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Effect of Daclatasvir and Sofosbuvir Therapy on Serum Levels of Angiogenic Factors: A Prospective Cohort Study

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Authors' contributions

This work was carried out in collaboration among all authors. All authors have contributed equally to this work either by designing, analysis and interpretation of data, drafting, revising critically or final approval of the revision to be published. All authors read and approved the final manuscript.

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ABSTRACT

Background: Direct-acting antiviral agents therapy is considered a breakthrough in hepatology due to high rates of sustained virologic response in all patients including those with decompensated cirrhosis. However, impact of Direct-acting antiviral agents-induced sustained virologic response on hepatocellular carcinoma development remains conflicting.
Aims: This study aimed at evaluating the change in circulating levels of vascular endothelial growth factor and transforming growth factor-β1, the main angiogenic factors involved in hepatocarcinogenesis process, in cirrhotic patients achieved sustained virologic response after Direct-acting antiviral agents therapy.
Study Design: This was a prospective, single-center, cohort study.
Place and Duration of Study: Patients were recruited from the outpatient clinic of National Liver Institute, which considered a tertiary referral center in Menoufia University, Egypt (September 2018 to February 2019).

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Methodology: Forty-five decompensated cirrhotic hepatitis C virus infected patients with no history of hepatocellular carcinoma participated in the study. All patients received 60mg oral daclatasvir and 400mg oral sofosbuvir once daily for 12 or 24 weeks with or without ribavirin. Serum levels of vascular endothelial growth factor and transforming growth factor-β1 were measured at baseline and 12 weeks after the end of therapy.
Results: The median serum levels of vascular endothelial growth factor showed a non-statistically significant increase (from 1123 ng/L to 1269 ng/L, *P* = 0.126). But, transforming growth factor-β1 median serum levels exhibited a non-statistically significant reduction (from 13.22 ng/ml to 12.44 ng/ml, *P* = 0.163) 12 weeks after treatment.
Conclusion: Our findings show direct-acting antiviral agents therapy do not affect vascular endothelial growth factor and transforming growth factor-β1 serum levels. But, a larger scale prospective cohort study on an extended follow-up period is recommended.

Keywords: Angiogenesis inducing agents; daclatasvir; hepatitis C chronic; sofosbuvir; sustained virologic response; hepatocarcinogenesis; vascular endothelial growth factor; transforming growth factor-B1.

1. INTRODUCTION

Chronic hepatitis C virus (HCV) infection is the prime causation of liver cirrhosis and hepatocellular carcinoma (HCC) [1] HCV accounts for about 27% of cirrhotic cases and about 25% of HCC cases worldwide, and over 350000 people die each year from hepatitis C-related liver diseases [2,3]. In Egypt, HCV infection has the greatest prevalence rate over the world and considered a major public health burden since it is the leading cause of end-stage liver disease, HCC, and hepatic related death [4]. The Egypt Demographic and Health Surveys (EDHS) measured HCV prevalence among the adult population aged 15–59 years was 14.7% in 2009 and 10.0% in 2015 substantially higher than global levels [5]. The currently available highly effective regimens of direct-acting antiviral agents (DAAs) have totally remodeled the panorama of hepatitis C therapy and promise a novel epoch in the treatment of HCV infection in a very brief period with great safety profile [6,7]. Oral combination of daclatasvir (DCV) an HCV nonstructural protein 5A (NS5A) replication complex inhibitor and sofosbuvir (SOF) a nucleotide analogue HCV nonstructural protein 5B (NS5B) polymerase inhibitor has powerful antiviral action and wide genotypic coverage achieving extremely elevated sustained virological response (SVR) rates nearly close to 100% in both treatment-naïve and treatment-experienced patients. These impressive results are expected to modulate the disease epidemiology and aid in achieving the goal of eliminating HCV infection in Egypt [8]. The regimen that was used in decompensated cirrhotic patients was sofosbuvir (400 mg, orally, once daily) plus daclatasvir (60 mg, orally, once daily) for 12 weeks. Weight-based ribavirin (1200 or 1000 mg/day if ≥75 or <75 kg body weight, respectively) was added to this regimen when treating cirrhotic patients and/or treatment-experienced patients who received prior interferon therapy. Treatment duration was extended to 24 weeks with addition of weight-based ribavirin only in treatment-experienced patients who failed to respond to sofosbuvir plus ribavirin regimen [9]. However, the impact of DAAs–induced SVR on HCC development remains conflicting and under debate [10]. In this respect, HCC was found to develop in an unexpectedly very high number of cirrhotic HCV infected patients within few weeks after beginning DAAs regimens suggesting that there is an association between exposure to DAAs and the emergence of HCC. Several authors have intimated that, there is a relation between DAAs treatment and hepatic neoplasia growth or dissemination and concluded that after DAA exposure, some patients with HCV are at increased risk of de novo HCC development, and other patients with a history of treated HCC are at increased risk of HCC recurrence [11-14]. While other opposite studies have confuted these findings and insisted that DAAs are protective against the development of HCC [15-17]. This highlights the need for further clinical evaluation for the patients achieved SVR after DAAs regimens.

