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Full Length Research Paper

Plasma osteopontin as a biomarker test in predicting liver fibrosis in Genotype 4 Chronic Hepatitis C infected patients

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Management of chronic Hepatitis C Virus (HCV) infection can be achieved by antiviral therapy based on staging of liver fibrosis. Liver biopsy is considered the reference standard in liver disease assessment, but it is an invasive procedure. Non invasive accurate tests are largely needed. Osteopontin is an important component of Extra Cellular Matrix (ECM) which promotes liver fibrosis. The relation between Osteopontin (OPN) level and hepatic fibrosis in chronic HCV infection has not been widely investigated. This study aimed to evaluate the role of plasma OPN in predicting hepatic fibrosis in chronic HCV infected patients. Forty chronic HCV patients and forty controls were included in this study. METAVIR score was used to stage liver fibrosis. Plasma OPN levels were determined using ELISA. There was a high significant difference between the patient group and controls as regards plasma OPN being 59.25±34.935 ng/ml versus 16.5±6.0914 ng/ml respectively; P<0.001. Regarding Osteopontin value, a high significant difference between insignificant and significant fibrosis was observed (34.5±12.34909 ng/ml vs 84±32.60027 ng/ml, respectively; p<0.001). A cut off value of 22.5 ng/ml was a possible cut off value to discriminate fibrosis from non-fibrosis giving a sensitivity of 90% and specificity of 95%. The cut off value of <50 ng/ml was a possible cut off value to discriminate insignificant hepatic fibrosis (F<2) from significant hepatic fibrosis (F≥2) giving 90% sensitivity and specificity. A cut off value of >60 ng/ml was a possible cut off value to discriminate advanced fibrosis (F3) from non advanced fibrosis (F1, F2) giving a sensitivity of 100.0% and a specificity of 47.2%. Plasma OPN can be used as a reliable test to predict fibrotic from non-fibrotic liver, and insignificant hepatic fibrosis from significant hepatic fibrosis.

Key words: Plasma osteopontin, liver fibrosis, chronic hepatitis C genotype 4, non invasive biomarkers.

INTRODUCTION

Hepatic fibrosis is a non specific reaction in response to chronic liver injury. The damage to hepatocytes causes activation of inflammatory cells and an abnormal deposition of extracellular matrix (ECM) in the "perisinusoidal space" between hepatocytes and sinusoidal endothelia (Schuppan and Afdhal, 2008).

Approximately 170 million people worldwide are suffering from chronic hepatitis C virus (HCV) infection, which is a major cause of chronic liver disease, cirrhosis, and primary hepatocellular carcinoma (HCC) (Schaefer et al., 2011). Egypt faces the largest burden of HCV infection in the world predominantly genotype 4 (Centers for Disease Control and Prevention (CDC), 2012).

In patients with chronic hepatitis C, precise staging of liver fibrosis is important not only for estimation of prognosis, but also for indication of antiviral therapy. Liver biopsy is considered the reference method for evaluation of liver fibrosis in chronic hepatitis C patients (Castera,

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2011). Liver biopsy is an invasive procedure that carries a risk of complications. It is now agreed that biopsy is an "imperfect gold standard" due to sampling errors, biopsy size (5 to 30 mm) and intra- and inter observer variability. 30% of patients may complain of pain, up to 3% have been noted to have complications severe enough to require hospitalization (Pasha et al., 1998) and a 0.01-0.3% rate of deaths has been reported (Strassburg and Manns, 2006). The risk benefit ratio of liver biopsy is insufficient to maintain it as a first line procedure, hence new and non-invasive criteria for evaluation of liver fibrosis are urgently needed (Poynard et al., 2008).

Osteopontin (OPN) is an arginine–glycine–aspartate (RGD)–containing acidic member of the small integrin–binding ligand N-linked glycoprotein (SIBLING) family of protein (Fisher et al., 2001).

Expression of OPN was highest in the liver in Kupffer cells, macrophages, and hepatic stellate cells, consistent with a role of OPN in the function of these cell types (Wang et al., 2000).

