



Serum VEGF as a Marker of progression of Hepatitis C Virus Induced liver Disease in Egyptian Patients

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Abstract:

Background: Hepatitis C virus (HCV) results in inflammatory liver damage. Angiogenesis has been reported to play an important role in the liver damage. Vascular endothelial growth factor (VEGF) is an important proangiogenic factor which induces endothelial cell proliferation, migration and survival.

Aim of the work: To evaluate VEGF as a marker of progression of HCV induced liver disease in Egyptian patients.

Methods: This study was conducted on 80 patients and 20 controls (Group5). The Patients were classified into 4 Groups: 20 with chronic hepatitis C infection with no evidence of cirrhosis by fibroscan (Group 1), 20 with Child A (Group 2), 20 with Child B (Group 3) and 20 with Child C liver cirrhosis (Group 4). Severity of liver disease was evaluated by the child Pugh score. Liver cirrhosis was diagnosed by ultrasound. Serum levels of VEGF were determined by enzyme linked immunosorbent assay (ELISA). HCV RNA quantification was performed by quantitative real time PCR.

Results: There was significant elevation of VEGF in HCV infected patients when compared to controls with p value < 0.001 . There was statistically significant difference in median VEGF among the studied groups with p value < 0.001 (median of VEGF in Groups 1, 2, 3, 4 and 5 was 544.4, 634.6, 1003, 1410 and 177 pg/ml, respectively). Significant correlation was noted between VEGF and serum albumin and prothrombin time.

Conclusion: serum VEGF can serve as a marker of progression of HCV induced liver disease. VEGF also correlates with hepatic synthetic function as reflected by serum albumin and prothrombin time.

Keywords: Chronic hepatitis C, liver cirrhosis, angiogenesis, VEGF

INTRODUCTION

Hepatitis C virus (HCV) infection is an important public health problem. In more than 80% of infected person, HCV infection can induce persistent hepatic injury which leads to disease progression from periportal inflammation to chronic hepatitis with bridging fibrosis, to frank cirrhosis and hepatocellular carcinoma (HCC).⁽¹⁾

Angiogenesis has been reported to play an important pathogenic role in liver damage during HCV infection⁽²⁾. The increased hepatic angiogenesis in chronic HCV could provide the molecular basis for liver carcinogenesis and contribute to the increased risk of HCC in patients with cirrhosis due to HCV⁽³⁾. Angiogenesis plays a major role in chronic inflammation. Accumulation of inflammatory infiltrate and development of fibrosis increase resistance of the tissue to blood flow and delivery of oxygen⁽⁴⁾. Under these circumstances angiogenesis switch occurs leading to up regulation of proangiogenic factors which are responsible for vascular remodeling and new vessel formation⁽⁵⁾.

Angiogenic growth factors are proteins that circulate in the blood stream. They are stored in platelets and inflammatory cells, and are sequestered within the extracellular matrix⁽⁶⁾. Vascular endothelial growth factor (VEGF) is a known marker of angiogenesis⁽⁷⁾. The serum levels of VEGF might be a useful predictor of the presence of HCC in patients with HCV-related liver cirrhosis⁽⁸⁾.

Determination of angiogenic factors and their soluble receptors in the sera of HCV-infected patients may represent an important tool for the follow-up of liver disease patients⁽⁹⁾⁽¹⁰⁾.

Clinical follow-up of liver disease often involves using invasive techniques such as biopsies, procedures that are not without risks. Therefore it is important to identify serum markers that may provide information

about the extent of disease progression without the need for biopsies⁽¹¹⁾⁽¹²⁾.

The aim of this work is to evaluate vascular endothelial growth factor (VEGF) as a marker of progression of chronic hepatitis C induced liver disease in Egyptian patients.