Angiogenesis has a crucial role in the development, progression, and dissemination of the extremely vascular solid tumor (HCC). When the precancerous lesions "dysplastic nodules" begin to evolve, angiogenesis is actuated and the process of angiogenesis carries on with the progression of the tumor [18,19]. Therefore, the circulating angiogenic regulators have been evaluated as predictive factors for cancer

development risk as well as diagnostic and/or prognostic factors in cancer patients, and measuring tumor angiogenesis is a possible predictor of aggressive tumor behavior [20,21]. Vascular endothelial growth factor (VEGF) is the most well established angiogenic factor in hepatic cancer. Levels of VEGF are significantly upgraded in early stages of HCC suggesting its importance in hepatocarcinogenesis and Serum VEGF level has been found to predict venous invasion and metastasis in HCC [22,23]. Moreover, high expression of VEGF mRNA in non-cancerous hepatic tissues correlated with increased HCC development risk and according to An et al. (2000), the nearer the non-cancerous liver cells were to cancerous cells, the stronger the VEGF expression they showed [24,25]. The versatile polypeptide cytokine transforming growth factor β1 (TGFβ1) controls many processes in the cell as proliferation and apoptosis [26]. TGFβ1 has a pivotal role as it serves as a chief tumor suppressor in premalignant cells and the beginning stages of carcinogenesis, while in the late phases of tumor it promotes cancer incursion and dissemination which means that, TGFβ1 acts as a double-edge blade as it is able to suppress growth in normal and premalignant epithelial cells. However, upon accumulation of genetic and epigenetic alterations in tumor cells, it switches to promotion of a proinvasive and prometastatic phenotype, accompanied by a progressive increase in the locally secreted TGFβ1 levels [27-29]. Regarding its primary effect, low levels of TGFβ1 are mandatory to start the carcinogenesis process, hence it can function as a serum predictor that altered in patients at increased risk of developing HCC even before the clinically detectable tumors emerge [30], and a strong association was found between a low serum TGF-β1 concentration and a higher risk of incidence HCC and indicated that serum TGF-β1 can distinguish between patients who are more likely to develop HCC from those with lower risk, especially among HCV-positive patients [31]. Alfa-fetoprotein (AFP) is a serum glycoprotein that was first recognized as a marker for HCC more than 40 years ago and has since been described to detect preclinical HCC [32]. When elevated, the AFP is 75-91% specific and values greater than 400 ng/mL are generally considered diagnostic of HCC in the proper clinical context, including appropriate radiologic findings [33].

In this context, this study is aimed at defining the change in the pattern of circulating levels of VEGF, TGFβ1, and AFP in a cohort of chronic HCV infected patients, genotype 4 receiving DAAs without history of HCC, in determining the effect of DAA-induced SVR on angiogenesis and hence on the hepatocarcinogenesis process.

