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Serum levels of vascular endothelial growth factor in non-alcoholic fatty liver disease

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Abstract

Background This study aimed to assess the significance of serum levels of vascular endothelial growth factor (VEGF) in non-alcoholic fatty liver disease (NAFLD).

Methods Sixty-seven consecutive NAFLD patients and 47 healthy controls who visited our liver clinics between May 2008 and December 2010 were included. The NAFLD diagnosis required elevated alanine aminotransferase and/or gamma-glutamyl transpeptidase levels, evidence of hepatic steatosis on ultrasound and/or liver histology, and exclusion of other causes of liver injury. Serum VEGF levels were determined by an enzyme immunoassay. Liver biopsy was obtained in 34 NAFLD patients. Histological lesions were scored by a liver histopathologist.

Results Serum VEGF levels tended to be lower in matched NAFLD patients than in healthy controls (296±146 vs. 365±186 pg/mL, P=0.092); levels in patients with non-alcoholic steatohepatitis (NASH) also tended to be lower than in those with simple fatty liver (FL) (279±149 vs. 359±190 pg/mL, P=0.095); while VEGF levels were significantly lower in NASH patients than in healthy controls (279±149 vs. 365±186 pg/mL, P=0.041). VEGF levels offered poor predictability for the differentiation between NAFLD patients and controls or between NASH and FL patients. However, patients with high VEGF levels (≥300 pg/mL) were significantly more likely to have FL, either in the total NAFLD population (67% vs. 35%, P=0.019) or in the 34 NAFLD patients with liver biopsy (57% vs. 15%, P=0.023), while those with high VEGF levels also had a significantly lower mean fibrosis score (0.7±0.9 vs. 1.6±1.0, P=0.017).

Conclusion Our data suggest that serum VEGF levels are equally high in healthy controls and in patients with simple fatty liver, but tend to decrease when NASH develops.

Keywords Nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, vascular endothelial growth factor, vascular endothelial growth factor receptors, angiogenesis markers

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Introduction

Non-alcoholic fatty liver disease (NAFLD) represents a major public health problem with a prevalence as high as 30% in many populations [1,2]. Liver biopsy is considered to be the gold standard for the assessment of the presence and severity of hepatic injury in NAFLD, but it is an invasive procedure that has also been associated with sampling errors [3,4]. Therefore, there is growing scientific interest in imaging studies and serum-based assays that aim to detect the presence of NAFLD, and to distinguish simple fatty liver (FL) from non-alcoholic steatohepatitis (NASH), the more severe and progressive type of NAFLD [5].

Considering that NASH is characterized by marked activation of inflammatory cells [6-9] and upregulation of several soluble inflammatory mediators, the role of different

cytokines and chemokines in NAFLD has been evaluated by several groups [10]. Liver inflammation, which is a key element of NASH, has been shown to be triggered by activated cytokines induced by lipotoxicity. Although the pathogenesis of NASH is still not fully understood, some studies suggested that angiogenesis might play a role in its progression [11,12]. Inflammatory cells are able to initiate angiogenesis through different pathways, thereby contributing to the formation of new vasculature in the liver [13,14]. Although angiogenesis has been well documented in patients with chronic viral hepatitis [15,16], information regarding angiogenesis in NAFLD is very limited.

Vascular endothelial growth factor (VEGF) is a proangiogenic factor implicated in the angiogenetic process. The aim of this study was to evaluate the possible significance of serum VEGF levels in patients with NAFLD.

Patients and methods

Sixty-seven consecutive patients with NAFLD who visited our outpatient liver clinics between May 2008 and December 2010 were included. The diagnosis of NAFLD was based on the following criteria: elevated alanine aminotransferase (ALT) and/or gamma-glutamyl transpeptidase (GGT) levels; evidence of hepatic steatosis on ultrasonography and/or liver histology; and exclusion of other causes of liver injury. In particular, all patients had negative serological markers for hepatitis B (HBsAg), hepatitis C (anti-HCV) and human immunodeficiency virus (anti-HIV), weekly alcohol consumption less than 210 g for men or 140 g for women, no use of potentially hepatotoxic agents, no evidence of metabolic or autoimmune liver disease, and absence of any known systemic disease with potential liver involvement. The history of alcohol use was taken from the patients and was confirmed by the patients' relatives or friends.