PATIENTS AND METHODS:

Patients:

The present study was carried on 80 patients with chronic hepatitis C (CHC). They included 48 males and 32 females recruited from internal medicine department and hepatology outpatients clinics of Ain-Shams University Hospitals. Their ages ranged from 20-56 years. 20 healthy controls matching the patients in their age and sex were involved in our study. They were randomly selected from blood donors from the blood bank, and from the subjects attending the outpatient clinics for pre-employment examination. Detailed medical history and full clinical examination were done to the control group to exclude any diseases. Laboratory investigations and abdominal ultrasound were done for the control group to exclude any liver diseases.

Exclusion criteria: none of the studied patients had another virus co infection, Schistosomiasis, history of significant alcohol consumption, HCC, any other malignancy, autoimmune disorders, other chronic diseases, receiving Interferon therapy or antiviral treatment.

The subjects were categorized into 5 groups: Group 1: included 20 patients with chronic hepatitis C viral infection attending the Hepatology outpatient clinic for assessment before receiving antiviral therapy with no evidence of cirrhosis by fibroscan (F0, 1 and 2). Group 2: included 20 patients with Child A liver cirrhosis. Group 3: included 20 patients with Child B liver cirrhosis. Group 4: included 20 patients with Child C liver cirrhosis. Liver cirrhosis was diagnosed by

clinical, laboratory and sonographic features. Group 5: included 20 healthy subjects as a control group with no clinical, laboratory or sonographic features of liver disease. An informed consent was obtained from all participants and the study was approved by the Ain Shams Medical Ethics Committee.

Methods:

All patients and healthy controls were subjected to the following:

- Complete medical history and clinical examination.
- Laboratory assessment : venous blood (8 ml) was withdrawn aseptically into a sterile disposable syringe from each patient and control, where 2 ml was placed in EDTA vacutainer for performing complete blood count (CBC), 2 ml of blood was collected on citrate for PT and INR determination, and 4 ml was collected in 2 plain vacutainers to be clotted and centrifuged (1500 g for 15 min) for HCV RNA copy number analysis and measurement of biochemical markers including AST, ALT, bilirubin, albumin, creatinine, BUN and VEGF. The laboratory work was conducted at Clinical Pathology Department.
- CBC was done using Coulter counter (T660) (Beckman. Coulter, California, USA).
- AST, ALT, bilirubin, albumin, creatinine and BUN were measured on Synchron CX9 autoanalyzer (Beckman Instruments Inc.; Scientific Instruments Division, Fullerton, CA 92634, 3100).
- PT and INR were determined on Diagnostica Stago (Asnieres, France)
- Additionally, serum HCV RNA level was quantified for CHC patients: HCV RNA was extracted from serum using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany),

then, the extract was added to Qiagen One Step RT-PCR Master Mix and real-time-Reverse Transcription Polymerase Chain Reaction (RT-PCR) was performed by Stratagene Mx3000P device (Corbett Research, Australia).

- Detection of Serum VEGF by Enzyme Linked Immunosorbent Assay (ELISA): Serum VEGF concentration (pg/ml) was evaluated using VEGF sandwich ELISA kit (BioSource International, Inc., USA), according to the instructions of the manufacturer. Briefly, sera or recombinant human VEGF, as standards, were added to plates coated with a polyclonal antibody against VEGF and incubated at room temperature for two hours. Optical density was determined using a Stat Fax 2100 microplate reader (Awareness Technology INC, USA) at 450nm with the lower end of sensitivity of the assay was 5 pg/ml and the upper limit was 1500 pg/ml. Samples with readings greater than the upper limit were diluted until their absorbance readings fall within the linear range of the assay.
- Abdominal Ultrasonography: Equipment used: Hitachi, EUB-5500. Measurements were performed after overnight fasting and the patient in supine position with emphasis on: Liver size (measuring the span of the right lobe in the mid clavicular line on oblique view and classified as shrunken <11 cm, average= 11-15 cm or enlarged >15 cm), liver echogenicity (bright or coarse echo pattern), splenic bi-polar diameter (longest axis) in cm (measured in a coronal plane, from the upper to the lower pole of the spleen which normally measures up to 12-13 cm). Portal vein diameter (mm) and patency were also determined. The normal PV is up to 13mm in

diameter. It was measured from the inner to the outer wall during suspended respiration.