2. MATERIALS AND METHODS

2.1 Study Design

This is a prospective, single-center, cohort study. Patients were recruited from the outpatient clinic of the National Liver Institute that is a tertiary referral center in Menoufia University, Egypt – recruited from September 2018 to February 2019. Our cohort study adheres to STROBE research reporting standards.

2.2 Patients

G*Power software program was used to calculate sample size. The study sample size was calculated to be 35 patients on effect size = 0.5, α–error probability = 0.05, and power = 0.8. However we conducted our study with 45 patients which raised the power of the study to 0.88. It is worth mentioning that, the sample size of this study was based on some previous studies [34-36].

Forty five decompensated cirrhotic patients with chronic HCV infection genotype 4, aged 18-65 years old were included in the study. Liver cirrhosis was proven by combination of clinical, laboratory, and ultrasound criteria. According to European Association for the Study of the Liver (EASL) guidelines, fibrosis stage must be assessed by non-invasive methods initially such as ultrasound, while liver biopsy reserved for cases where there is uncertainty [6].

The exclusion criteria were the following: patients with prior history of liver transplantation or hepatocellular carcinoma, co-infection with HIV or HBV, pregnant or lactating females, and finally refusal to participate in the study.

2.3 Methodology

Demographic and clinical data of the participants (n=45) were collected including age, gender, weight, height, and body mass index (BMI). The initial evaluation of the patients included full clinical examination, HBsAg, quantitative PCR for HCV-RNA, and abdominal ultrasound by available methods.

Either the ultrasound detection of hepatic focal lesion or elevation of AFP level above 400 ng/ml, raised the suspicion of HCC. AFP elevation is 75-91% specific for the diagnosis of HCC, but values greater than 400 ng/mL are generally considered diagnostic of HCC [32].

Lab parameters including complete blood cell count, total and direct bilirubin, ALT, AST, albumin, INR, urea, creatinine, and serum AFP were assessed at baseline and 12 weeks after the end of antiviral treatment.

All patients received antiviral therapy according to the Egyptian protocol for the treatment of HCV infection. The recommended regimen was oral daclatasvir and sofosbuvir at a dose of 60mg once daily and 400mg once daily, respectively for 12 or 24 weeks with or without addition of ribavirin, according to guidelines and medical decision.

Abdominal ultrasound was performed on subjects at week 4, at the end of treatment and at 12 weeks after the end of the treatment.

HCV-RNA quantification was made by real- time PCR, with a limit of detection of 15 IU/ml to assess the virological response at week 4, at the end of treatment and at 12 weeks after the end of the treatment. Sustained virological response (SVR) to therapy was defined as undetectable serum HCV- RNA viral load 12 weeks after completion of HCV therapy (SVR12).

Blood samples were collected into vacutainers by venipuncture, then delivered into plastic tubes, which were centrifugated at 4,500 g for 10 min. The separated serum was kept in a deep freezer at – 80ºC until the time of analysis. The serum levels of VEGF and TGFβ1 were measured at base line and 12 weeks after the end of treatment using specific ELIZA commercial kits (SunRedBio, Shanghi, China. Catalogue No. : 201-12-0081 and 201-12-5480, respectively). All measurements were done according to the manufacturer‘s specifications using a Multiskan FC with incubator microplate reader (USA).

2.4 Statistical Analysis

Categorical data are presented as numbers (percentages). Continuous data are presented as mean ± SD and medians (ranges). Kolmogorov-Smirnov test was used to assess the normality of data. Normally distributed variables were analyzed using paired t-test and non-normally distributed variables were analyzed using the Wilcoxon signed-rank test.

Statistical analysis was performed using IBM SPSS Statistical package version 22.0 (IBM Corp; USA, 2013). The level of statistical significance was set at $P < 0.05$ using a two-tailed test.

