

Association of Serum Vascular Endothelial Growth Factor (VEGF) Levels with Nonalcoholic Fatty Liver Disease

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Abstract: Background. Nonalcoholic fatty liver disease (NAFLD) is a growing problem due to the epidemic of obesity. Although the pathogenesis of NAFLD is still not fully understood, some studies suggested that angiogenesis might play a role in its progression. Vascular endothelial growth factor (VEGF) is a main proangiogenic factor implicated in angiogenesis. **Aim.** To assess the significance of serum VEGF levels in NAFLD patients. **Methods.** Cross-sectional study of 40 consecutive NAFLD patients and 40 healthy controls who came to Adam Malik General Hospital Medan between May 2018 and July 2018 were included. The NAFLD diagnosis required evidence of hepatic steatosis on ultrasound and exclusion of other causes of liver injury. Serum VEGF levels were determined by an enzyme immunoassay Quantikine Human VEGF-ELISA kit. Univariate analyses, bivariate analyses (T independent test and Mann-Whitney test) with SPSS version 22. Statistical significant if $p < 0,05$. **Results.** From a total of 40 patients, 18 patients (45%) were men with an average age of 44,6 years. Serum VEGF levels were significantly higher in NAFLD patients compared to healthy controls (315,8 pg/mL vs 214,75 pg/mL, $p = 0,002$). Levels of AST, ALT, fasting blood glucose, total cholesterol, triglyceride, HDL cholesterol and LDL cholesterol were significant difference between NAFLD patients and healthy controls ($p < 0,001$). There were no significant difference of serum VEGF levels in NAFLD patients based on age, sex and Body Mass Index (BMI).

Conclusion. Our result showed that serum VEGF levels significant higher in NAFLD patients. This suggest that angiogenesis could play an important role in the pathogenesis of NAFLD.

Keyword. NAFLD, angiogenesis, VEGF

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I. Introduction

The liver is one of the principle regulators of lipid in the body. Nonalcoholic fatty liver disease is a hepatic manifestation of the metabolic syndrome.¹ NAFLD is a growing problem due to the epidemic of obesity.² The definition of NAFLD requires that (a) there is evidence of hepatic steatosis, either by imaging or by histology and (b) there are no causes for secondary hepatic fat accumulation such as significant alcohol consumption, viral hepatitis, drugs, endocrine or hereditary disorder.^{3,4} Steatosis is the presence of lipid within the cytoplasm of hepatocytes greater than 5% of the liver's weight or found in greater than 5% of hepatocytes histologically.⁵ The spectrum of NAFLD is a continuum ranging from simple steatosis to NASH and finally cirrhosis.⁶ The excessive alcohol consumption defined as > 30 gr/day for men and 20 gr/day for women.⁵ The pathogenesis and progression of NAFLD are not entirely clear.⁶ Some studies suggested that angiogenesis play an important role in the progression of NAFLD.^{7,8} Angiogenesis is a critical step in tissue damage, healing and vascular remodeling. Disruption of the liver vascular architecture has been linked to progression to fibrosis and cirrhosis.⁹ Vascular endothelial growth factor is the key factor in the induction of angiogenesis.^{10,11} Liver biopsy is considered to be gold standard for the diagnosis, but it is an invasive procedure.³ Therefore, there is growing scientific interest in imaging studies and serum-based assays that aim to detect the presence of NAFLD. The aim of this study was to evaluate the possible significance of serum VEGF levels in NAFLD patients.

II. Material And Methods

This cross-sectional observational study was carried out on patients of Department of Internal Medicine at Adam Malik General Hospital from May 2018 to July 2018. A total 80 adult subjects (both males and females) of aged ≥ 18 , years were for in this study.

Study Design: Cross-sectional observational study

Study Location: This was a tertiary care teaching hospital based study done in Department of Internal Medicine, at Adam Malik General Hospital.

Study Duration: May 2018 to July 2018.

Sample size: 40 NAFLD patients, 40 healthy controls.

Sample size calculation: The sample size was estimated by unpaired two-samples t-test formula.

We assumed that the confidence interval of 5% and confidence level of 95%. The sample size actually obtained for this study was 10 for each group. We planned to include 40 NAFLD patients, 40 healthy controls.

