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Role of Serum Nitric Oxide in Prediction of Hepatocellular Carcinoma in Hepatitis C Patients Treated with Direct - Acting Antiviral Agents

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&Authors' contributions:

Basha OM, Elsadek, Shokry MM, Elsaid HH, Farag AA and these authors are contributed equally to this work.

Keywords:

Advanced cirrhosis; Direct-acting antiviral agents; Hepatocellular carcinoma; Nitic oxide

Abbreviations:

AFP: Alpha feto-protein; AASLD: The American Association for the study of Liver Diseases; CPC: Child-Pugh class; CHC: Chronic hepatitis C; CT = Computed tomography; DAC: Daclatasvir; DAAs: Direct-acting antiviral agents; FDA: Food and Drug Administration; HCV: Hepatitis C viral; HCC: Hepatocellular carcinoma; iNOS: Inducible Nitric Oxide Synthase; NO: Nitric Oxide; NS5A: Nonstructural protein 5A; NS5B: Nonstructural protein 5B; NS3: Nonstructural protein 3; sNO: Serum Nitric Oxide; RBV: Ribavirin; SOF: Sofosbuvir; SVR: Sustained viral response rate; BCLC: The Barcelona Clinic Liver Cancer

1. Abstract

1.1. Background: Hepatocellular Carcinoma (HCC) is the 6th most common malignancy globally and it's the most common tumor which originates primarily in the liver, the aim of this study is to find out the connection between increased serum nitric oxide (sNO) and its HCC predictive value in patients who received direct-acting antiviral agents (DAAs).

1.2. Methods: In this study we randomized 72 patients with chronic hepatitis C virus (HCV) infection who were attempted to be treated with sofosbuvir (SOF) / daclatasvir (DAC) ± Ribavirin for 12 weeks and we assessed sNO level before and after treatment.

1.3. Results: Among chronic hepatitis C (CHC) patients who received DAAs therapy, HCC developed in significantly higher frequency among cirrhotic patients with advanced disease (28.6%) than those with early cirrhosis (23.1%), and never in non-cirrhotic patients ($p<0.001$). There was a significant value of sNO in the diagnosis of HCC, especially regarding its post treatment level. At a cut-off ≥ 361.24 µmol/l, post treatment level of sNO had a sensitivity of 100%, specificity of 100%, positive predictive value of 100% and negative predictive value of 100% with an accuracy of 100% ($P<0.001$).

1.4. Conclusion: sNO level increases significantly after DAAs therapy in advanced cirrhosis, and this increase is significantly associated with the development of HCC.

2. Background

Hepatitis C virus (HCV) infection is still a great public health problem, with a global seropositive rate of about 2.5% (177.5 million people) and a global viremic rate of nearly 1.7% (118.9 million positive cases) [1]. Worldwide, about 350 000 people die yearly from HCV-related chronic liver disease including hepatocellular carcinoma (HCC) [2].

Egypt has the heaviest HCV burden worldwide. However, over the past two decades, HCV prevalence among Egyptians was noticeably decreasing, which was attributed to the mass treatment with interferon then the successful mass treatment campaigns using oral direct-acting antiviral agents (DAAs) [3].

After its Food and Drug Administration (FDA) approval in December 2013, DAAs have replaced interferon therapy for HCV treatment. The first drug in DAAs revolution was sofosbuvir (SOF), a nucleotide Nonstructural protein 5B (NS5B) polymerase inhibitor that has a pan genotypic activity, a high barrier to resistance, and an excellent tolerability [4]. Daclatasvir (DCV) is a Nonstructural protein 5A (NS5A) replication complex inhibitor which was FDA approved in July 2015. It has a high pan genotypic antiviral potency, good tolerability and limited drug-drug interactions [5]. Combined therapy of HCV infection using SOF and DCV with or without ribavirin (RBV) is associated with a sustained virologic response (SVR) rate of >90% [5].

About 25%of HCC individuals are associated with long term HCV infection globally. HCC is the 9th common malignant tumor in females and 5th common cancer in males. HCC is the 2ndmost lethal cancer globally [6].