Criteria suggestive of chronic liver disease and cirrhosis are: increased liver echogenicity, irregular liver margins, attenuation of intrahepatic portal and hepatic veins, relative enlargement of caudate lobe and atrophy of right lobe (ratio of caudate/ right lobe in cirrhosis >0.65), presence of periportal thickening and presence of ascites.

- Transient Elastography (Fibroscan): was performed for Group 1 patients using fibroscan 502 equipment.
- According to the degree of decompensation in liver function, the study patients were categorized into Child's class A, B, and C according to Child pugh's score⁽¹³⁾.

Statistical methods: Statistical analysis was performed using IBM SPSS statistics (V. 20.0, IBM Corp., USA, 2011). Data were expressed as Mean \pm SD for quantitative parametric measures in addition to median percentiles for quantitative non-parametric measures and both number and percentage for categorized data. Wilcoxon Rank Sum test was used for comparison between two independent groups for non-parametric data. Comparison between more than 2 patient groups for non-parametric data was done using Kruskal Wallis test. Comparison between more than 2 patient groups for parametric data was done using Analysis of Variance (ANOVA). The multiple comparison using TUKEY'S Test was also used to investigate the possible statistical significance between each 2 groups. Ranked Spearman correlation test was used to study the possible association between each of the two variables among each group for non-

parametric data. The best cut off for differentiation between different stages of liver cirrhosis was determined from the ROC curves. A p value of less than 0.05 was considered significant.

RESULTS

Eighty CHC patients and twenty healthy control persons were included in this study. The median of the VEGF level showed a high statistically significant elevation in CHC patients with or without cirrhosis when compared to healthy control with *p value* < 0.001 .

Serum VEGF level was compared in the 5 groups showing a statistically highly significant difference between patients with liver cirrhosis of different classes: Child A (Group 2), Child B (Group 3) and Child C (Group 4) with median VEGF (634.6, 1003.1, 1410 respectively) and *p value* <0.001 , being higher in Child C (Group 4) than Child A (Group 2) and Child B (Group 3), and higher in Child B (Group 3) than Child A (Group 2). Furthermore, there was a statistically highly significant difference as regards serum VEGF between patients without cirrhosis (Group 1) and patients of Child B liver cirrhosis (Group 3) with *p value* <0.01 , being higher in (Group 3) patients. In addition there is a statistically significant difference between patients without cirrhosis (Group 1) and patients of Child C (Group 4) as regards VEGF with *p value* < 0.05 , being higher in (Group 4) patients. However, there was no statistically significant difference between patients without cirrhosis (Group 1) and patients of Child A (Group 2) as regards serum VEGF (544.4 vs 634.6) with *p value* > 0.05 (Table 1, Figure1).

There was a highly significant difference among the five groups as regards platelet count, serum albumin and hemoglobin, being lowest in patients of Child B (Group 3) and Child C (Group 4) compared to other groups with p value < 0.001 . Also, there is a highly significant difference between the five groups of the study regarding serum AST, ALT, total and direct bilirubin, being highest in patients of Child B (Group 3) and Child C (Group 4) with p value < 0.001 . On the other hand, there is no statistically significant difference between the studied groups as regards WBCs with p value > 0.05 . Moreover, there was no statistically significant difference between the four studied groups regarding HCV RNA copy with p value > 0.05 (Table 1). Our results showed highly significant correlation between VEGF and serum albumin ($r=-0.613$), and also between VEGF and PT and INR ($r=-0.570$) ($r=0.539$) respectively. In addition, a significant correlation was found between VEGF and AST, WBCs, hemoglobin, total and direct bilirubin ($r=0.438$) ($r=0.348$), ($r=-0.332$), ($r=0.575$), ($r=0.492$) respectively. On the other hand, no statistically significant correlation was found between serum VEGF and ALT, platelet count, creatinine, BUN, Na, K, PCR and age (Table 2).