3. RESULTS

3.1 Demographic Data

Out of 54 eligible patients, nine patients were dropped out because of non-compliance or non-achieving a virological response. Only 45 patients were included in the ultimate analysis (Fig. 1).

Study subjects are uniformly distributed in terms of sex (51.1% female vs 48.9% male) and the mean age is 53±7.15 years. The detailed baseline demographic data of the participants is shown in Table 1.

3.2 Change in Laboratory Parameters after DAA Therapy

All study participants are confirmed to achieve SVR12 with no detected lesions by surveillance ultrasound. There was a statistically significant improvement in the liver function tests including serum direct bilirubin, ALT, and AST 12 weeks after treatment, while the platelet count, total bilirubin, serum albumin, prothrombin concentration, and INR showed non-statistically significant improvement 12 weeks after treatment. It is noteworthy mentioning that, there was a significant reduction in hemoglobin level 12 weeks at the end of the treatment, while there was a significant increase in renal function tests (serum creatinine and blood urea) (Table 2).

3.3 Change in AFP and Angiogenic Factor Levels after the End of the Treatment

There is a non-statistically significant increase in median serum levels of VEGF 12 weeks after DAAs therapy with reference to the median baseline levels (from 1123 ng/L to 1269 ng/L, $P = 0.126$)(Table 3; Fig. 2). And, the median AFP serum levels showed non-significant increase after DAAs treatment when compared with the median baseline levels (from 4.42 ng/ml to 4.50 ng/ml, $P = 0.531$)(Table 3; Fig. 3). In contrast, the median serum levels of TGFβ1 exhibits non-

statistically significant decrease 12 weeks after treatment as compared to its median baseline levels (from 13.22 ng/ml to 12.44 ng/ml, *P* = 0.163) (Table 3; Fig. 4)

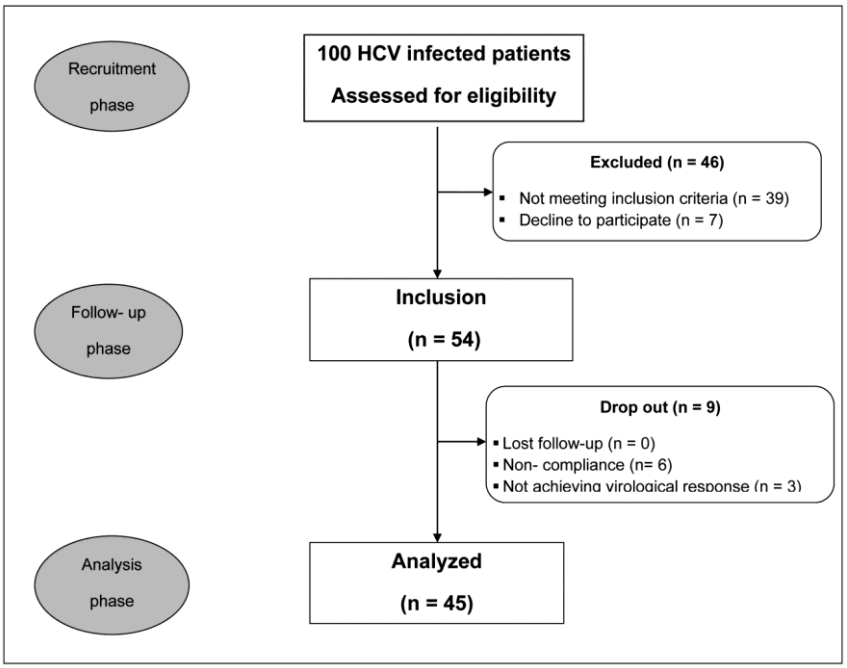

Fig. 1. Flow chart of study participants

Table 1. Base line demographic data for all patients (n=45)

Parameter		Value
Gender	Male	48.9%
	female	51.1%
Age,(years)	Mean	53.69±7.15

Variables are presented as numbers (percentages) for categorical data or mean for continuous data