Nitric oxide (NO) is a small free radical signaling molecule which is implicated in hepatic physiology and patho-physiology. It participates in several physiological pathways in almost every tissue and organ. It activates a vitally wide spectrum of functions, including modulating blood pressure, memory formation and neurotransmission, regulation of tumoricidal and bactericidal actions of macrophages, and hepatic regeneration [7]. The inducible NOS (iNOS) are one of the most important mediators, in pathological events as gene mutation and DNA destruction in the setting of HCV infection, which can lead to inflammation, fibrosis and HCC. HCV core protein, Nonstructural protein 3 (NS3) is responsible for induction of fibrosis in human B-lymphocytes of HepG2 cells causing DNA destruction. This stimulates DNA mutation leading to HCV pathogenesis and oncogenesis [8].

3. Methods

3.1. Study Design and Setting

This prospective study was carried out on randomized 72Egyptian patients presenting to the Hepatology Outpatient Clinic, Internal Medicine Department, and Zagazig University Hospitals suffering from chronic HCV infection. All participants were treated with DAAs agents for 12 weeks; all 72 patients received DAAs in the form of SOF 400mg per day and DCV 60 mg per day with or without ribavirin 400 mg twice daily, for 12 weeks. All participants were followed for 6 months after end of HCV therapy, for development of hepatic focal lesions.

3.2. Study Population and Recruitment

All patients were grouped into two main groups as follows: group (I) including 32 chronic hepatitis C (CHC) patients without cirrhosis group (II) including 40 HCV patients with cirrhosis, who were then subdivided into 2 sub-groups; IIA comprising 26 early cirrhotic patients with Child-Pugh class (CPC) A [9] and IIB comprising 14 patients with advanced liver cirrhosis of CPC B.

Exclusion criteria included pregnancy, age below 18 years, CPC C liver cirrhosis, previously diagnosed HCC and end stage renal disease. Cardiac patients who receive nitrates were also excluded.

Clinical data collection and follow-up

All patients were classified according to CP score [9] by clinical, laboratory and radiological methods.

1. Complete history taking, and full clinical examination were done for all grouped patients.
2. Laboratory investigations: were done for all patients before and after receiving DAAs agents as follows:

A. Routine laboratory investigations:

a. Complete blood picture (CBC)

b. Liver function tests including serum albumin, transaminases and total bilirubin

c. International normalized ratio (INR)

d. Alpha- fetoprotein (AFP) level

e. Hepatitis B and C virus markers

f. HCV PCR by real time PCR

B. Specific laboratory investigations

Determination of (NO) by Enzyme-linked Immunosorbent Assay (ELISA) kit provided by Bioassay Technology Laboratory Company.

3. The imaging scans were done for all patients in the form of: pelvi-abdominal ultrasonography (to detect focal lesion development), fibro scan (to detect cirrhosis by F4 reading), triphasic computerized tomography after treatment only to confirm the presence of HCC.

4. Statistical Analysis

By using SPSS 20.0 for windows all data were analyzed (SPSS Inc., Chicago, Illinois, USA). Continuous variables were expressed as mean ± SD, while the categorical variables were expressed as numbers (and percentages). The appropriate testes were used for example: Chi-square ($\chi 2$) test, Mann-Whitney U (MW) test, independent student t (t) test. $P<0.05$ was considered statistically significant. The specificity, the sensitivity and area under the curve (AUC) of serum markers were determined by receiver operating characteristic (ROC) statistics.

5. Results

Among 72 patients with chronic HCV infection in the final analyses, 28 (87.5%) males and 4 (12.5%) females were included in group I, and 22 (84.6%) males and 4 (15.4%) females in group IIA. 12 (85.7%) males and 2 (14.3%) females in group IIB, without a significant

statistical difference (p = 0.95). The mean age in group I was53.50 ± 8.81, 52.23 ± 8.15 in group IIA and 50.29 ± 5.01 in group IIB without a significant statistical difference (p = 0.45)

Comparing serum levels of AFP between three studied groups before and after treatment.

In non-cirrhotic group I AFP baseline range before treatment was 1.08 - 15.7 ng/ml (median 4.11), while in cirrhotic group IIA it was 1.79 - 37.4 ng/ml (median 8.25) and in cirrhotic group IIB it was 2.5 - 23ng/ml (median 10.3) (P=0.01). In non-cirrhotic group I AFP baseline range after treatment was 2.01 - 18.09 ng/ml (median 11.22), while in cirrhotic group IIA it was 4.2 – 3241 ng/ml (median 171.3) and in cirrhotic group IIB it was 6 – 3267 ng/ml (median 342.4) (P=0.008) (Table 1).