OPN is expressed in macrophages and related cells at sites of hepatic injury contributing to the host response to infection or injury. Because OPN stimulates cell migration, it may act as a chemotactic factor in the recruitment of macrophages to sites of liver injury (Ramaiah and Rittling, 2007).

HSC has been regarded as the main producer of ECM. Under injury stimuli, quiescent HSC trans differentiates into its activated form, myofibroblast, with increased proliferation, enhanced resistance to apoptosis and the ability of generating a large amount of collagen (Xiao et al., 2012).

OPN is an important component of ECM which promotes liver fibrosis and has been reported as a biomarker for its severity (Pritchett et al., 2012). It induces ECM accumulation by binding to type I collagen, fibronectin, and osteocalcin, contributing to tissue fibrotic process (Suzuki et al., 2005). Therefore this study was carried out to evaluate the level of plasma OPN as a non-invasive biomarker in predicting hepatic fibrosis in chronic hepatitis C patients.

MATERIALS AND METHODS

Patients

Eighty sex and age matched persons attending the outpatient clinics at Ain Shams University Hospital during the period from October 2012 to April 2013 were divided into 2 groups of 40 persons each.

Group I included 40 patients with chronic HCV infection Genotype 4, while Group II included 40 healthy subjects negative for HCV antibody and HB surface antigen coming for pre-employment check up or for blood donation at the blood bank. Fibroscan was done to the control subjects to exclude any patient with hepatic fibrosis.

Diagnosis of chronic hepatitis C infection and liver fibrosis

The diagnosis of chronic hepatitis C infection and liver fibrosis in group I was based on clinical features, laboratory test, Hepatitis C virus Antibody (HCV Ab) by ELISA, HCV RNA by quantitative polymerase chain reaction (PCR), genotype testing, diagnostic imaging and presence of chronic hepatitis in liver biopsy.

A written informed consent was obtained from all enrolled subjects in this study, and the study was approved by the medical ethical committee in Ain Shams University.

Exclusion criteria

Patients with history of alcohol consumption (>80 g/day for >5 years), hepatitis B virus infection, autoimmune liver diseases, hepatocellular carcinoma (HCC), ischemic heart disease, diabetes, body mass index (BMI) ≥ 30, osteoporosis diagnosed by Dual Energy X-ray Absorbtiometry (DEXA) scan, liver cirrhosis, bilharzial, metabolic liver diseases, patients with previous interferon therapy and pregnant females were all excluded from the study. Patients with any stage of liver fibrosis were excluded from the control group by Fibroscan.

Tools of the study

All patients and control subjects were subjected to the following:

1. Full medical history and clinical examination.
2. Electrocardiography (ECG).
3. Laboratory investigations including: complete blood, liver profile, kidney and thyroid profiles, serum Anti-Nuclear Antibody (ANA) by standard laboratory test. Hepatitis Viral markers: Hepatitis B virus Surface Antigen (HBsAg) by ELISA, Hepatitis C virus Antibody (HCV Ab) by ELISA. HCV RNA by quantitative polymerase chain reaction (PCR) and genotype test were done for Group I patients.
4. Pelviabdominal Ultrasonography equipment: Hitachi, EUB-5500. Measurements were performed after overnight fasting and the patient in supine position with emphasis on: liver size, liver echogenicity, splenic bi-polar diameter (longest axis), portal vein diameter (mm) and patency. Criteria suggestive of chronic liver disease and cirrhosis include: increased liver echogenicity, irregular liver margins, attenuation of intrahepatic portal and hepatic veins, presence of periportal thickening, relative enlargement of caudate lobe and atrophy of right lobe (ratio of caudate/right lobe in cirrhosis >0.65) (Bates, 2004).
5. Liver biopsy: Ultrasonography-guided liver biopsy was done for chronic hepatitis C patients only. Liver biopsies were performed using disposable semiautomatic 18-

Table 1. Comparison between Group I and Group II as regard plasma Osteopontin (OPN).

