

High sensitive CRP and pentraxine 3 as noninvasive biomarkers of nonalcoholic fatty liver disease

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Abstract. – **INTRODUCTION:** Nonalcoholic fatty liver disease (NAFLD) has become the most common hepatic disease. Liver biopsy is the gold standard for the diagnosis of NAFLD. To overcome the problems with liver biopsy many studies are being performed to find noninvasive methods for the evaluation of hepatic status.

AIM: This study aims to study to role of high sensitive CRP and pentraxine 3 in the setting of NAFLD

PATIENTS AND METHODS: thirty two NAFLD cases and 34 controls were enrolled. All subjects were studied clinically and blood was drawn for para-clinical studies. Liver biopsy was performed for all cases. Levels of hs-CRP and pentraxine were analyzed to find any significant difference for the stages of steatosis and fibrosis based on pathologic findings.

RESULTS: Hs-CRP level was higher in nonalcoholic steatohepatitis (NASH) cases versus non-NASH cases. Its level was also increased in higher levels of fibrosis. Pentraxine 3 had no efficacy in differentiating different levels of NAFLD and fibrosis.

CONCLUSIONS: Hs-CRP can be used in combination with other biomarkers in the noninvasive evaluation of NAFLD.

Key Words:

Non-alcoholic Fatty Liver Disease, Biological Markers, C-reactive protein, Pentraxine.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is defined as a spectrum of chronic pathological changes in liver, ranging from pure asymptomatic hepatic steatosis to a potentially progressive nonalcoholic steatohepatitis (NASH), fibrosis and, finally, cirrhosis which can lead to hepatocellular carcinoma (HCC)^{1,2}. Prevalence of

NAFLD is reported as 25-30% of general population in western countries, ranked first among chronic liver diseases worldwide³⁻⁵. This prevalence is nearly 10% in general population of Asia⁶. NAFLD is more common in patients with metabolic syndrome^{7,8}. This figure reaches up to 57.5-74% in obese population⁹. Nineteen percent of obese people are diagnosed with steatohepatitis⁹. Diagnosis and management of these patients has a huge financial burden on health authorities worldwide and in Iran¹⁰.

Determining stage of NAFLD is essential for identifying prognosis and treatment decisions. Distinguishing pure steatosis from steatohepatitis is an important issue, as pure steatosis has benign prognosis while steatohepatitis can potentially lead to liver fibrosis^{2,11,12}. Lipid accumulation in hepatocytes can lead to inflammation within them. Accordingly, significant fibrosis can cause cirrhosis over a period of 10-20 years¹³, but the pathophysiology is not well understood yet¹⁴. 2-3% of NAFLD cases can progress to NASH¹⁵. In 25% of cases, NASH can progress to cirrhosis and its' accompanied related complications. So, early diagnosis of NAFLD is of great value. Currently, percutaneous liver biopsy remains the gold standard in evaluating liver histology and diagnosis of NAFLD. However, there are some limitations regarding performing biopsy such as being invasive, expensive, carrying sampling errors^{16,17} (only 1/50,000 of the whole liver is examined via biopsy)¹³, having inter and intra-observer disparities of 10 to 20% is reported in assessing samples causing either under or over estimation of the problem^{13,18}, needing hospitalization as well as liver biopsy associated risks like pain, hypotension, intra-peritoneal bleeding and damage to biliary system¹⁴. So, it can't be considered as a suitable

screening method¹⁹. Various noninvasive methods have been used for a definite diagnosis including clinical findings, blood markers, radiologic studies (ultrasound, MRI, elastography)^{20,21} but, these are not standardized yet in diagnosing the severity of fibrosis accurately⁹. Importance of evaluating serum biomarkers as noninvasive tests in distinguishing simple hepatic steatosis from NASH has been discussed in several clinical studies²²⁻²⁴. However, positive role of serum biomarkers for predicting severity of liver fibrosis has not been proven, yet. Consequently, it demonstrates a substantial need for developing an effective screening test to differentiate NASH from simple steatosis in order to determine those who are at increased risk for liver fibrosis prior progression to an advanced condition.

There are a growing number of evidences on the correlation of increasing serum biomarkers with severity of NAFLD. NAFLD is a mild chronic inflammatory disease and one could assume that this inflammatory process can increase systemic markers of inflammation. High-sensitivity C-reactive protein (hs-CRP) and pentraxin-3 (PTX-3) are acute phase proteins made in the liver in response to inflammatory processes²⁴. Recently the efficacy of these biomarkers for diagnosing NASH and any possible correlation between level of them with status of liver inflammation and fibrosis has been evaluated^{23,24}. However, there is not enough evidences regarding changes in hs-CRP and PTX-3 levels in NAFLD or NASH cases. We have tried to re-evaluate these biomarkers role in the context of noninvasive management of NAFLD.