Table 1: AFP among the three studied subgroups before and after treatment

		Group I (n=32)	Group IIA (n=26)	Group IIB (n=14)	test	P	LSD
Pre	AFP(ng/mL) Mean ± SD Median (Range)	5.89 ± 4.66 4.11 (1.08-15.7)	14.59 ± 11.87 8.25 (1.79-37.4)	10.37 ± 8.25 10.3 (2.5 – 23)	K 8.67	0.01 *	0.01*¹ 0.008**² 0.25 NS³
Post	AFP(ng/mL) Mean ± SD Median (Range)	10.45 ± 5.28 11.22(2.01-18.09)	693.11 ±1271.9 171.3(4.2-3241)	769.83±1285.7 342.4 (6-3267)	K 8.99	0.008 **	<0.001**¹ <0.001**² 0.81 NS³
P!		0.12 NS	<0.001**	<0.001**			

SD: Stander deviation
K: Kruskal Wallis test
NS: Non significant (P>0.05) *: Significant (P<0.05) **: Highly significant (P<0.05)
LSD: P1: No cirrhosis versus Child A P2: No cirrhosis versus Child B P3: Child A versus Child B
AFP: Alpha feto-protein

5.1. New Development of Focal Lesions (HCC) After Treatment among the Three Studied Groups

Focal lesions have been diagnosed after treatment in 10 cirrhotic patients (13.89% of all participants) and were all proved to be HCC. Out of these 10 HCC patients, six patients belonged to group IIA (6/26, 23.1%), four patients belonged to group IIB (4/14, 28.6%), while no cases were diagnosed in non-cirrhotic group I (P=0.009) (Table 2, Figure 1).

5.2. Serum Levels of Sno between Three Studied Groups before and After Treatment

Before treatment, sNO baseline in non-cirrhotic group I was 2.06-81.14µmol/l (median 7.42). In the same group sNO baseline after treatment was 1.91-81.37µmol/l (median 9.97) (P=0.13). In group IIA sNO baseline before treatment was 2.29-135.91µmol/l (median 25.3), while after treatment sNO baseline was 3.62-729.66µmol/l (median 117.3) (P<0.001). In group IIB sNO baseline before treatment was 4.5-146.78µmol/l (median 45.6), while sNO baseline after treatment in the same groups was 13.52-823.01µmol/l (median 154.32) (P<0.001). Generally, there were statistically significant differences in sNO level between the different groups both before treatment (P=0.04) and after treatment (P<0.001), being higher in patients with more advanced disease (Table 3).

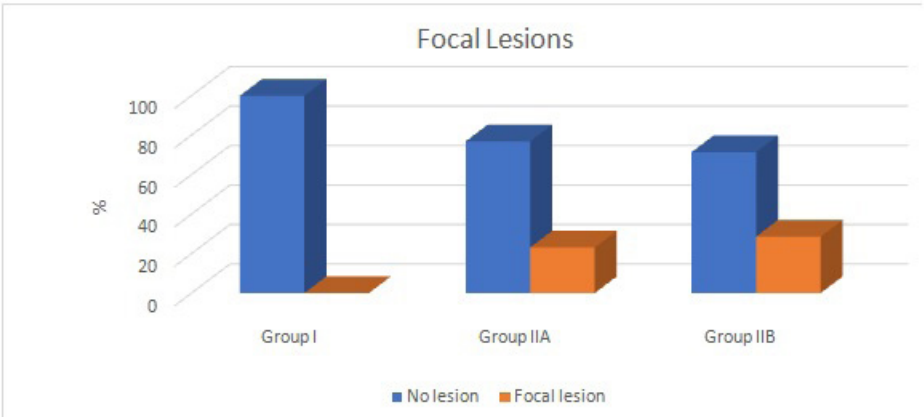


Figure 1: Frequency of focal lesions among the three studied groups.

Table 2: New development of focal lesions (HCC) after treatment among the three studied groups

Variable	Group I (n=32)		Group IIA (n=26)		Group IIB (n=14)		χ2	P
	No	%	No	%	No	%		
No focal lesions	32	100	20	76.9	10	71.4	9.52	0.009 **
Focal lesions	0	0	6	23.1	4	28.6		
χ2: Chi square test **: Highly significant (P<0.01)								