Variable		Mean±SD	Range	z	P
OPN (ng/ml)	GI (20)	59.25±34.935	15-150	-4.881	0.000
	GII (20)	16.5±6.0914	7.5-27.5		

gauge true cut needle. The core biopsies were more than 10 mm in length and contained at least 8 portal tracts and were routinely stained with hematoxylin-eosin stain. The histological features were analyzed according to the histological activity index (HAI), where maximum inflammation grade was scored 18, HAI: 0 means no inflammation, HAI: 1-4, minimal inflammation; HAI: 5-8, mild inflammation; HAI: 9-12, moderate inflammation; HAI: 13-18, marked inflammation. Scores <9/18 was termed mild inflammation, while HAI≥9 was termed severe inflammation (Montazeri et al., 2005). METAVIR score was used to stage liver fibrosis (F0-F4) that was scored on a five point scale (F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, portal fibrosis with numerous septa; F4, cirrhosis) (Rousselet et al., 2005). The presence of stage F<2 was termed insignificant (no or minimal) fibrosis; whereas the presence of stages F≥2 was termed significant fibrosis.

6. Transient Elastography (Fibroscan): was performed for Group II subjects to confirm absence of any fibrosis using fibroscan 502 equipment. The patient lies on his back with his arm raised behind his head. The physician applies water based gel to the skin, places the probe with a slight pressure and examines the right lobe of liver through the intercostal space.

The examination includes 10 consecutive measurements made to the same location. The result is delivered at the end of the examination, in a form of a number in kilopascals (kPa). Limitation is linked to the presence of ascites, obesity and any flares of hepatitis. Fibroscan is contraindicated in patients with active implantable devices such as pacemakers and defibrillators and on wounds who are excluded from our study.

7. Measurement of Plasma Osteopontin (OPN) level: A 5-mL sample of peripheral blood was taken from each patient one hour before performing liver biopsy and anticoagulated by ethylene diaminetetraacetic acid (EDTA). Plasma was separated by centrifuging blood samples at 1000 g for 15 minutes at room temperature within 30 min from collection. Plasma was aliquoted and preserved at -20°C for subsequent assay. Repeated freezing and thawing was avoided. Plasma Osteopontin was assayed using commercially available Quantikine® ELISA kit, according to the manufacturer instructions (R&D systems, DosToo, Minneopolis, MN, USA) which has the minimum detectable dose of OPN ranging from 0.006 to 0.024 ng/ml.

Principle

Quantikine® ELISA employs the quantitative sandwich enzyme immunoassay technique.

Statistical analysis

Analysis of the data was done by IBM computer using SPSS (Statistical Package for Social Science) version 16. Data were expressed as Mean ± SD for quantitative parametric measures, in addition to Median Percentiles for quantitative non-parametric measures and both number and percentage for categorized data. Student t test was used for comparison between two independent mean groups, whereas Wilcoxon Rank Sum test was used for comparison between two independent groups. Ranked Spearman correlation test was used to study the possible association between each two variables among each group, while Chi-square test was used to study the association between each 2 variables. The probability of error at 0.05 was considered significant while at 0.01 it was highly significant.

Diagnostic validity test was used to show sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy of the test. The ROC (receiver operating characteristics) curve was constructed to obtain the most sensitive and specific cutoff for each technique and to evaluate the most discriminating markers between the compared groups, of which AUC can also be calculated.

RESULTS

Group I included 40 patients with chronic hepatitis C virus (Genotype 4) infection (20 males and 20 females), whose ages ranged from 30 years up to 56 years, with a mean of 44.45±6.7237 years. Group II included 40 healthy volunteers (20 males and 20 females), whose ages ranged from 30 years to 55 years, with a mean of 40.8±7.5645 years representing controls. Table 1 shows comparison regarding OPN between the two groups. There was a high significant difference between the studied groups as regard plasma OPN: 59.25±34.935 ng/ml in Group I versus 16.5±6.0914 ng/ml in Group II (p<0.01) (Table 1).

As regards the stage of the fibrosis in patients of Group I, according to METAVIR score, 20 patients (50%) showed minimal fibrosis (F1), 16 patients (40%) showed

Table 2. Comparison between mild inflammation (G<9) and severe inflammation (G≥9) (HAI) in Group I as regard Osteopontin value.