Subjects & selection method: The study population was drawn from consecutive NAFLD patients who presented to Adam Malik General Hospital from May 2018 to July 2018. Forty healthy controls matched for age and sex were also enrolled. Controls were either subjects who visited the hospital for routine examinations during the same period or hospital staff members. Controls had normal glucose metabolism and liver biochemistry and no evidence hepatic steatosis on abdominal ultrasound.

Inclusion criteria:

1. NAFLD patients. The NAFLD diagnosis required evidence of hepatic steatosis on ultrasound and exclusion of other causes of liver injury.
2. Either sex.
3. Aged \geq 18 years.

Exclusion criteria:

1. Pregnant women
2. Aged $<$ 18 years.
3. Patients with previous history of hepatitis B or hepatitis C.
4. Patients with a history of alcohol consumption $>$ 30 gr/day for man and $>$ 20 gr/day for woman.

Procedure methodology

After written informed consent was obtained, a well-designed questionnaire was used to collect the data of the recruited patients retrospectively. The questionnaire included socio-demographic characteristics such as age, gender, height, weight, concomitant disease and alcohol consumption. Height and weight were measured using standardized method. The body mass index (BMI) was calculated as the weight in kilograms divided by height in meters squared. Laboratory investigations such as complete blood count, liver enzyme alanine aminotransferase (ALT), aspartate aminotransferase (AST), HBsAg, anti-HCV, FBG, total cholesterol, triglyceride, HDL cholesterol and LDL cholesterol levels. All lipid parameters were quantified on samples collected in the fasting state. Blood samples were taken by phlebotomy of venous blood and inserted into vacutainer tube. The blood is centrifuged for 10 minutes to obtain serum. The obtained serum is stored at -80°C until it is used for enzyme-linked immunosorbent assay (ELISA) examination. Serum VEGF levels were determined by an enzyme immunoassay Quantikine Human VEGF-ELISA kit.

Statistical analysis

Data was analyzed using SPSS version 22 (SPSS Inc., Chicago, IL). Statistical analysis was performed using unpaired t-test for comparisons of quantitative variables between groups and confirmed by nonparametric Mann-Whitney test. The level $p < 0.05$ was considered as the cutoff value or significance.

III. Result

Table no 1 presents the general characteristic of subjects with and without NAFLD. There were 80 subjects were enrolled during this study that consist of 40 NAFLD patients and 40 people as healthy controls. The mean age of NAFLD patients was 47,3 years. Gender was 45% males. According to BMI, 80% of NAFLD patients were obese. Forty-five percent of the NAFLD patients had type 2 diabetes mellitus.

Table no 1: Characteristics of NAFLD patients and healthy controls

	NAFLD	
	Yes	No
Age, mean (SD), year	47,3 (13,4)	44,6 (12,7)
Sex, n (%)		
Males	18 (45)	18 (45)
Females	22 (55)	22 (55)
BMI, n (%)		
Obese	32 (80)	15 (37,5)
Normal weight-overweight	8 (20)	25 (62,5)
Comorbidity, n (%)		
Type 2 diabetes mellitus	18 (45)	-
Obesity	5 (12,5)	-

Hypertension	1 (2,5)	-
Others	16 (40)	-

Table no 2 shows metabolic parameters of NAFLD patients and controls. Fasting blood glucose, 142.5 (90-245) mg/dL, 98 (79-137) mg/dL. AST, 35,45±9,76, 26,5± 8,18 U/L. ALT 49,95 ± 16,09, 29,68 ± 6,65 U/L. Total cholesterol, 229,5 (188-294) mg/dL, 173 (142-184) mg/dL, triglyceride 167(100-256) mg/dL, 88 (77-136) mg/dL, HDL cholesterol, 40.5 (25-56) mg/dL, 60 (34-66) mg/dL, LDL cholesterol, 154 (110-223) mg/dL, 125 (90-223) mg/dL, respectively of NAFLD patients and controls. The difference in the values of all parameters in respect of two groups were statistically significant (p<0.05).