Table 3: sNO among the two studied groups pre & post treatment

Variable		Group I (n=32)	Group IIA (n=26)	Group IIB (n=14)	K	P	LSD
Pre	sNO (µmol/l) Mean ± SD Median Range	 18.06 ± 21.84 7.42 2.06 – 81.14	 30.84 ± 42.79 25.3 2.29 – 135.91	 53.75 ± 60.80 45.6 4.5 – 146.78	2.97	0.04*	0.01*[1] 0.006**[2] 0.31 NS[3]
Post	sNO(µmol/l) Mean ± SD Median Range	 19.77 ± 22.03 9.97 1.91 – 81.37	 164.30 ± 285.74 117.3 3.62 – 729.66	 265.72±364.35 154.32 13.52 – 823.01	16.2	<0.001 **	<0.001**[1] <0.001**[2] 0.03* [3]
SD: Stander deviation K: Kruskal Wallis test NS: Non significant (P>0.05) *: Significant (P<0.05) **: Highly Significant (P<0.05) LSD: P1:No cirrhosis versus Child A P2: No cirrhosis versus Child B P3: Child A versus Child B sNO: Serum Nitric Oxide							

5.3. Sno & AFP among the Cases Who Had HCC and Cases Who Hadn't Pre & Post Treatment

Serum levels of sNO and AFP both pre- and post- treatment in patients with HCC (n=10) were compared to those without (n=62). sNO level in non-HCC group before treatment was 2.06-81.14µmol/l (median=7.46) while sNO level in the same group after treatment was 1.91-81.37µmol/l (median=11.75) (P=0.07). In HCC group, sNO baseline before treatment was 11.01-146.78µmol/l (median=135.91) while sNO baseline after treatment was 641.11-823.01µmol/l (median=729.66) (P<0.001). Also, there were statistically significant differences in sNO levels between the two different groups, both before and after treatment (P<0.001). The AFP level in non-HCC group before treatment was 1.08-37.4ng/ml (median=4.7) and it was 2.01-273.1ng/ml (median=11.22) after treatment (P=0.06). In HCC group, AFP baseline before treatment was 7.20-32.2ng/ml (median=23) and after treatment it was 7.23-3267ng/ml (median=2961) (P<0.001). in addition, there were statistically significant differences between the two different groups as regard AFP levels both before and after treatment (P<0.001) (Table 4).

5.4. Validity of Sno & AFP in Prediction of HCC among the Studied Cases

The sensitivity of pre-treatment sNO at cut off ≥71.98 µmol/l in HCC prediction was 80%, the specificity was 96.8% and the accuracy was 94.4% with positive predictive value of 80%, negative predictive value of 96%, and area under the curve of 0.92 (P<0.001). In comparison with sNO, the sensitivity of pre-treatment AFP at cut off ≥12.95ng/ml was 80%, the specificity was 77.4%, the accuracy was 77.8 % and the area under the curve was 0.86 (0.001). Also, the sensitivity of post-treatment sNO at cut off ≥361.24 µmol/l was 100%, the specificity was 100% and the accuracy was 100% with positive predictive value of 100%, negative predictive value of 100% and the area under the curve of 1 (P<0.001). In comparison with sNO, the sensitivity of post-treatment AFP at cut off ≥134.4 ng/ml was 70%, the specificity was 95.2%, the accuracy was 91.7% and the area under the curve was 0.91 (Table 5, Figure 1).

Table 4: sNO & AFP among the cases who had HCC and cases who hadn't pre & post treatment

