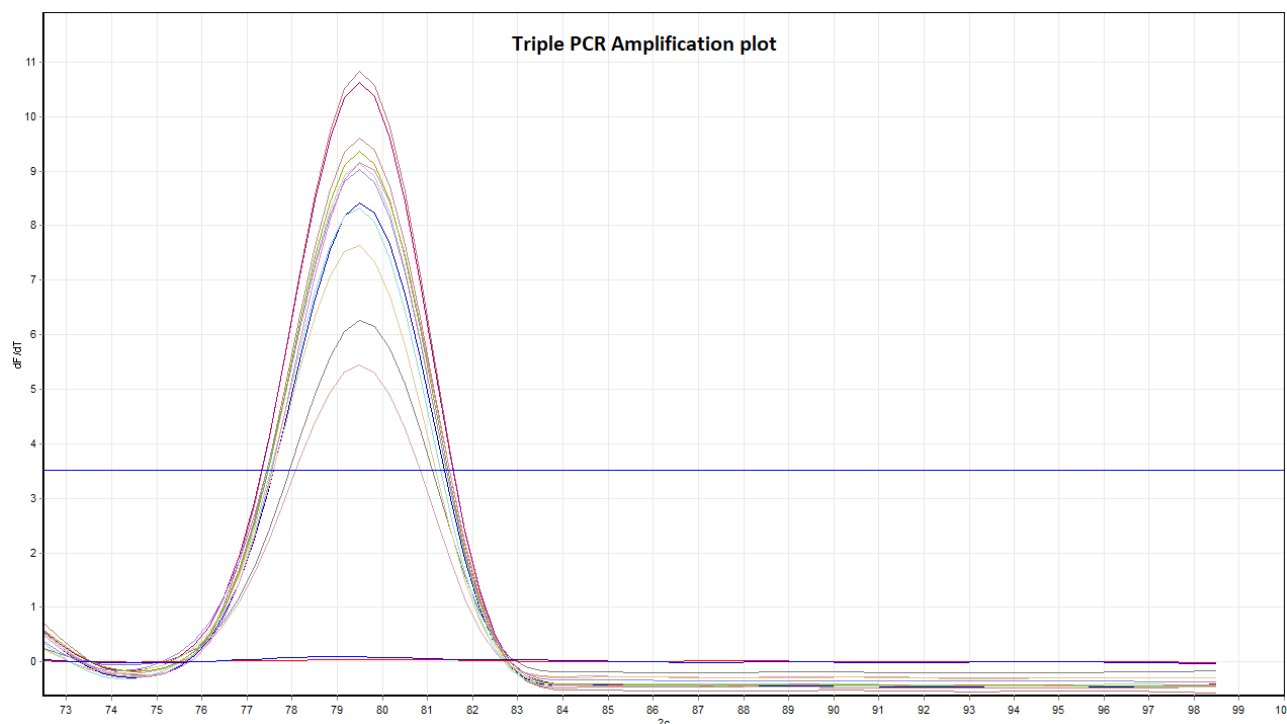


Figure 1 Triple PCR Amplification plot.

studies evaluating potential role of PMNC HCV PCR had been conducted on patients treated with combination regimen<sup>[22-24]</sup>.

The present study was designed to assess triple PCR assay for HCV RNA in the serum and peripheral blood mononuclear cells as predictive factor for SVR after treatment with different direct acting antiviral regimens.

As the current concern is HCV eradication, so the presence of relapsers might represent a hidden septic focus in the community threatening the national plans. Replication of HCV quasispecies in the PBMCs might be the most acceptable theory justifying viral persistence or reinfection<sup>[25]</sup>. Despite the relatively frequent studies in the interferon era, the role of HCV in PBMCs in relapse prediction had not yet studied thoroughly in the current DDAs era<sup>[22-24]</sup>. El-Awady et al. 2003 had established a settlement that the non-existence of both negative and positive strands in patients with resolved serum HCV PCR RNA is the only certified grantee of treatment fair response<sup>[23]</sup>.

To our knowledge, this is the second Egyptian study essentially directed to foretelling HCV relapse post DAAs therapies relying on intracellular PBMCs replication. Firstly, was the study of Alzahaby et al, 2017 who had reported in their small sized sample that presence of HCV RNA positive strand in PBMCs is associated with high relapse rate (41.7%), whereas the presence of HCV RNA (negative replicative and positive genomic strands) in PBMCs was associated with the highest relapse rate (45.45%)<sup>[26]</sup>.

The current study, on larger sized sample, had reexamined the linkage between HCV presence at PBMCs and SVR post DAAs. SVR was achieved in 92.2% of the patients with two negative strands, while five of the six relapsers (83.8%) were with positive HCV PCR at PBMCs. None of the patients with two positive strands in PBMCs had reached SVR, whereas 50% of those with positive and negative strands were relapsers.