The aim of the study was to investigate clinical usefulness of hs-CRP and PTX-3 plasma levels in differentiation of stages of NAFLD.

Patients and Methods

Population

This case-control study was based on data from a total of 66 Iranian cases (32 with biopsy proven NAFLD and 34 healthy control subjects) at Mazandaran Medical University, Imam Khomeini Hospital, Sari, Iran, between 2011 and 2012. The study was approved by the Institutional Review Board and all patients gave written informed consent for using their clinical data solely for research purposes before participation. Demographic, laboratory and clinical data were obtained from the study population. All our cases

were defined to have ultrasound confirmed fatty liver change and aminotransferases twice normal. The exclusion criteria for the cases included serological evidence of liver diseases including viral hepatitis B (positive HBsAg and/or anti HBc), chronic hepatitis C, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, hemochromatosis, Wilson's disease, recent history of consuming hepatotoxic drugs and drugs intervening with serum PTX-3 level (amiodarone, diltiazem, tamoxifen, glucocorticoids, statins)²⁴⁻²⁶, history of past or current ethanol consumption assessed by patient's medical history (more than 20 grams per day for men, more than 10 grams per day for women). None of cases had evidences of hepatic failure like ascites, hepatic encephalopathy, bleeding esophageal varices or any other considerable hepatic complication. Subjects with a history of chronic inflammatory disease, heart failure and autoimmune rheumatologic disease – which could increase PTX-3 level^{24,27,28} – were not recruited in our study. All NAFLD cases were undergone liver biopsy.

Control subjects were selected from non-obese patients who had normal liver ultrasound and aminotransferase levels and did not have diabetes mellitus, hyperlipidemia or positive markers of HBV or HCV. Clinical and para-clinical features of cases and controls were blinded entirely for investigators while performing validation essays and subsequent analysis.

Laboratory Evaluation

After completing medical assessment including a detailed history and physical examination, 10 ml whole blood samples were obtained from cases and controls and collected in sterile tubes. Then, plasma was extracted by centrifugation at 1500 rpm for 20 minutes at room temperature and stored frozen at -80°C . Hs-CRP and PTX-3 were quantitatively measured using special sandwich ELISA method described below. We also checked fasting serum samples (12 hours) for measuring metabolic variables (liver enzymes, blood sugar, liver function tests and lipid profile). All NAFLD cases were hospitalized and undergone liver biopsy using an automatic liver biopsy needle (Bard, gauge #16).

PTX3 and hs-CRP Measurement

The PTX3 kit (AdipoBioscience, Inc, Santa Clara, CA, USA) was used for quantitative measurement of PTX3, based on sandwich ELISA

method, according to company's instructions, using ELISA reader model ELX 500 (Biotech, Ft. Lauderdale, FL, USA) with a sensitivity of 0.1 ng/ml. Hs-CRP kit (DRG, Germany) was used for quantitative measurement of hs-CRP, using ELISA sandwich method and streptavidin conjugated specific monoclonal antibody, using ELISA reader model ELX500 made in Biomedical Company, USA (Waltham, MA, USA) with a sensitivity of 0.1 mg/l.

Liver Histopathology

All liver biopsy specimens were fixed in formalin, processed and stained with hematoxylin-eosin to be graded based on a standardized approach. Histological exam and scoring of all specimens were done by two experienced pathologists who were blinded to the clinical findings. As most studies in the literature have used two criteria for the diagnosis and classification of NAFLD cases, we have also used both systems^{29,30}. Diagnosis of steatosis was established based on presence of macrovesicular fat in at least 5% of hepatocytes in liver biopsies²⁹. Steatohepatitis criteria based on Brunt and Kleiner NAS activity score was defined as the following: hepatocellular steatosis, lobular inflammation, hepatocellular ballooning, perisinusoidal and peri-cellular fibrosis in zone 3 of hepatic acini³¹. Hepatic steatosis was graded based on percentage of hepatocytes containing macrovesicular fat as follows: Grade 0: No evidence of steatosis, grade 1: < 33% of hepatocytes contain macrovesicular fat, grade 2: 33-66% of hepatocytes contain macrovesicular fat, grade 3: > 66% of hepatocytes contain macrovesicular fat. Fibrosis stages were evaluated as following four-point scale: stage 0: No fibrosis, stage 1: perivenular or perisinusoidal fibrosis in zone 3, stage 2: pericellular and periportal fibrosis, stage 3: septal/bridging fibrosis, stage 4: cirrhosis. According to NAS score cases were classified into three subgroups: simple steatosis, borderline NASH and definite NASH. According to the more recent literature, cases were categorized into NASH and non-NASH groups^{24,30}. All data have been analyzed in cases in two different subclasses based on above mentioned categories.