Demographic characteristics and medical history were recorded for all patients, together with any history of known arterial hypertension or the presence of diabetes mellitus, diagnosed on the basis of antidiabetic treatment and/or fasting glucose >126 mg/dL on more than one occasion.

Forty-seven healthy controls matched for age, sex and body mass index (BMI) with 47 of the aforementioned 67 patients were also enrolled. Controls were either subjects who visited the outpatient clinics of our hospital for routine examinations during the same period, or hospital staff members. Controls had normal glucose metabolism and liver biochemistry and no evidence of hepatic steatosis on abdominal ultrasound.

The study was approved by the Hippokratio Hospital Institutional Review Board.

Anthropometric assessments

Participants' body weight was measured with a digital scale (Seca Robusta 813, Hamburg, Germany) to the nearest 100 g and height was measured to the nearest 0.5 cm. Waist circumference (WC) was tape-measured to the nearest 0.1 cm. Increased WC was defined as >102 cm for men and >88 cm for women.

Laboratory markers

The laboratory data recorded included complete blood count, prothrombin time, urea, creatinine, urate, liver enzymes (ALT, aspartate aminotransferase, alkaline phosphatase, and GGT), total protein, albumin, as well as detection of HBsAg, anti-HBc, anti-HBs, anti-HCV, anti-HIV, and liver autoantibodies.

Serum VEGF levels were measured using a commercially available sensitive enzyme-linked immunosorbent assay (ELISA, Quantikine/immunoassay kit, R&D Systems, Minneapolis, MN, USA). The intra- and inter-assay coefficients of variation were <7% and <10%, respectively. Serum levels of caspase-generated fragments of keratin-18 (K18) as well as of and soluble Fas (sFas) were also measured by ELISA based immunoassays (M30-Apoptosense ELISA assay, PEVIVA, Alexis, Grünwald, Germany and Human Fas/TNFRSF6 Quantikine ELISA Kit, R&D Systems, Minneapolis, MN, USA, respectively).

Definitions

Patients were considered obese if they had a BMI ≥30 kg/m^2. Metabolic syndrome was defined according to the National Cholesterol Education Program: Adult Treatment Panel III criteria [17].

Patients with NAFLD were classified into those with FL and those with NASH, according to the widely accepted histological criteria described below and/or to a recently published formula based on serum K18 and sFas levels, shown to correctly classify 88% of NAFLD patients [18].

Transient elastography (TE)

Liver stiffness was measured (in kPa) using the standard probe of TE (Fibroscan, Echosens, France) in 48 of the 67 patients, but the results were reliable in only 46. The result was considered reliable if 10 successful measurements were obtained, with a success ratio >60% and a ratio of interquartile range to mean stiffness <30%. For patients who underwent both TE and liver biopsy, liver stiffness measurement was performed a few hours before liver biopsy in most or within 4 weeks before or after liver biopsy in some patients.

Liver histology

Adequate liver biopsies were obtained in 34 of the 67 NAFLD patients. Histological lesions were classified according

to the Brunt classification by one blinded liver histopathologist (DT). A liver biopsy was considered to be adequate if at least 6 portal tracts were identified and the specimen length was ≥1.5 cm. The diagnosis of NASH was made according to the Brunt classification criteria [19], as modified by Kleiner *et al* [20]. Global grading of necroinflammatory activity and staging of fibrosis were assessed according to Brunt *et al* [19]. Severity of steatosis and NAFLD activity score were evaluated according to Kleiner *et al* [20].