Table 2. Serum laboratory data for all patients at base line and after treatment

Parameter		Base line before DAA treatment	After the end of DAA treatment	Wilcoxon signed-rank test	*P*-value
Hb (g/dl)	Mean	13.47±1.53	11.37±1.33	5.73	0.001
Platelet count (U/L)	Mean	169.89±88.10	172.11±74.42	0.48	0.63
Total bilirubin (mg/dl)	Mean	0.98±0.55	0.79±0.35	1.17	0.243
Direct bilirubin (mg/dl)	Mean	0.45±0.32	0.22±0.13	3.7	0.001
ALT (U/L)	Mean	64.04±42.02	26.67±10.90	5.4	0.001
AST(U/L)	Mean	61.42±35.38	23.67±11.77	6.5	0.001
Albumin (G/dl)	Mean	3.92±0.58	4.48±3.62	0.299	0.76
Prothrombin concentration (%)	Mean	75.67±15.49	79.09±10.83	0.84	0.4
INR	Mean	1.20±0.26	1.13± 0.13	1.2	0.245
Urea (mg/dl)	Mean	26.20±6.85	37.02±10.36	5.1	0.001
Creatinine (mg/dl)	Mean	0.77±0.20	0.96±0.21	4.2	0.001

ALT: alanine aminotransferase; AST: aspartate aminotransferase; DAAs: direct acting antiviral agents; Hb: hemoglobin; INR: international normalized ratio

Table 3. Serum levels of selected biological markers for all patients at base line and after treatment

Parameter		Base line before DAA treatment	After the end of DAA treatment	Wilcoxon signed-rank test	*P*-value
VEGF (ng/L)	Mean	1329.53 ± 583.06	1421.80 ± 637.626	1.529	0.126
	Median	1123.00	1269.00		
AFP (ng/ml)	Mean	5.53 ± 4.02	5.40 ± 3.99	0.626	0.531
	Median	4.42	4.50		
TGF-β1 (ng/ml)	Mean	15.74±8.24	13.49±5.80	1.394	0.163
	Median	13.22	12.44		

AFP: alpha-fetoprotein; TGF-β1: transforming growth factor-β1; VEGF: vascular endothelial growth factor

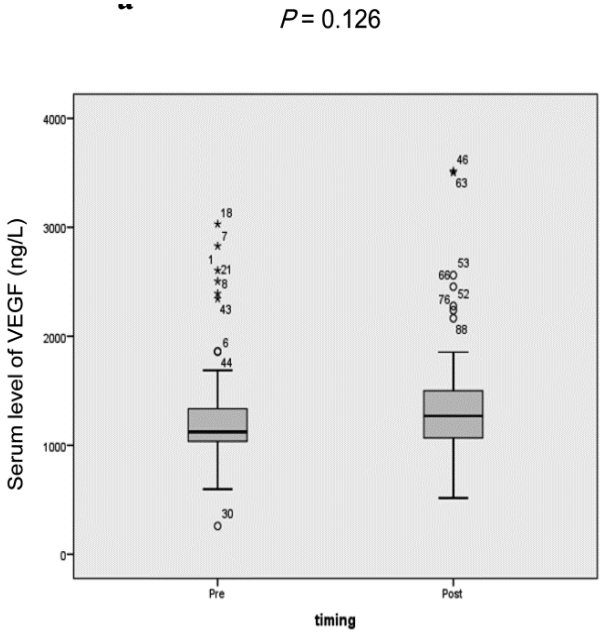

Fig. 2. Vascular endothelial growth factor (VEGF) serum levels at baseline (pre), and 12 weeks after treatment (post)