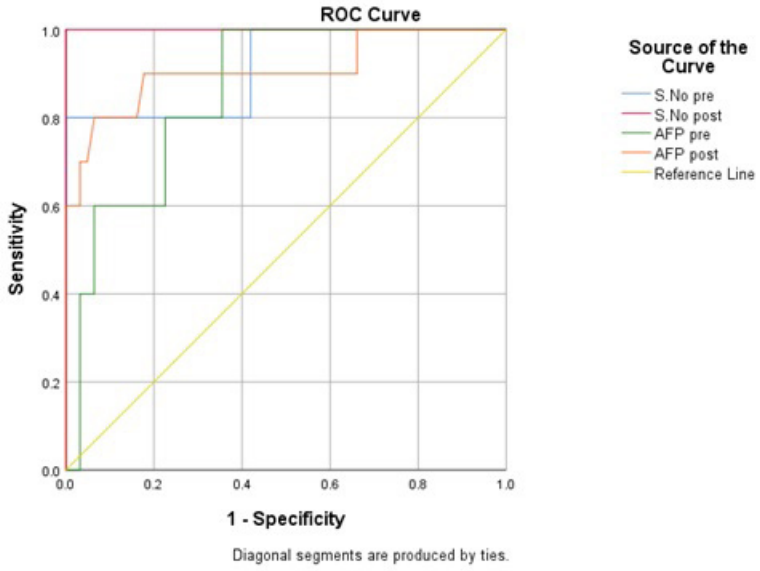
Variable		No lesions (n=62)	Focal lesions (n=10)	MW	p
Pre	sNO(µmol/l) Mean ± SD Median Range	16.89 ± 19.77 7.46 2.06 – 81.14	108.49 ± 52.69 135.91 11.01 – 146.78	4.20	<0.001 **
Post	sNO(µmol/l) Mean ± SD Median Range	19.44 ± 18.82 11.75 1.91 – 81.37	729.34 ± 77.89 729.66 641.11 – 823.01	5.05	<0.001 **
P!		0.07 NS	<0.001**		
Pre	AFP(ng/mL) Mean ± SD Median Range	8.24 ± 8.24 4.7 1.08 – 37.4	20.18 ± 9.44 23 7.20 – 32.2	3.62	<0.001 **
Post	AFP(ng/mL) Mean ± SD Median Range	23.49 ± 54.99 11.22 2.01 – 273.1	1924.12 ± 1596.27 2961 7.23 – 3267	4.12	<0.001 **
P!		0.06 NS	<0.001**		

SD: Stander deviation
MW: Mann Whitney test
NS: non-significant (P>0.05) *: Significant (P<0.05) **: Highly Significant (P<0.05)
AFP: Alpha feto-protein
sNO: Serum Nitric Oxide

Table 5: Validity of sNo & AFP in prediction of HCC among the studied cases

Variable	Time	Cutoff Value	AUC	95% CI	Sensitivity	Specificity	+PV	-PV	Accuracy	p-value
sNO	Pre	≥71.98	0.92	0.81-1	80	96.8	80	97	94.4	<0.001**
	Post	≥361.24	1	1-1	100	100	100	100	100	<0.001**
AFP	Pre	≥12.95	0.86	0.76-0.96	80	77.4	36.4	96	77.8	<0.001**
	Post	≥134.4	0.91	0.78-1	70	95.2	70	95	91.7	<0.001**

AUC: area under the curve
CI: confidence interval.
AFP: Alpha feto-protein
sNO: Serum Nitric Oxide



ROC: receiver operating characteristic

Figure 2: Validity of sNO& AFP in prediction of HCC among the studied cases

6. Discussion

Chronic HCV infection is the most common cause of HCC in Egypt. The prevalence of CHC complicated with HCC in Egypt is about 22% [10]. We had conducted this prospective study on 72 CHC patients, aiming at evaluating the predictive value of sNO in HCC development after receiving DAAs therapy.

In our study we had examined all our patients by fibro scan, and abdominal ultrasonography and we found that 40 patients (55.6%) had liver cirrhosis, 26 (36.1%) of CHC A and 14 (19.5%) of CHC B. None of participants in our study harbored any detectable hepatic focal lesions prior to DAAs therapy. In this study, 10 patients (14%) had developed HCC after DAAs therapy that was confirmed by abdominal triphasic CT. Our study shows that no focal lesions were discovered in non-cirrhotic group, while all patients who developed HCC belonged to cirrhotic groups.

The world data are in an extensive debate between negation and confirmation about the role of DAAs in HCC development. But first we must differentiate between the conception of HCC recurrence and HCC occurrence, HCC occurrence is defined as new discovery of focal lesion in hepatic patient without any past medical history of hepatic malignancy (HCC de novo), while HCC recurrence is defined as reappearance of focal lesions in hepatic patients with previous past history of eradicated HCC.

The first spark began by Conti et al [11] when they published a report at 2016 and described an HCC occurrence rate of 3.2% at 24 weeks follow up after receiving DAAs regimen among 344 cirrhotic patients. Also, Reig et al [12] monitored 58 patients with previous HCC who received DAAs for 12 weeks and reported a 27.6% recurrence rate of HCC. Similarly, Nakao et al [13] informed that in 242 cirrhotic patients treated with DAAs regimen, HCC recurrence rate was 1.7% after 12 months and 7% after 24 months follow up. At the end, Hassany et al [14] reported that HCC recurrence was detected in 42% of 62 patients who received DAAs regimen after eliminating HCC successfully, 80% of them developed recurrence within 24 weeks of treatment onset.