Statistical analysis

Quantitative variables with normal distribution were expressed as mean values ± standard deviation (SD) and those with abnormal distribution as median values (range). Statistical analysis was performed using the Mann-Whitney test for comparisons of quantitative variables between groups, Spearman's coefficient for correlations of quantitative variables, and a two-tailed Fisher's exact test for qualitative data. The accuracy of VEGF levels for predicting early (stage: 0-1) or advanced (stage: 2-4) liver disease was assessed by the area under the receiver operating characteristic curve (AUROC). A two-tailed P-value of <0.05 was considered to be statistically significant.

Results

Of the 67 NAFLD patients, 21 (31%) were diagnosed with FL and 46 (69%) with NASH. FL and NASH were diagnosed in 11 (32%) and 23 (68%) of the 34 patients who underwent liver biopsy, and in 10 (30%) and 23 (70%) of the remaining 33 patients without a liver biopsy according to the K-18/sFas formula. In the 34 patients with a liver biopsy, the accuracy of the K-18/sFas formula in differentiating between the histological presence of FL and NASH was 91% (31/34).

Baseline patient characteristics are shown in Table 1. Patients with NASH had higher WC (107±10 vs. 103±13 cm, P=0.016)

Table 1 Demographic, anthropometric, clinical and laboratory characteristics of the study population

Characteristic	Patients with fatty liver (n=21)	Patients with NASH (n=46)	P	Matched patients with NAFLD* (n=47)	Healthy controls (n=47)	P
Age, years	43±9	44±11	0.425	46±12	47±13	0.777
Sex, males, n (%)	11 (52)	34 (74)	0.099	27 (57)	27 (57)	>0.999
Body mass index, kg/m^2	27±3	30±4	0.060	27±4	28±4	0.054
Waist circumference, cm	99±7	106±10	0.016	104±10	100±10	0.097
Smoking, n (%)			0.229			0.046
Current	3 (62)	14 (30)		10 (22)	20 (43)	
Never	13	21		26 (57)	16 (35)	
History	5	11		11 (24)	11 (24)	
Concomitant diseases, n (%)			0.426			0.007
Diabetes, n (%)	1 (5)	0 (0)		1 (2)	1 (2)	
Arterial hypertension, n (%)	0 (0)	2 (4)		2 (4)	5 (11)	
Coronary artery disease, n (%)	1 (5)	0 (0)		0 (0)	1 (2)	
Dyslipidemia, n (%)	7 (33)	22 (48)		22 (47)	2 (4)	
Other, n (%)	1 (5)	1 (5)		4 (9)	6 (13)	
ALT, IU/L (ULN=40)	61±29	69±30	0.342	73±33	31±19	<0.001
AST, IU/L (ULN=40)	35±15	39±13	0.234	44±21	23±6	<0.001
GGT, U/L (ULN=50)	72 (16-168)	39 (21-753)	0.072	45 (17-753)	18 (5-58)	<0.001
Cholesterol, mg/dL - Total	239±51	209±41	0.053	222±47	246±35	0.106
- HDL	50±12	43±9	0.060	48±12	66±28	0.227
- LDL	154±34	133±31	0.151	142±33	154±43	0.425
Triglycerides, mg/dL	155±121	192±86	0.069	159±76	131±103	0.283

*Patients with NAFLD matched to healthy controls for age, gender and body mass index

NASH, non-alcoholic steatohepatitis; NAFLD, non-alcoholic fatty liver disease; ULN, upper limit of normal; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; HDL, high-density lipoprotein; LDL, low-density lipoprotein

compared to patients with FL. Though the differences were not statistically significant, NASH patients also tended to have higher BMI (30±4 vs. 29±5 kg/m^2, P=0.06), higher triglyceride levels (175±75 vs. 155±104 mg/dL, P=0.069) and lower HDL levels (44±10 vs. 51±13 mg/dL, P=0.060) compared to those with FL.