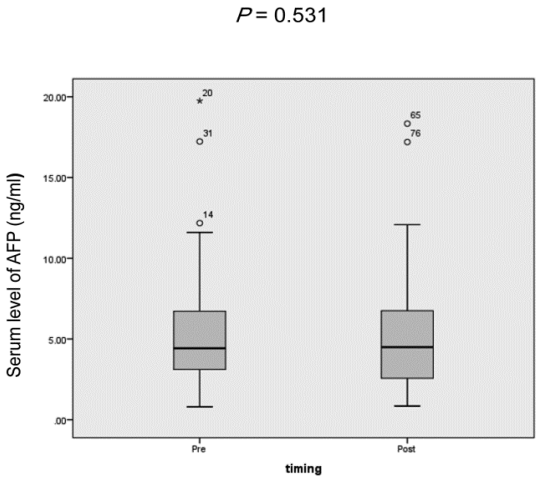

Fig. 3. Alpha-fetoprotein (AFP) serum levels at baseline (pre), and 12 weeks after treatment (post)

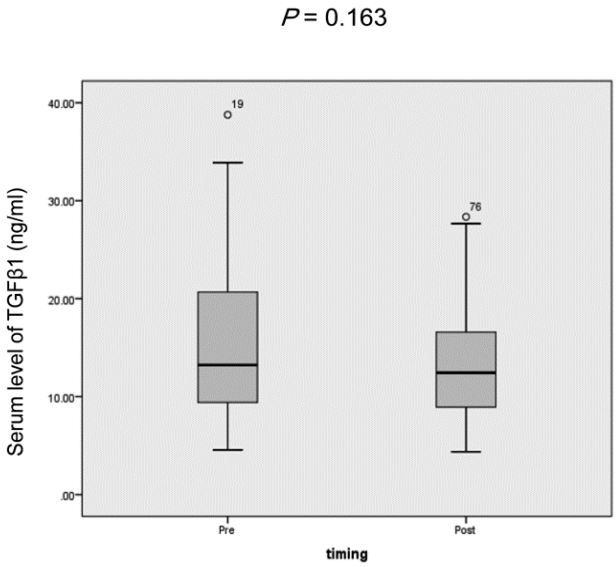

Fig. 4. Transforming growth factor β1 (TGFβ1) serum levels at baseline (pre), and 12 weeks after treatment (post)

4. DISCUSSION

DAAs therapy is currently known to be one of the master advances in clinical hepatology because of high rates of SVR in patients with different stages of HCV infection including those having decompensated cirrhosis [37]. By the advent of DAA medications; the long-awaited solution for HCV infection, both hepatologists and patients raised their aspirations and expectations not only to eradicate the viremia but also to enhance the liver functions and to diminish the allied complications such as HCC. However, reporting of the cases with dramatic relapse or "*de novo*" occurrence of malignant hepatoma within a few weeks of the DAAs treatment have collapsed this general climate of fervor and generated much scientific dispute. Since then, this topic has been recently a subject of several controversial publications that did not put an end to this debate [10,38]. The absence of large prospective studies about this issue keeps it an open puzzling question. Since, there is no decisive evidence yet from randomized clinical trials neither to confirm nor to reject the potential oncogenic effect of DAAs, this risk should not be underestimated.

Our data showed that, the circulating levels of VEGF increase non-significantly, whereas the circulating levels of TGFβ1 decrease non-significantly both here 12 weeks after the end of DAAs therapy. Concerning VEGF, the main angiogenic growth factor in HCC, Villani et al. (2016) demonstrated that during DAAs treatment and up to the end of the treatment, the levels of VEGF become temporary elevated until reverted to the baseline after the cessation of antiviral medications which indicates limited effect of DAAs on angiogenesis [39]. Similarly, Facilli et al. (2018) have reported that, VEGF levels increased from baseline to the end of antiviral therapy and maintained elevated for three months after the cessation of antiviral medications [40].

Ali et al. (2004) and Watanabe et al. (2016) concluded that the decrease in TGFβ1 levels could be the primary marker for hepatic neoplasia, and the decreased levels of this biomarker may suggest initiation of the process of hepatocarcinogenesis, where the increase in TGFβ1 serum levels may contribute to the advanced stages of hepatic carcinoma [31,41]. According to this hypothesis and in the insight of our results, we did not find that DAAs therapy has profound effect on hepatocarcinogenesis process, since TGFβ1 levels didn't significantly affected.