Table no 2: Metabolic parameters of NAFLD patients with NAFLD and healthy controls

Laboratorium characteristic	NAFLD		p
	Yes (n=40)	No (n=40)	
Fasting Blood Glucose, median (min-max), mg/dL	142,5 (90-245)	98 (79-137)	<0,001
AST, mean ±SD, U/L	35,45 ±9,76	26,5 (8,18)	<0,001
ALT, mean ±SD, U/L	49,95 ± 16,09	29,68 (6,65)	<0,001
Total cholesterol, median (min-max), mg/dL	229,5(188-294)	173(142-184)	<0,001
Triglyceride, median (min-max), mg/dL	167 (100-256)	88 (77-136)	<0,001
HDL cholesterol, median (min-max), mg/dL	40,5 (25-56)	60 (34-66)	<0,001
LDL cholesterol, median (min-max), mg/dL	154 (110-223)	125(90-223)	<0,001

Table no 3 shows the serum VEGF levels in NAFLD patients and healthy controls. There was significant difference of serum VEGF levels between NAFLD patients and healthy controls (p=0,002). NAFLD patients had higher serum VEGF levels compared to healthy controls (315,8 vs 214,75 pg/mL).

Table no 3 :Characteristic of patient with NAFLD and healthy

	NAFLD patients	Healthy controls	p
Mean, pg/mL	444,39	233,36	0,002
Standard deviation, pg/mL	397,98	95,18	
Median, pg/mL	315,8	214,75	
Min-max, pg/mL	59,9-1934,3	67,9 -435,1	

Table no 4 shows serum VEGF levels of 40 NAFLD patients. We split our NAFLD patients according to their sex, age and BMI. The difference in the serum VEGF levels in respect of the groups were not statistically significant (p>0.05). Serum VEGF levels tended to be higher in females than in males, in patients older than 47 years of age and in obese patients as compared to normal weight and overweight patients.

Table no 4 :Serum VEGF levels in NAFLD patients according to sex, age and BMI

	N	VEGF, median (min-max), pg/mL	P
Sex			
Males	18	301,95 (59,9-695,8)	0,232
Females	22	384,65 (74,5-1934,3)	
Age			
> 47 years	22	351,9 (59,9-1822,4)	0,838
≤ 47 years	18	315,8 (74,5-1934,3)	
BMI			
Obese	32	323,75 (59,9-1934,3)	0,361
Normal weight-overweight	8	271,1 (133,7-645,5)	

IV. Discussion

The rationale of this study was to comprehend the potential relationship between angiogenic cytokines VEGF and NAFLD. Angiogenesis is known to play a role in the pathophysiology of NAFLD. Mean age of subjects with NAFLD was 47,3 years. Papageorgiou *et al* were found mean age NAFLD patient was 46 years meanwhile Demirci *et al* in Turkey found median age patient with NAFLD was 46,6 years.^{12,13} Prevalence of NAFLD increases with age. Prevalence NAFLD is 26% in people aged 40-59 years.¹⁴

In this study, the most common comorbidity in NAFLD patient was type 2 diabetes mellitus (45%). Another comorbidities were dyslipidemia (12,5%) and hypertension (2,5%). Elsheikh *et al* found prevalence comorbidities in NAFLD patients were type 2 diabetes mellitus 54%, dyslipidemia 67%, hypertension 77%.¹⁵ Lonardo *et al* found liver fat content in patients with type 2 diabetes mellitus were 80% higher than controls.¹⁶ This study found that 80% of NAFLD patients were obese. Obesity is an independent risk factor for NAFLD. A study found the prevalence of NAFLD in patients who are severely obese was more than 90%.⁴ There is a linear relationship between NAFLD and metabolic syndrome.¹

Alanin transaminase enzyme and AST enzyme are marker of liver injury. NAFLD is the most common cause of elevated serum levels of liver enzymes on routine examination. In NAFLD, we can find abnormal liver function test with mild to moderate elevation (1,5 to 4 fold) of ALT and AST levels and greater elevation of ALT than AST (AST/ALT: < 1).¹⁷ We found ALT and AST were significant higher in NAFLD patients ($p < 0,001$). This results were same with Sanyal *et al* found that AST and ALT levels were significant higher in NAFLD patients.¹⁸ Papageorgiou *et al* also found AST and ALT levels were significant higher in NAFLD patients ($p < 0,001$).¹²

According to the Adult Treatment Panel (ATP III) guidelines, the lipid profile and FBG are components of metabolic syndrome.¹⁹ This study found levels of total cholesterol, triglycerida, HDL cholesterol and LDL cholesterol were significant higher in NAFLD patients ($p < 0,001$). This results were consistent with Rao *et al*, who found lipid profile levels were significant higher in NAFLD patients.²⁰ Yilmaz *et al* found levels of total cholesterol and LDL cholesterol were significant higher in NAFLD patients but there was no significant difference in HDL cholesterol levels.²¹