In the 47 NAFLD patients who were matched to healthy controls, VEGF levels were lower in patients than in controls (296±146 vs. 365±186 pg/mL) but the difference did not reach statistical significance (P=0.092). Given that no difference in the VEGF levels was found between patients with FL and controls (336±136 vs. 365±186 pg/mL, P=0.532), the previous finding was attributed to patients with NASH. In particular, patients with NASH had significantly lower VEGF levels compared to healthy subjects (279±149 vs. 365±186 pg/mL, P=0.041). In addition, serum VEGF levels tended to be lower in the 46 patients with NASH compared to the 21 patients with FL (289±147 vs. 359±190 pg/mL, P=0.095) (Fig. 1). A similar trend in serum VEGF levels was also observed in the 34 patients who underwent liver biopsy (FL: 326±130 vs. NASH: 253±149 pg/mL, P=0.098). These data suggest that serum VEGF levels tend to decrease with the progression from simple steatosis to steatohepatitis.

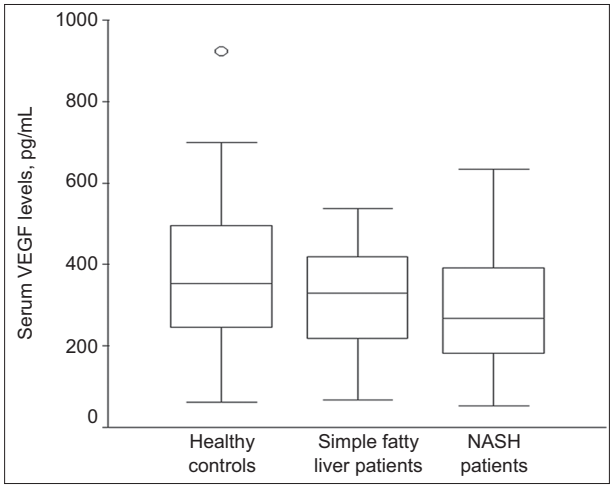

Figure 1 Serum levels of vascular endothelial growth factor (VEGF) in healthy controls and in patients with simple fatty liver or non-alcoholic steatohepatitis (NASH)
Controls vs. NASH: P=0.041, fatty liver vs. NASH: P=0.095. Box and whisker plots express medians, and interquartile and overall ranges. The outlying values are plotted individually

Serum VEGF levels were not able to differentiate effectively between NAFLD patients and controls (AUROC: 0.601, 95% CI 0.486-0.715; P=0.092) (Fig. 2A). In the 67 NAFLD patients, serum VEGF levels also could not differentiate between FL and NASH patients (AUROC: 0.628, 95% CI 0.485-0.770; P=0.095) (Fig. 2B). In the 34 NAFLD patients with liver biopsy, VEGF levels were numerically but not statistically higher in patients with an early stage of fibrosis (0-1) than in those with an advanced stage (2-4) (302±141 vs. 239±149 pg/mL, P=0.129), while there was no significant correlation between VEGF levels and fibrosis stage (r=-0.268, P=0.125).

When we split our NAFLD patients according to their median VEGF value, patients with high (≥300 pg/mL) VEGF levels were found to have FL significantly more frequently, both in the total NAFLD population (67% vs. 35%, P=0.019) and in the 34 NAFLD patients with liver biopsy (57% vs. 15%, P=0.023). Patients with high VEGF levels also had a lower mean fibrosis score (0.7±0.9 vs. 1.6±1.0, P=0.017) (Table 2).

No significant correlation was observed between serum VEGF levels and age (r=0.104, P=0.271), BMI (r=-0.109, P=0.247), sex (r=0.090, P=0.342), liver inflammation expressed by histological grading (r=-0,116, P=0.513), hepatic steatosis (r=0.182, P=0.319) or liver stiffness at elastography (r=-0.117, P=0.430). Additionally, no significant correlation was found between serum VEGF levels and cholesterol (r=0.176, P=0.526), triglyceride (r=0.064, P=0.616) or GGT values (r=0.064, P=0.526). Interestingly, serum VEGF levels had a significant inverse correlation with aspartate aminotransferase (r=-0.262, P=0.007) and ALT values (r=-0.275, P=0.005).