Abd Alla and El Awady 2017 in their way to validate PBMCs PCR as a diagnostic test for intracellular HCV when SRT-PCR is negative, had also tested sofosbuvir plus ladipasvir treatment responses relying

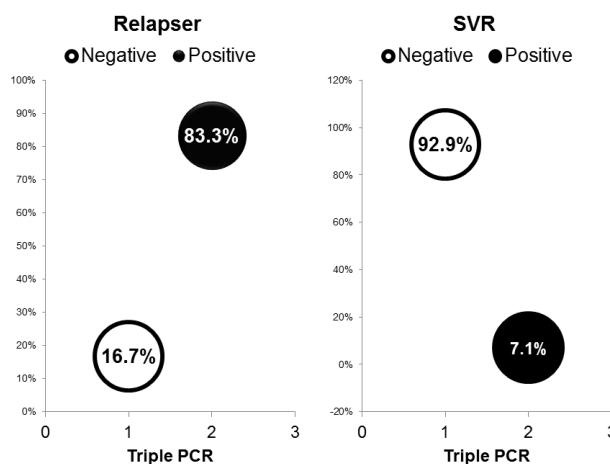


Figure 2 Triple PCR of HCV RNA in both responders and relapsers

on PBMCs HCV strands detection are respectively recognized more often in naïve and experienced patients. They declared that antisense and sense strands were respectively recognized essentially in naïve and experienced patients, with likely 18.02% relapsing rate<sup>[9]</sup>.

HCV relapse might be a consequence of continuous or recurrent intracellular PBMCs viral replication. Occult hepatitis C (negative viremia with positive HCV PCR RNA in PBMCs and/ or liver tissue) prevalence had been investigated among SVR achievers post DAAs<sup>[7-8]</sup>. The occurrence of HCV in PBMCs was assigned a presence of 25% chronic HCV patients treated with the combination of sofosbuvir and daclatasvir for 3 months<sup>[7-8]</sup>.

Recently, Mekky et al in their large cohort Egyptian multicenter study searching for OCI prevalence among SVR achieved patients following DAAs therapies had detected HCV-RNA in PBMCs of 50 cases of their population (3.9%)<sup>[27]</sup>.

According to the postulations of Ingotlet al in 2013, the recognition of negative strand in PBMCs before treatment may be suggested as a potential marker of good treatment response, while detection

of negative strand at the end of therapy is a predictor of relapse<sup>[28]</sup>. Consequently, abolition of HCV strands replication in PMBCs is only credit grantee of prohibiting seroconversion<sup>[9]</sup>.

Univariate analysis of the two patient groups data had ascertained the role of triple HCV RNA negativity among others (high platelet counts, high INR, low serum creatinine, low HCV RNA viral load, low MELD score, and absence of esophageal varices); to be the main factors affecting good response to DAAs.

Likewise, Al-zahaby et al, 2017 had identified low albumin, elevated bilirubin and elevated INR which represent parameters of decompensated cirrhosis as predictors of low SVR rate<sup>[26]</sup>. These agreed with Charlton et al. 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<sup>[29]</sup>.

Nevertheless, multivariate regression analysis had proved that the negativity of triple HCV RNA was the most momentous predictor for achieving SVR after treatment with different regimens of directly acting antivirals drugs.

Conclusively, reliance on HCV presence on PBMNCs at end of treatment with DAAs might perfectly fortunes the occurrence of relapse, accordingly new strategies might be implied as timing, and regimen modifications. Consequently, a combined serum and PBMNCs HCV PCR might be a recommended new pattern of evaluation at end of DAAs therapies.

#### Ethical approval

The study was conformed to ethical guidelines of 1975 Declaration of Helsinki. The study was reviewed and approved by ethical committee at the National Liver Institute, Menoufia University, Egypt.

#### Informed consent

An informed written consent was obtained from all individual participants included in the study.

#### Author contribution

A: Study Design: El-Sayed Ibrahim and Sabry Moawad Abdelmageed;

B: Data Collection: El-Sayed Ibrahim, Eman Abdelsameea;

C: Statistical Analysis: Ayman Alsebaey;

D: Data Interpretation: Maha Elsabaawy, Eman Abdelsameea, Ayman Alsebaey, Abd-ElAleem A.El-Gendy and Sabry Moawad Abdelmageed;

E: Manuscript Preparation: Maha Elsabaawy, Eman Abdelsameea, El-Sayed Ibrahim, Sabry, Moawad Abdelmageed, Ayman Alsebaey and Abd-ElAleem A.El-Gendy;

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