On the opposite side large studies had another opinion, Kanwal et al [15] followed up 22500 CHC patients and Iaonnou et al [16] examined 62354 patients after receiving DAAs and achieving a sustained virologic response (SVR) and they did not find any significant increase in HCC incidence rate than that reported in CHC patients not treated with DAAs.

In the current study, the presence of baseline cirrhosis was a significant risk factor for development of HCC (25% vs. 0%, P<0.001). This conception was confirmed by Romano et al [17] who studied 3917 CHC patients with DAAs therapy and informed that HCC occurrence rate in cirrhotic patients was 3.61%, while it was 0.46% in non-cirrhotic.

It seems that following CHC patient with DAAs, it is very important to find the reliable markers that could predict the HCC development. The results in our study informed that there was a statistically high significant difference in AFP levels before and after treatment between the different groups (P=0.01, P=0.008, respectively) as AFP level is much higher in cirrhotic groups. Also, there was highly statistically significant differences in AFP levels before and after treatment between patients with HCC and those without (P<0.001) as AFP level was higher in presence of HCC. These results were correlated with published data, as Gambarian et al [18] concluded that AFP as a single test is fair enough for detection of HCC in cirrhotic individuals, also in 2002 Nguyen et al informed [19] that AFP level more than 200 ng/ml is diagnostic for HCC individuals with hepatic masses and HCV cirrhosis. On the other hand, the American Association for the study of Liver Diseases (AASLD) no longer recommends AFP as a diagnostic marker for HCC because of low sensitivity and specificity [20]. Also, The Barcelona Clinic Liver Cancer (BCLC) did not include AFP assessment in its classification system as a prognostic indictor for HCC at 2009 [21]. This makes AFP no longer satisfactory for HCC diagnosis and prognosis with the rise of the term of (AFP-negative HCC) nowadays, and so the need of novel biomarker for diagnosis of HCC is mandatory.

NO is a small free radical, lipophilic gas with different biological effects in cell injury and carcinogenesis [22]. Its production is mediated mainly by iNOS which plays an important role in angiogenesis and metastasis of tumor cells [23].

Interestingly, our study displayed that CHC cirrhotic patients who developed HCC showed significantly higher levels of NO post treatment than CHC patients without cirrhosis who did not developed HCC. Similar to our results, Zhang et al [24] showed that NO level increases in individuals with HCC due to over expression of eNOS and iNOS in tumor tissues. Also, Moussa et al [25] reported higher levels in plasma nitrites/nitrates in CHC patients with HCC. But Zhou et al [26] assessed NO levels in tumor tissues and non-cancerous liver tissue in individuals with HCC and reported that the NO levels were significantly higher in the non-malignant tissue than those in the malignant tissue. This difference is attributed to sampling difference between serum and cancerous tissue, and to the fact that the half-life of endogenous NO is extremely short.

Our study revealed that CHC cirrhotic patients (Child class B) with HCC development showed increase in sNO level more than CHC patients (Child class A) and more than CHC patients without cirrhosis, that was significant regarding both pretreatment levels (P0.04) and post treatment levels (P<0.001). These data informed about the significant value of sNO in the diagnosis and prediction of HCC, especially regarding its post treatment level. At a cut-off ≥ 361.24 μmol/l, post treatment level of sNO had a sensitivity of 100%, specificity of 100%, positive predictive value of 100% and negative predictive value of 100% with an accuracy of 100% for HCC detection (P<0.001).

On the other hand, the diagnostic performance of post treatment level of AFP in HCC patients (at a cut-off ≥ 134 ng/ml) in our results was less significant (sensitivity, specificity, positive predictive value, and negative predictive value were 70%, 95.5%, 70%, and 95.2%, respectively).

This was in agreement with another Egyptian study by Eissa et al [27] that was conducted on CHC patients to find out the predictive value of Sno for diagnosis of HCC without relation to DAAs therapy. Eissa et al [27] reported an acceptable prognostic role of sNO in combination with AFP in the diagnosis of HCC. In that regard, the sensitivity and specificity of sNO were 74% and 88.98%, respectively, and the sensitivity and specificity of AFP were 52% and 100%, respectively (with a sensitivity of 82% for the combined measurement of sNO and AFP).

7. Conclusion

The rate of HCC development in CHC patients is more among cirrhotic patients especially with advanced disease. sNO level increases significantly after DAAs therapy, and this increase is significantly associated with the development of HCC. The significance of sNO for HCC prediction is much higher than that of AFP.

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