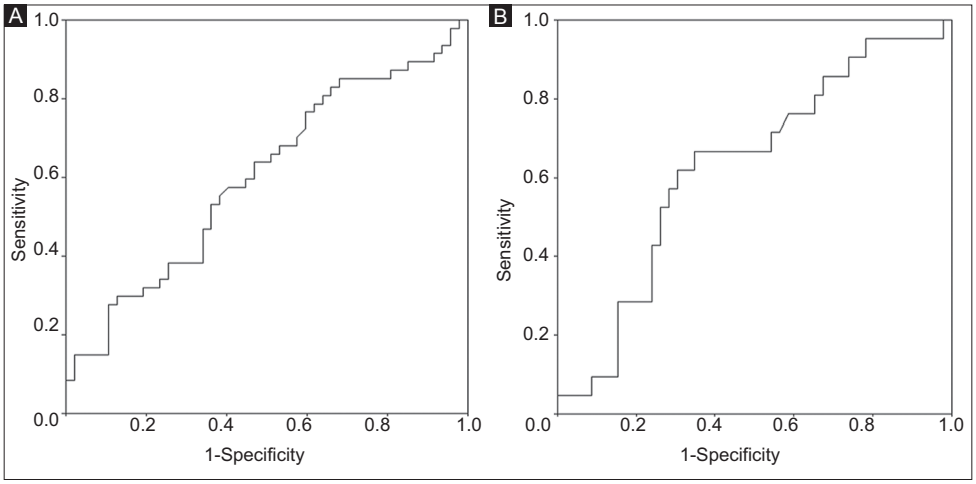

Figure 2 (A) Area under the receiving operating characteristic curve (AUROC) of serum levels of vascular endothelial growth factor for the discrimination between patients with non-alcoholic fatty liver disease and healthy controls (AUROC: 0.601, 95% CI 0.486-0.715; P=0.092) (B) or between patients with simple fatty liver and those with non-alcoholic steatohepatitis (AUROC: 0.628, 95% CI 0.485-0.770; P=0.095)

Table 2 Vascular endothelial growth factor (VEGF) serum levels in patients with non-alcoholic fatty liver disease (NAFLD) in relation to their characteristics

Characteristic	High VEGF (≥300 pg/mL) (n=30)	Low VEGF (<300 pg/mL) (n=37)	P
Age, years	44±10	46±12	0.566
Sex, males (%)	18 (60)	27 (73)	0.303
Body mass index, kg/m^2	29±4	31±4	0.221
Waist circumference, cm	105±10	108±12	0.549
Smoking, n (%)			0.056
Current	9 (30)	8 (22)	
Never	18 (60)	16 (43)	
History	3 (10)	13 (35)	
Concomitant diseases, n (%)			0.492
None	15 (50)	14 (38)	
Diabetes	1 (3)	0 (0)	
Arterial hypertension	0 (0)	2 (5)	
Coronary artery disease	0 (0)	1 (3)	
Dyslipidemia	12 (40)	17 (46)	
Other	2 (7)	3 (8)	
ALT, IU/L (ULN=40)	56±26	74±30	0.078
AST, IU/L (ULN=40)	35±15	40±13	0.245
GGT, U/L (ULN=50)	91±77	89±37	0.113
Cholesterol, mg/dL - Total	224±47	214±45	0.112
- HDL	50±11	43±10	0.066
- LDL	142±34	137±34	0.589
Triglycerides, mg/dL	161±86	195±104	0.390
Liver stiffness by elastography, kPa	6±2	8±6	0.150
NAFLD type, n (%)			0.019
Simple fatty liver	14 (67)	16 (35)	
Non-alcoholic steatohepatitis	7 (33)	30 (65)	
NAFLD type according to liver biopsy, n (%)			0.023
Simple fatty liver	8 (57)	3 (15)	
Non-alcoholic steatohepatitis	6 (43)	17 (85)	
Histological grading	0.7±0.8	1.1±0.87	0.180
Fibrosis score	0.7±0.9	1.6±1.0	0.017
Severity of fibrosis, n (%)			0.079
Stage 0-1	11 (79)	9 (45)	
Stage 2-4	3 (21)	11 (55)	
Steatosis score	1.7±0.9	1.8±0.9	0.112