Receiver Operating Characteristic (ROC) Curve Analysis. was used to establish the specificity and sensitivity of using serum VEGF as a marker of progression of HCV induced liver disease. The cut-off value of serum VEGF level that could distinguish chronic patients without cirrhosis from

cirrhotic patients was >641.1 pg/mL, with sensitivity and specificity of 71.67% and 80%, respectively, positive predictive value 91.5%, with AUC: 0.833 (Figure 2A). Within the cirrhotic group, the cut-off value of serum VEGF level that could distinguish patients with child C from patients with child B was >1179 pg/ml, with sensitivity and specificity of 85% and 71.3%, respectively, positive predictive value 73.93% , with AUC : 0.78 (Figure 2B). Additionally, the cut-off value of serum VEGF level that could distinguish patients with child B from patients with child A was >683.5 pg/ml with sensitivity and specificity of 80.9% and 84.3% respectively, positive predictive value 85%, with AUC: 0.898 (Figure 2C). However, VEGF failed to differentiate between patients without cirrhosis and patients with Child A ($p>0.05$) with sensitivity and specificity of 50% and 80.3%, respectively, and AUC: 0.525 (Figure 2D).

Table (1): Laboratory data for the 5 studied groups:

Parameters	No Cirrhosis (Group1) (N=20)	Child A (Group2) (N=20)	Child B (Group3) (N=20)	Child C (Group 4) (N=20)	Controls (Group5) (N=20)	P
Hb (gm/dL)•	14±1.7	13.2±1.7	11.6±2.7	10.8±1.6	15.1±1.1	<0.001
WBCs(10 ⁹ /L)•	7.4±1.5	6.2±2.1	6.6±1.6	7.8±3.5	8.0±0.9	0.632
Platelet count (10 ⁹ /L)•	243.5±71.6	214.8±67.0	191.1±97.8	149.1±37.1	343.1±119.3	<0.001
ALT (IU/L)•	42.3±17.1	39.8±12.9	57.5±32.7	52.3±28.3	15.4±3.9	<0.001
AST (IU/L)•	47.0±23.4	48.0±27.3	78.6±34.9	99.9±59.4	20.9±6.0	<0.001
Albumin (gm/dL)•	4.2±0.3	3.9±0.5	2.7±0.3	2.2±0.5	4.9±0.1	<0.001
Total bilirubin (mg/dL)•	1.0±0.7	0.7±0.3	1.7±0.6	3.0±1.7	0.8±0.2	<0.001
PT(sec)•	12.5±0.8	13.3±1.5	15.5±2.1	20.1±2.7	12.0±0.8	<0.001
HCV-PCR (RNA X10 ³ IU/mL)*	666(360 - 1120)	630 (366 – 1178)	877 (740 – 1360)	1055 (970 – 1460)	-	0.08
VEGF (pg/ml)*	544.4 (417.65 – 641.1)	634.6 (316.1 – 683.5)	1003.1 (844.1 – 1619)	1410 (1259.5 – 1743)	177 (67.7 – 335)	<0.001 ^a

P>0.05= Non significant; P<0.01= Highly significant.

^aVEGF: Patients without cirrhosis versus patients with Child A, p>0.05; patients with Child A versus patients with Child B , p<0.001; patients with Child B versus patients with Child C, p<0.05; patients without cirrhosis versus controls, p<0.001; patients with Child A versus controls, p<0.001; patients with Child B versus controls, p<0.001; patients with Child C versus controls, p<0.001.

•Data were presented as mean±SD and compared together using ANOVA test.

*Data were presented as Median and IQR and compared together using Kruskal Wallis test.