Variable		Range	Mean±SD	Z	P
OPN (ng/ml)	G<9 (34)	15-125	54.8529±30.07115	-0.955	0.339
	G≥9 (6)	47.5-150	84.1667±57.13653		

Table 3. Comparison between insignificant fibrosis (F<2) and significant fibrosis (F≥2) (Metavir) in Group I as regard Osteopontin value.

Variable		Range	Mean±SD	z	P
OPN (ng/ml)	F<2 (20)	15-55	34.5±12.34909	-3.6	0.000*
	F≥2 (20)	47.5-150	84±32.60027		

Table 4. Correlation between OPN and different parameters in Group I.

Variable	Plasma Osteopontin	
	r	P-value
Age	-0.025	0.877
WBCs	-0.288	0.072
HBG	-0.083	0.611
PLT COUNT	-0.095	0.559
AST	0.178	0.273
ALT	0.461	0.003*
PT	-0.016	0.924
Total Bil	0.248	0.123
Direct Bil	-0.094	0.563
ALP	0.199	0.218
Serum Albumin	-0.174	0.283
Serum Creatinine	-0.186	0.250
Blood Urea	0.161	0.322
FBS	0.193	0.232
TSH	0.092	0.574
AFP	0.142	0.381
PCR	0.231	0.189
METAVIR (Activity)	0.288	0.072
METAVIR (Fibrosis)	0.535	0.000*
HAI G	0.457	0.003*
HAI F	0.496	0.001*

moderate fibrosis (F2) and 4 patients (10%) showed advanced fibrosis (F3).

By HAI, in Group I, 34 patients (85%) showed mild inflammation (G<9) while 6 patients (15%) showed severe inflammation (G> 9). There was no statistically significant difference between mild and severe inflammation as regards Osteopontin value (Table 2), while there was a high statistically significant difference between insignificant and significant fibrosis as regards Osteopontin value (Table 3). There was a statistically positive significant correlation between OPN and fibrosis stage (Table 4).

There were significant correlation between serum Osteopontin level and ALT, grade of inflammation by HAI

Table 5. Cutoff values of OPN for discriminating between non-fibrotic liver, significant fibrosis and advanced fibrosis.

Variable	Cutoff value	Sensitivity	Specificity	PPV	NPV
No fibrosis vs F1, F2, F3	<22.5 ng/ml	90%	95%	94.7%	90.5%
Significant fibrosis F ≥2 vs F0, F1	>50 ng/ml	90%	90%	90%	90%
Advanced fibrosis F3 vs F0, F1, F2	>60.02	100.0	47.2	17.4	100.0

Table 6. Characteristics of the studied subjects as regard the gender.

Gender	Frequency	Percent
Female	40	50
Male	40	50
Total	80	100

score, stage of liver fibrosis by both HAI and METAVIR scores in Group I (Table 4). After doing multivariate analysis using parameters with significant correlation with OPN, ALT was the only parameter that showed significant value.

The AUROC for OPN as a predictor of hepatic fibrosis was 0.944, with a cut off value of 22.5 ng/ml as a possible cutoff value to discriminate fibrosis from non-fibrotic, giving a sensitivity of 90.0%, specificity of 95.0%, PPV of 94.7%, NPV of 90.5%, and efficiency of 92.5% (Table 5, Figure 1). The AUROC for OPN as a predictor of the degree of hepatic fibrosis was 0.922, with a cutoff value of 50 ng/ml as a possible cutoff value to discriminate insignificant hepatic fibrosis (F<2) from significant hepatic fibrosis (F≥2) giving a sensitivity of 90.0%, specificity of 90.0%, PPV of 90%, NPV of 90%, and efficiency of 90% (Table 5, Figure 2).

The AUROC for OPN as a predictor of advanced fibrosis was 0.517, with a cut-off value of 60.02 ng/ml as a possible cut-off value to discriminate advanced fibrosis (≥F3) from non advanced fibrosis (F1, F2), giving a sensitivity of 100%, specificity of 47.2%, PPV of 17.4%, NPV of 100**%** (Table 5, Figure 3).