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; ULN, upper limit of normal; HDL, high-density lipoprotein; LDL, low-density lipoprotein

Discussion

Like all chronic liver diseases, NAFLD is characterized by a wide spectrum of inflammation and fibrosis. NASH, the most severe form of NAFLD, can progress to cirrhosis and may eventually lead to hepatocellular carcinoma [21-23]. The two-hit hypothesis, based on the initial intrahepatic accumulation of triglycerides followed at a second stage by inflammatory

mediators, gut endotoxin, mitochondrial dysfunction and endoplasmic reticulum stress causing liver injury, has provided some explanation for the pathogenesis of NASH, but has recently been challenged. Currently, the pathophysiology of NAFLD is considered to be multifactorial, including a relation between changes in microvasculature and the progression of fibrosis [24-27]. Tissue damage from both fat accumulation and lipotoxicity results in reduced sinusoidal perfusion and changes in sinusoidal architecture. Additionally, several cytokines activated by lipotoxicity further induce the migration of inflammatory cells, which contribute to angiogenesis in the inflammatory foci [28]. During disease progression, fatty hepatocytes, tissue inflammation and perisinusoidal fibrosis constrict the sinusoidal lumens and impair the sinusoidal perfusion, leading to hypoxia [28,29]. All these events provoking liver damage can initiate angiogenesis [26]. As a consequence, angiogenesis leading to new vasculature formation may have prognostic value in disease progression. The idea that interfering with angiogenesis might be a potential target to avoid progression of liver disease has stimulated research into several markers for angiogenesis in chronic liver diseases.

Angiogenesis has been documented in cases of viral hepatitis, but information regarding angiogenesis in NAFLD is very limited. Angiogenesis in chronic hepatitis C has been reported to be induced more frequently than in patients with chronic hepatitis B or healthy controls [30]. Salcedo *et al* concluded that serum VEGF, angiopoietin 2 and tyrosine kinase 2 levels could be useful as noninvasive markers of response to therapy and disease progression in patients with chronic hepatitis C [31]. Moreover, enhanced angiogenesis has been described in association with hepatocellular carcinoma in patients with chronic hepatitis and cirrhosis [32,33]. In our study, VEGF serum levels did not differ between patients with FL and healthy controls, but were found to be lower in NASH patients compared to healthy controls (P=0.041) and tended to be lower in patients with NASH compared to those with FL (P=0.098). These findings suggest that serum VEGF levels tend to decrease during the progression from healthy liver or simple steatosis to steatohepatitis.