Table (2): Correlation between VEGF levels and the different parameters:

	VEGF(pg/ml)	
	R	P-value
WBCS (10 ⁹ /L)	0.348	<0.05
Hb (gm/dL)	-0.332	<0.05
Plt (10 ⁹ /L)	-0.061	0.708
PT (sec)	0.570	<0.001
INR	0.539	<0.001
AST (IU/l)	0.438	<0.05
ALT (IU/l)	0.248	0.122
Total bilirubin (mg/dL)	0.575	<0.001
Direct Bilirubin (mg/dl)	0.492	<0.001
Albumin (gm/dL)	-0.618	<0.001
HCV-PCR (RNA X10 ³ IU/mL)	0.078	0.634

P>0.05= Non significant; P<0.05= Significant; P<0.01= Highly significant.

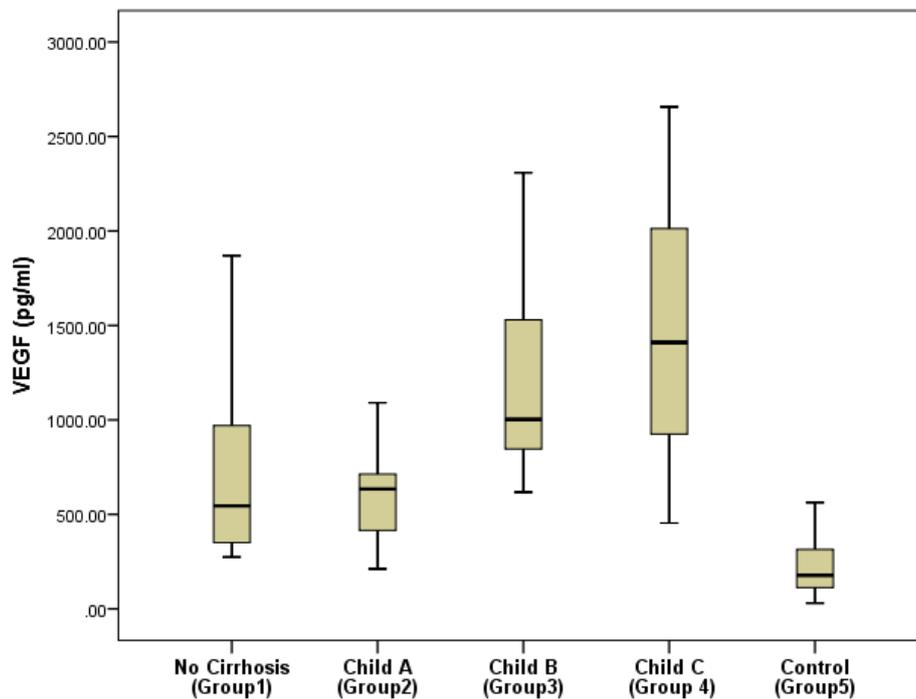
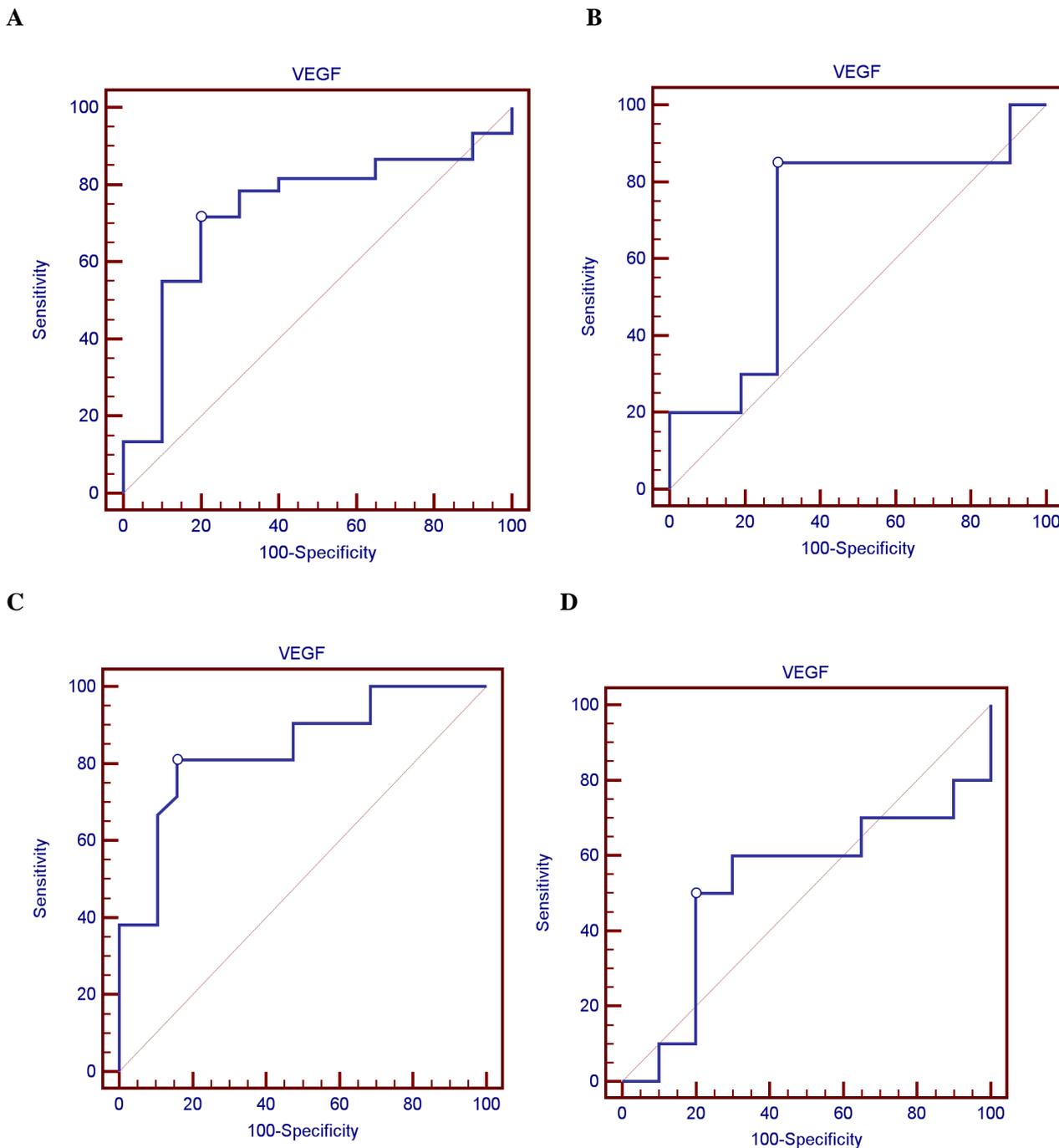