Like another chronic liver disease, NAFLD is characterized by inflammation and fibrosis. NAFLD has been considered a condition with a “two-hit” process of pathogenesis since 1998 when Day and James first proposed this hypothesis. The first hit is the development of hepatic steatosis via accumulation of triglycerides in hepatocytes, which increases the vulnerability of the liver to “second hits”. The second hit includes oxidative stress (free radical formation due to excessive fatty acid oxidation), cardiolipin (present on inner mitochondrial membrane) peroxidation leading to mitochondrial dysfunction and more reactive oxygen species formation, endoplasmic reticulum stress, proinflammatory cytokines and gut-derived bacterial endotoxin.^{1,22} Tissue damage related both to lipotoxicity and to fat accumulation results in reduced sinusoidal perfusion as well as changes in sinusoidal architecture. Cytokines activated by lipotoxicity mediates the migration of the inflammatory cells leading to inflammation. As the disease progress, fatty hepatocytes and inflammation impaired sinusoidal perfusion leading to hypoxia. All of these events provoking liver damage can innate angiogenesis. Hypoxia activates angiogenesis as a results of signaling mediated by hypoxia-inducible factors (HIFs). HIFs activate transcription of VEGF gene.²³

Most of data on angiogenesis in NAFLD come from animal models while the few studies on serum VEGF levels in NAFLD patients have been controversial.²⁴ We found serum VEGF levels was significantly higher in NAFLD patients ($p < 0,001$). Coulonet *al* reported that serum VEGF levels was significantly higher in NAFLD patients ($p = 0,03$).⁷ Tarantino *et al* also found serum VEGF levels was significantly higher in NAFLD patients ($p < 0,0001$).⁸ On the other hand, Papageorgiou *et al* found that no significant difference in serum VEGF levels, but significantly lower serum VEGF levels in NASH patients.¹² Yilmaz *et al* reported no significant difference in serum VEGF levels in NAFLD patients compared to healthy controls.²¹ The reason for conflicting results between studies could probably be found in the diversity of the patient populations and their clinical characteristics (differences in BMI and inclusion or exclusion criteria). Confounding factors like age, sex and comorbidities. Another reason was due to the differences in laboratory techniques. ELISA kits from different manufacturers can measure substantial differences in serum levels of cytokine, due to the use of different sets of antibodies with different affinities for the same antigen.^{8,25}

No statistically significant difference of serum VEGF levels was observed when we split our NAFLD patients according to their sex ($p = 0,232$), age ($p = 0,838$) and BMI ($p = 0,361$). We found that serum VEGF levels tended to higher in females than in males. Malamitsi *et al* found that serum VEGF levels in females showed 49% higher serum VEGF levels than males ($p = 0,01$).²⁶ In the adult, angiogenesis occurs infrequently. Exceptions are found in the female reproductive system. Estrogens increase the expression of VEGF mRNA in uterus.²⁷ Although women have menstrual cycles, it seemed capillaries in males are more likely to develop than in females.^{10,26}

VEGF is important for adipose tissue development and maintenance. The expansion of adipose tissue is linked to the development of its vasculature.²⁸ Elfving *et al* found obese patients showed significantly increased serum VEGF levels.²⁹ This study found no significant difference serum VEGF levels in obese NAFLD patients compared to patients with normal weight-overweight ($p = 0,361$) although serum VEGF levels tended to higher in obese patients. This is in accordance with a number of other studies (Kahl *et al* and Minelli *et al*) where no correlation was found.^{30,31} This study found no significant difference serum VEGF levels in young NAFLD patients (≤ 47 years) compared to NAFLD patients older than 47 years of age ($p = 0,838$). Using the cut off point at age 47 (the mean age of NAFLD patients). Similarly, Ventriglia *et al* found no significant difference serum VEGF levels according to age.³²

The limitations of our study merit comment. First, the relatively small sample size limits the generalizability of our conclusions. The second, this study was in Asia, the results might be different according to ethnicity. The third, this study neither assessed the hepatic expression of VEGF nor measured VEGF receptors, which may give important information about the role of VEGF in the pathogenesis of NAFLD.

V. Conclusion

The rationale of our study was to comprehend the potential relationship between VEGF and NAFLD patients. Our result showed that serum VEGF levels significant higher in NAFLD patients. This suggest that angiogenesis could play an important role in the pathogenesis of NAFLD. To confirm our observations, larger validation analyses and longitudinal prospective studies are obviously necessary.

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