DISCUSSION

OPN has been reported to play a multifaceted role in several physiologic and patho-physiological functions. The role of OPN in inflammation, immunity, bone remodeling, kidney stone formation, oncogenesis, angiogenesis and cancer progression, vascular calcification, and apoptosis has been investigated. With regards to inflammation, OPN is involved in several inflammatory and immune responses (Denhardt et al., 2001). Expression of OPN was highest in the liver in Kupffer cells, macrophages, and hepatic stellate cells, consistent with a role of OPN in the function of these cell types (Wang et al., 2000). OPN is an important component of ECM which promotes liver fibrosis and has been reported as a biomarker for its severity (Pritchett et al., 2012). It induces ECM accumulation by binding to type I collagen, fibronectin, and osteocalcin, contributing to tissue fibrotic process (Suzuki et al., 2005).

In this study, we found that the plasma OPN levels in chronic hepatitis C (CHC) patients (genotype 4) were significantly elevated compared to those in healthy controls. This could be attributed to the excessive production of OPN in damaged liver and the imbalance between its production and clearance that leads to increased circulating OPN levels (Honsawek et al., 2010).

Comparing plasma OPN levels with different stages of fibrosis, we found that plasma OPN levels were significantly increased in patients with significant fibrosis (F≥2) than those with minimal or no fibrosis (F<2). This finding was in agreement with that of Syn et al. (2011) who stated that OPN promotes the progression of fibrosis in nonalcoholic steatohepatitis. These results could be explained by the suggestion that recruitment and activation of the inflammatory and immune cells by OPN enhance hepatic inflammation, which in turn may activate HSCs and fibrogenesis (Syn et al., 2011). Also it goes in agreement with the study of Lee et al. (2004) who found that recombinant OPN directly induced the type I collagen production and type II transforming growth factor-β receptor mRNA and protein in a dose-dependent manner in HSCs, supporting the role of OPN in hepatic fibrosis. Our results are also similar to the results of Huang et al. (2010) who found significant increase in the OPN concentrations in HCV infected individuals with extensive fibrosis and inflammation.

This study revealed significant positive correlation between OPN and stages of hepatic fibrosis by both METAVIR and HAI scoring systems. This finding goes with that of Patouraux et al. (2012) who stated that OPN

Table 7. Clinical and demographic data of both groups.

Variable	Group I		Group II	
	Range	Mean±SD	Range	Mean±SD
Age (years)	30-56	44.45±6.7237	30-55	40.8±7.5645
WBCs/mm^3	4000-10700	6345±1780.146	5600-10000	7925±1379.884
HBG (gm/dl)	10.8-15.7	12.81±1.27316	12.5-14	13.1305±0.43027
PLT (x10^3/mm^3)	137000-309000	225950±52782.05	200000-340000	268000±36070.11
AST (IU/L)	27-57	40.6±7.5491	15-23	18.05±2.2589
ALT (IU/L)	25-77	45.45±14.4786	20-30	25.05±3.1867
PT (sec)	16-18	16.8±0.7678	8.8-14.7	12.645±1.8961
Albumin (gm/dl)	3.2-4.5	4.065±0.34378	3.5-4.9	4.125±0.43027
Creatinine (mg/dl)	0.4-1.1	0.767±0.17965	0.6-0.9	0.766±0.10252
FBS	76-118	92.45±11.727	90-105	96.35±5.0812
TBil (mg/dl)	0.39-1.9	0.7945±0.3441	0.2-0.9	0.497±0.2133
DBil (mg/dl)	0.01-1	0.299±0.2374	0-0.19	0.0855±0.0556
ALP	30-284	114.85±71.7725	30-55	41.1±6.7893
TSH	0.4-2.7	1.354±0.65624	0.88-3.3	2.1385±0.8125
AFP (ng/ml)	0.8-8.76	3.6735±2.40214	0.1-2.1	0.6±0.52915
OPN	15-150	59.25±34.935	7.5-27.5	16.5±6.914
PCR	61-796009	90327.6±192582.7	-	-
G (HAI)	3-9	5.6±2.1619	-	-
F (Met)	1-3	1.6±0.6805	-	-