Figure 1: Box-plot chart showing difference between serum VEGF levels among the 5 studied groups.



Figure(2): Roc curves

- A)** For discriminating CHC patients without cirrhosis from cirrhotic patients with AUC 0.833 and best cut off > 641.1 pg/ml.
- B)** For discriminating patients with child C from patients with child B with AUC 0.78 and best cut off > 1179 pg/ml.
- C)** For discriminating patients with child B from patients with child A with AUC 0.898 and best cut off >683 pg/ml.
- D)** For discriminating patients with child A from patients without cirrhosis with AUC 0.525.

DISCUSSION

Vascular endothelial growth factor (VEGF) is one of the most important pro-angiogenic factors which induces endothelial cell proliferation, migration and survival⁽⁷⁾. Among the factors contributing to liver damage during CHC, angiogenesis has been reported to play a significant pathogenic role. Angiogenesis has been suggested to represent a risk factor for HCC in patients with CHC⁽¹⁴⁾. Therefore, we examined the circulating VEGF levels in the sera of chronic HCV-infected patients to assess their significance as non-invasive way to monitor the disease progression.

The results of this study clearly demonstrate a highly significant increase of serum VEGF level in chronically HCV infected patients compared to healthy controls. These findings are in line and supported by results of *Helaly and Abou Shama*⁽¹⁵⁾ and *Janczewska-Kazek et al*⁽¹⁶⁾. Moreover, *Sieghart et al.*,⁽¹⁷⁾ found dramatically and statistically significant elevation in the serum levels of VEGF in HCV infected subjects in comparison with healthy subjects.

Our findings are supported by the results obtained by *Hassan et al.*,⁽¹⁸⁾ who reported increased VEGF immunostaining of liver biopsy specimens from HCV-infected subjects. Indeed they suggested that VEGF production is stimulated by HCV infection through a mechanism including the stabilization of hypoxia inducible factor-1 α (HIF-1 α)⁽¹⁸⁾. Additionally, *Abe et al.*,⁽¹⁹⁾ demonstrated that HCV core protein has the distinct potential to up regulate and sustain HIF-1 α expression under hypoxia, thereby contributing to increased VEGF expression, through the HCV core/NF-kB axis⁽⁷⁾. Moreover, *Abe et al.*,⁽¹⁹⁾ suggested that the distinct angiogenic potential of HCV core protein under hypoxia might help transformed cells survive in the hypoxic environment of the cirrhotic liver by supplying them with oxygen and nutrients. Also, HCV-infected hepatocytes secrete VEGF, which induces a localized depolarization of hepatocytes that

promotes viral transmission between adjacent hepatocytes⁽²⁰⁾.

On the other hand, our results are in disagreement with *Assy et al.*,⁽²¹⁾ and *Genesca et al.*,⁽²²⁾ who found mean VEGF serum levels in cirrhotic patients significantly lower than that of healthy controls explaining their results that this may reflect the decrease in the hepatic regenerative activity and the decreased platelet count found in cirrhotic patients. Also, *Mukoza, et al.*,⁽⁸⁾ found there was no significant difference between the control group and the CHC group as regards VEGF.

The result of the present study revealed significant difference in median VEGF in different stages of liver cirrhosis being highest in Child C and lowest in patients without cirrhosis, however there was no significant difference regarding serum VEGF between Child A patients and those without cirrhosis, this could be attributed to the small sample size.. These results indicate that there is strong correlation between VEGF and the severity of liver disease which may be attributed to the release of VEGF from the damaged hepatocytes. Serum VEGF measurement may therefore be a useful non-invasive and simple clinical test for patient stratification.

This is in concordance with many previous studies which showed positive correlation between serum VEGF and grade of liver cirrhosis supporting the critical role played by angiogenesis in chronic liver disease⁽²³⁾⁽²⁴⁾. Results of the current study agree with the results obtained by *Kishta et al.*,⁽²⁴⁾ who demonstrated in their study an over expression of VEGF mRNA in liver tissue of cirrhotic patients and that was proportional to the stage of cirrhosis . Moreover, *El Shayeb et al.*,⁽²⁵⁾ found that serum VEGF reflects well the degree of hepatic dysfunction and there is a significant positive correlations between VEGF and Child Pugh's score. They also recommended that serial measurement of VEGF might

be of diagnostic and predictive value for occurrence of HCC in patients with chronic HCV induced liver cirrhosis.

In contrary to our results, *Assy et al.*,⁽²¹⁾ found no significant difference in serum VEGF levels among the different Child-Pugh's classes. Also, *Shi et al.*⁽²⁶⁾ reported in their study that expression of VEGF in patients of Child A and Child B was significantly higher than Child C patients and they suggested that this may reflect the status of liver compensation in cirrhotic patient. Moreover, *Brdoskey et al.*⁽²⁷⁾ found that in the early stages of fibrosis, the production of VEGF and the neovascularization increases; whereas in the late stages cirrhotic nodules in hepatitis C patients are characterized by decreased density of micro vasclature and decreased VEGF production.