Most of the data on angiogenesis in NAFLD come from animal models, while the few reports on serum VEGF levels in NAFLD patients have been controversial. Kitade *et al* showed that angiogenesis is involved in the development of NASH-related liver fibrosis and carcinogenesis in leptin-deficient rats [34]. In a rat model, it was also shown that renin inhibition may have favorable effects on liver fibrogenesis in NASH, through inhibition of angiotensin-II, tumor growth factor β and VEGF [35]. More recently, Yang *et al* suggested that anti-VEGF receptor agents ameliorate hepatic venous dysregulation, microcirculatory dysfunction, splanchnic venous pooling and ascites in NASH cirrhotic rats [36]. In humans, Amarapurkar *et al* reported that immunohistochemical VEGF hepatic expression was seen in 29% of NASH patients or 46% of patients with chronic liver disease of various etiologies, being more common in the early stages of fibrosis [37]. Yilmaz *et al* reported no significant difference in serum VEGF levels, but significantly lower serum levels of soluble VEGF receptor 1 in NAFLD patients compared to healthy controls, with lower soluble VEGF receptor 1 levels being associated with increased fibrosis [38]. On the other hand, Coulon *et al* found that serum VEGF serum levels were significantly higher in patients with FL compared to healthy controls, but only relatively higher in patients with NASH compared to healthy controls. In the same study, the concentration of soluble VEGF receptor 1 was significantly higher in the serum of FL and NASH patients compared to controls [39]. Moreover, Tarantino *et al* showed that serum VEGF levels were higher in NASH patients compared to patients with FL or healthy controls, but serum VEGF level was not a useful marker for differentiation between FL and NASH patients [40]. More recently, Ciupinska-Kajor *et al* reported that the immunohistochemical hepatic expression of VEGF was higher in simple steatosis and borderline NASH in severely obese patients and in NASH in non-obese patients with NAFLD [41]. In NASH, centrilobular arteries and increased microvessel density are more commonly detected in advanced fibrotic stages, suggesting a possible association between neoangiogenesis and NASH progression to cirrhosis [42].

In addition to the existing controversial data, our results also suggest that serum VEGF levels cannot accurately differentiate healthy controls from NAFLD patients, or patients with simple fatty liver from those with NASH. However, our finding of higher serum VEGF levels in healthy controls, or even perhaps in patients with FL, compared to those with NASH may seem strange, since increased angiogenesis is usually thought to be present in more advanced liver disease, as mentioned above. One explanation for such discrepant findings may be the type of assay used to determine serum VEGF levels. Only free VEGF was measured in our study, which means that the detected VEGF levels may, at least in some cases, underestimate the circulating VEGF levels in serum. In addition, we neither assessed the hepatic expression of VEGF nor measured VEGF receptors, which may offer important information about the role of VEGF in the pathogenesis of NAFLD. Another reason for these conflicting results could be the heterogeneity of patients and controls among the different studies. Finally, angiogenesis can be useful early in the course of NAFLD, reflecting the autohealing ability of the liver. However, beyond a critical point, angiogenesis may become injurious itself and VEGF inhibitors could predominate, thus resulting in a reduction in circulating VEGF levels. Alternatively, our results could reflect a possible dichotomous effect of VEGF on NAFLD progression, similar to that reported for adipose tissue dysfunction [43].

In conclusion, serum VEGF levels offer poor predictability in differentiating healthy controls from NAFLD patients or patients with FL from those with NASH. However, our data suggest that serum VEGF levels are similarly high in healthy controls and patients with FL and tend to decrease when NASH develops. This is a challenging finding, because it may indicate a dichotomous effect of VEGF levels in NAFLD; this question needs to be further evaluated in larger studies in order to clarify the role of VEGF in the pathogenesis of NAFLD.

Summary Box

What is already known:

- Non-alcoholic steatohepatitis (NASH) is characterized by marked elevation of inflammatory cells
- Angiogenesis might play a role in the progression of NASH
- Vascular endothelial growth factor (VEGF) is a proangiogenic factor implicated in the angiogenetic process
- In the human setting, the few reports on serum VEGF levels in patients with non-alcoholic fatty liver disease (NAFLD) are controversial

What the new findings are:

- VEGF levels cannot reliably differentiate between patients with NAFLD and healthy controls or between patients with simple fatty liver (FL) and NASH
- Serum levels of VEGF are lower in patients with NASH than in healthy controls and relatively lower in NASH than in patients with FL
- NAFLD patients with high VEGF levels (≥300 pg/mL) have a lower incidence of NASH and a lower mean fibrosis score

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