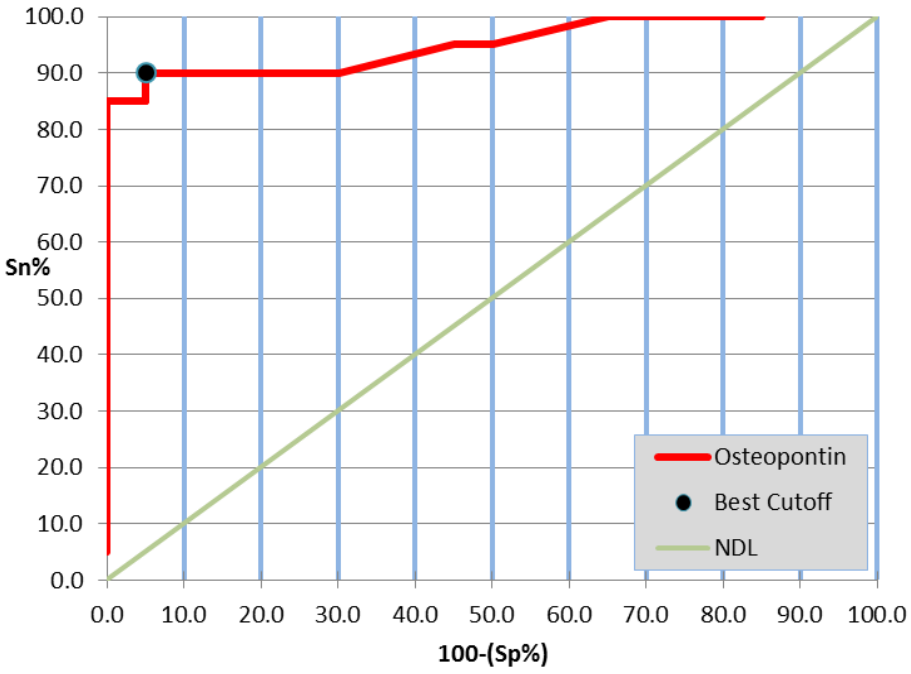

Figure 1. ROC curve analysis showing the diagnostic performance of Osteopontin for discriminating patients with hepatic fibrosis from those without fibrosis.