Highly significant positive correlation between serum VEGF and serum AST was observed in all groups of patients. This was in accordance with the work of *Talaat*⁽²⁾ who found clear correlation between liver enzymes, which are surrogate markers of liver disease, and serum VEGF, supporting the claim that hepatocellular damage leads to marked VEGF release into the blood stream. However, in the current study, there was no significant correlation between ALT and serum VEGF in all groups. *Mukozy et al.*,⁽⁸⁾ found no significant correlation between VEGF levels and the degree of hepatic dysfunction. This could be explained by the fluctuations that occur in liver enzymes during the disease period⁽²⁸⁾. Also it is well known that in liver cirrhosis due to viral hepatitis serum levels of AST is usually higher than ALT with higher AST/ALT ratio⁽²⁹⁾. Absence of any elevation does not rule out significant injury or hepatic fibrosis. Liver enzyme tests do not reveal the true status of hepatic function⁽³⁰⁾.

In the present study, we found a highly significant negative correlation between serum VEGF and serum albumin. This was in accordance with *Yao et al.*⁽³¹⁾

who demonstrated that maintaining the serum albumin at a higher level attenuating endogenous VEGF expression. In contrast, *Assy et al.*⁽²¹⁾ found no significant correlation between serum VEGF and serum albumin and they suggested that serum VEGF didn't reflect hepatic synthetic function.

Principally, serum VEGF is a combination of both VEGF released from platelets and the circulating plasma VEGF. Platelets may normally contain a certain amount of VEGF in their granules. Therefore, serum VEGF level can be influenced by platelet count⁽³²⁾. In the current work, we found no significant correlation between serum VEGF and platelet count. Similarly, *Assy et al.*⁽²¹⁾ observed no significant correlation between platelet count and serum VEGF suggesting that VEGF is not stored in platelets only. Although, *Kim et al.*⁽³³⁾ found a significant correlation between serum VEGF and platelet count in HCC patients, they didn't find a similar correlation in liver cirrhosis patients. They suggested that serum VEGF/platelet count might be used as an indicator of the development of HCC in patients with liver cirrhosis during their follow up. On the other hand, *Gunsilus and Gastl*,⁽³⁴⁾ found a signyficant correlation between peripheral platelet count and absolute value of serum levels of VEGF in their work. Similarly, *Genesca et al.*⁽²²⁾ reported that the circulating VEGF levels were significantly lower in patients with liver cirrhosis than in control group and they attributed this to the low platelet count found in cirrhotic patients.

The use of serum levels of angiogenic factors as prognostic biomarkers presents several advantages. First, the technique is virtually non-invasive. Secondly, the determination procedure is easy: protocolized methods, such as sandwich enzyme-linked immunosorbent assays (ELISAs) of most factors are commercially available⁽³⁵⁾.

CONCLUSION

Circulating VEGF level in patients of CHC can serve as an indicator of the progression of liver disease. It may reflect the hepatic synthetic function in patients with liver cirrhosis due to its correlation with serum albumin and prothrombin time. It may serve as serum marker that may provide information about the extent of disease progression without the need for biopsies. Further studies on larger scale are recommended to confirm our results.

Declaration of Interest

The authors declare that no funding or grant was received for the study. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article

ABBREVIATIONS

ALT: alanine aminotransferase; AST: aspartate aminotransferase;; BUN: blood urea nitrogen; CBC: complete blood count ; CHC: chronic hepatitis C; EDTA: Ethylenediaminetetraacetic acid; ELISA: enzyme-linked immunosorbent assay; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HIF-1 α : hypoxia inducible factor-1 α ; IBM: International Business Machines; INR: international normalized ratio; K: potassium mRNA: Messenger RNA; Na: sodium; NF-kB: nuclear factor -kB; PCR: polymerase chain reaction; PT: prothrombin time; RNA: Ribonucleic acid; SPSS: Statistical Package for the Social Sciences; VEGF: vascular endothelial growth factor; WBCs: white blood cells

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