Concerning the reduction in haemoglobin serum levels, in contrast to our findings, another study reported no significant difference was noted regarding pre- and post- treatment haemoglobin (Hb) levels [42]. Conversely, El Sagheer et al. (2018) reported a significant decrease in hemoglobin level from 13.6 before treatment down to 12.7 g/dl after [43]. Another study reported a significant change in hemoglobin level

after DAAs treatment ($p < 0.001$) [44]. Also our results showed that the SVR was associated with a significant increase in renal function tests contrary to Nevola et al. (2020) who reported that patients who eliminated HCV by DAAs had an improvement of serum creatinine compared to the baseline value. The reason of dissimilarities observed in the two studies on negative predictors of improvement in renal function are unclear [45].

Given the observational nature of our study, we did not provide the biological mechanisms by which DAAs treatment may influence the angiogenic factors levels. However the real mechanisms by which DAAs therapy can affect angiogenic factors levels is still unclear and not fully elucidated [46].

One of the worth noting learned lessons is that delaying the treatment of HCV-infection until progression to cirrhotic grade might be associated with a substantial oncogenic hazard despite the resolution of viral infection [47]. Even though the insignificant effect of DAAs on AFP and angiogenic factor levels, we still recommend HCC surveillance for DAAs treated cirrhotic patients in the immediate post-SVR context, which is not yet currently recommended [48], as both European Association for the Study of the Liver (EASL) and American Association for The Study of Liver Diseases (AASLD) guidelines recommend HCC surveillance for DAAs treated patients every six month for indefinite duration but not in the immediate post-SVR context [6,49].

The strength of this study comes from the prospective design, whereas previous studies are of retrospective nature. And, to the best of our knowledge this is a priming study addressing the effect of DAAs therapy on TGFβ1 levels.

Additionally, subjects in this study are derived from a single, national health care center with relatively unvarying antiviral treatment-regime and are in the same cirrhotic grade minimizing the geographical, ethnical and clinic-pathologic heterogeneity within the studied group of patients. Such heterogeneity could be an important limitation in the previous studies.

This study's data is helpful for many countries because HCV genotype 4 defines 12%-15% of absolute global HCV infection and exists in North Africa, Middle East, South Asia, and central sub-Saharan Africa countries such as The Central African Republic, The Democratic Republic of Congo, Gabon, Chad and Equatorial Guinea, in addition to Eastern region of Africa like Ethiopia [50,51].

This study has some limitations which include brief average follow-up time that limits long term interpretation of our findings. Another limitation is lack of comparable control group of patients who had not received antiviral therapy, as the ideal method to examine whether DAAs treatment affect angiogenic factors serum levels is to randomize patients to treatment with DAAs *vs* no treatment or placebo and then assess the change in the angiogenic factors levels in each group before and after. However, such study design would be unethical regarding the clearly established benefits of DAAs treatment for all HCV-infected patients in the recommendations of both EASL and AASLD [6,49].

5. CONCLUSION

DAA-induced SVR does not significantly affect VEGF and TGFβ1 serum levels – two critical factors in hepatocarcinogenesis. Consequently, showing safety of DAAs, without angiogenic and oncogenic risk of DAA therapy. This data here needs further study, analyzing larger scale prospective cohort in additionally extended follow-up period.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products in commercial promotion but for the advancement of science. Also, the research is not funded by the producing company - rather it is funded by personal efforts of authors.

CONSENT

Authors declare that written informed consent was obtained from approving authority for publication of this research.

ETHICAL APPROVAL

The study protocol was in accordance with the ethical standards of the 1975 declaration of Helsinki and was approved by the institutional review board of the National Liver Institute (NLI

IRB 00003413) on February 4, 2018; IRB Protocol Number: 00132/2018.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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