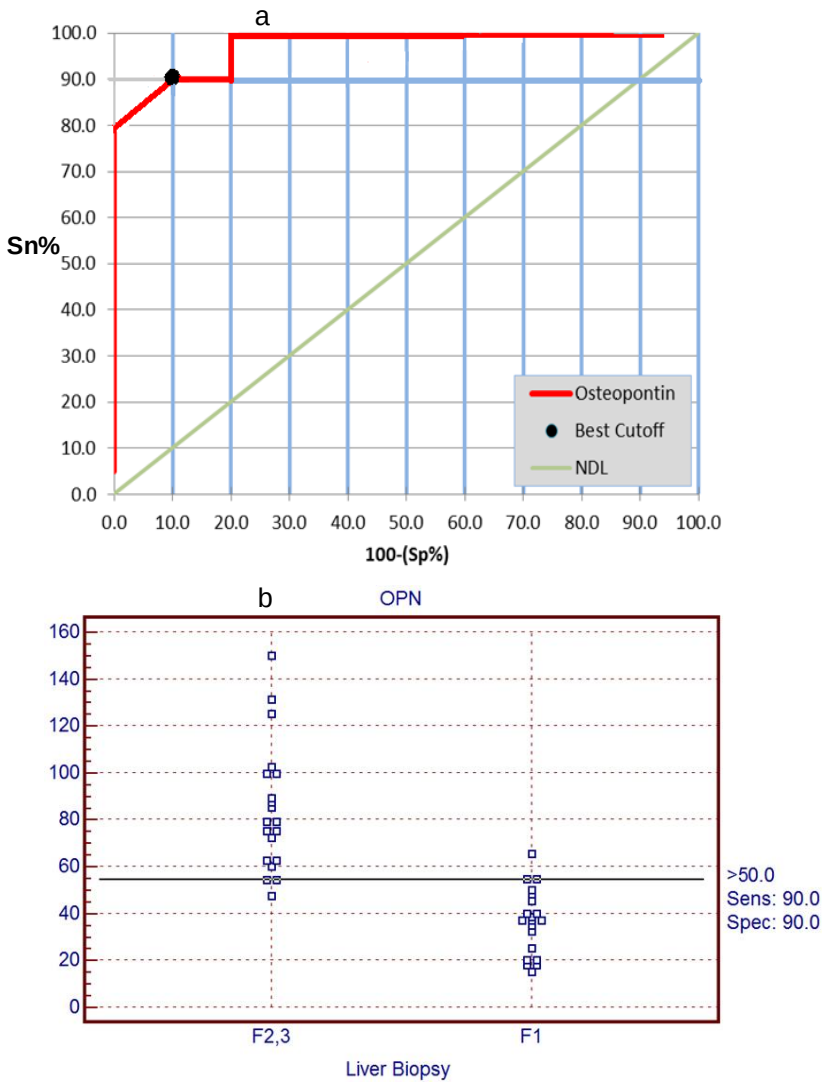

Figure 2a and b. ROC curve analysis showing the diagnostic performance of Osteopontin for discriminating insignificant fibrosis (F<2) from significant fibrosis (F≥2).

level in liver, adipose tissue and serum was correlated with fibrosis in patients with alcoholic liver disease. It was also in agreement with the study of Whitington et al. (2005) who reported that expression of OPN correlates with portal biliary proliferation and fibrosis in biliary atresia, and was in agreement with Kawashima et al. (1999) who have shown that OPN is expressed in activated Kupffer cells, hepatic macrophages and stellate cells in the necrotic areas in the liver of carbon tetrachloride (CCl4)-intoxicated rats which suggests that OPN plays an important role in the recruitment of inflammation in the liver supporting the role of OPN in the fibrogenesis of liver.

Our results agree with those of Huang et al. (2010) who found significant correlation between OPN concentrations and the stage of liver fibrosis in HCV infected patients. Cao and Liu (2006) found that OPN is a key component of the extracellular matrix (ECM) that is associated with the fibrotic process during tissue remodeling. OPN might be a central pathway of hepatic satellite cell (HSC) activation which is largely responsible for the development of liver-fibrosis in the liver by increasing the ECM deposition in the sub-endothelial space between the hepatocytes and endothelial cells (Cheng and Mahato, 2007). Using cDNA microarray, Lee et al. (2004) reported that OPN incubation activated HSCs *in vitro* and led to cell proliferation and migration.

Our results showed statistically significant correlation between the OPN level and the grade of inflammation measured by the HAI score. The results are in agreement with those of Huang et al. (2010) who found significant correlation between the OPN concentration and HAI

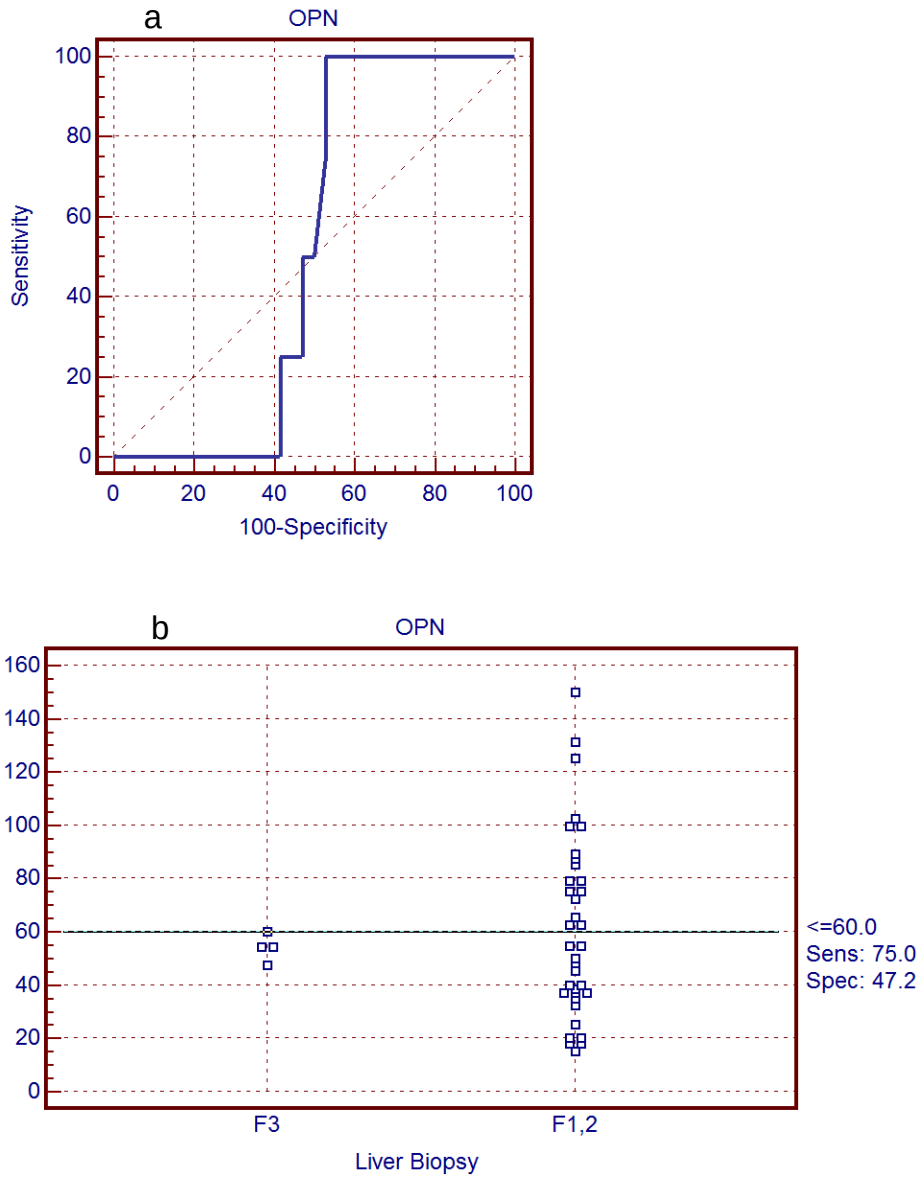

Figure 3a and b. ROC curve analysis showing the diagnostic performance of Osteopontin for discriminating advanced fibrosis (F<2) from non-advanced fibrosis (F≥2).

score in subjects infected with the HCV. This could be explained by the recent researches that proved that OPN is aTh1 cytokine, which plays an important role in the pathogenesis of various inflammatory and autoimmune diseases (Cantor and Shinohara, 2009). The role of OPN in inflammatory liver diseases such as alcoholic and nonalcoholic liver disease and T-cell-mediated hepatitis was also suggested recently (Ramaiah and Rittling, 2007). Although our results show insignificant difference between patients with severe inflammation and mild inflammation as regard OPN levels, plasma OPN levels in patients with severe inflammation shows Mean±SD: 84.1667±57.13653, which are obviously higher than those with mild inflammation with Mean±SD: 54.8529±30.07115. This could be explained by the limited sample size enrolled in our study, that is, 6 patients with severe inflammation (G≥9).

On constructing receiver operator characteristic curve (ROC), we found that the cutoff value for diagnosis of liver fibrosis was 22.5 ng/ml with AUC of 0.944, sensitivity of 90% and specificity of 95%. Also, on constructing ROC for prediction of significant hepatic fibrosis (F≥2), cutoff value was 50 ng/ml with AUC of 0.922, sensitivity of 90% and specificity of 90%. A cut off value of >60 ng/ml was a possible cut off value to discriminate advanced fibrosis (F3) from non advanced fibrosis (F1, F2) giving a

sensitivity of 100% and specificity of 47.2.

It can be concluded that OPN was correlated with the stage of hepatic fibrosis in chronic HCV-related liver fibrosis suggesting that OPN could be used as a non-invasive biomarker in predicting hepatic fibrosis in chronic hepatitis C virus infection, in order to restrict the use of liver biopsy complementary to plasma OPN level to those patients with significant fibrosis F≥2 with cut off value of OPN 50 ng/ml.

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