Statistical Analysis

Descriptive statistics was reported as frequencies and percentages, or means and standard deviations. Data was analyzed using SPSS, version 19 (SPSS Inc., Chicago, IL, USA). An indepen-

dent *t*-test was used for univariate analysis to compare the scores of each of the measures and parameters between groups. Pearson chi-square was used to analyze differences of nonparametric variables between groups. A *p*-value of less than 0.05 was considered statistically significant.

Initially, all variables were checked for any difference between cases and controls; then the significant differences were looked for within cases between NASH and non-NASH based on Kleiner and also between three groups of NAS score. For the last part, analysis was performed in the NAS score groups putting the first 2 groups of simple steatosis and borderline NASH versus definite NASH.

Results

Clinical, demographic and biochemical data of patients (n=32) and controls (n=34) are summarized in Table I. Two patients were excluded from our study; one did not fulfill the diagnostic criteria for NAFLD in the biopsy specimen and another was diagnosed as having autoimmune hepatitis. Histopathological findings of liver biopsies are illustrated in Table II. According to Kleiner et al 32 patients with NAFLD were further classified into two subgroups as having NASH (n=20, 62.5%) and Non-NASH (n=12, 37.5%). Classification of patients based on NAS score showed that 10 patients had simple steatosis, 10 had borderline NASH and 12 had definite NASH. The clinical, demographic and biochemical data of NAFLD subgroups according to NAS score and Kleiner et al criteria are demonstrated in Table III and Figure 1.

Initially, all variables were checked for any difference between cases and controls; then the significant differences were looked for within cases between NASH and non-NASH based on Kleiner et al, and also between three groups of NAS score. For the last part, analysis was performed in the NAS score groups putting the first 2 groups of simple steatosis and borderline NASH versus definite NASH.

Demographic Data of Patients and Controls

Marked higher BMI was observed in patients in comparison with control subjects (*p* = 0.000); age of patients was significantly higher than controls, these were due to intentional selection of younger non-obese controls (*p* = 0.003) (Table I).

Table I. Clinical and biochemical data of study population.

Variable	Cases N = 32 mean ± SD	Controls N = 34 mean ± SD	p value
Age (year)	43.56 ± 13.52	34.79 ± 9.42	0.003
Sex (n)	M:19 F:13	M:10 F:24	0.025
Weight (kg)	80.03 ± 13.41	61.74 ± 10.59	0.000
Height (cm)	165.75 ± 8.97	165.44 ± 8.73	0.888
BMI (kg/m ²)	29.19 ± 4.39	22.39 ± 2.90	0.000
ALT (U/ml)	45.56 ± 16.8	18.35 ± 4.06	0.000
AST (U/ml)	75.25 ± 31.89	14.97 ± 5.12	0.000
ALP	206.16 ± 82.08	198.72 ± 79.87	0.782
Platelet (× 1000/ml)	258.95 ± 118.35	324.01 ± 67.23	0.875
FBS (mg/dl)	103.03 ± 23.12	83.59 ± 11.78	0.024
TG (mg/dl)	189.72 ± 95.28	143.18 ± 38.54	0.041
Cholesterol (g/dl)	192.19 ± 36.03	127.90 ± 24.79	0.034
HDL (mg/dl)	46.94 ± 9.76	47.27 ± 6.03	0.674
LDL (mg/dl)	115.91 ± 46.20	73.61 ± 27.82	0.036
Hs-CRP (mg/l)	0.029 ± 0.021	0.021 ± 0.027	0.210
Pentraxine (ng/ml)	1.751 ± 0.76	1.599 ± 0.51	0.344

Within the subgroups, mean age of NASH cases were higher than non-NASH but it was not statistically significant ($p = 0.24$) (Table III). Furthermore, considering their mean BMI, we did not find any significant difference of BMI within NAFLD subgroups (Table III).

Biochemical Data of Patients and Controls

As expected, mean AST and ALT levels in the case group were significantly higher than control group ($p = 0.00$) (Table I). Two by two comparisons within the patient group according to NAS score showed that between simple steatosis and borderline NASH there was not statistically significant differences in studied biochemical data.

Table II. Histopathological findings of liver biopsies.

Variable	NASH n (%)	Non-NASH n (%)
Lobular inflammation	11 (55)	9 (45)
ballooning	6 (30)	14 (70)
Perisinusoidal fibrosis	7 (35)	13 (65)
Pericellular fibrosis	7 (35)	13 (65)
Steatosis		
Grade 1	7 (35)	7 (58.3)
Grade 2	9 (45)	5 (41.7)
Grade 3	4 (20)	0
Fibrosis		
Stage 0	13 (65)	10 (83.3)
Stage 1	3 (15)	1 (8.3)
Stage 2	1 (5)	1 (8.3)
Stage 3	3 (15)	0

Comparison among simple steatosis and definite NASH had the same trend. Comparison between borderline and definite NASH revealed that cholesterol level in definite NASH was significantly higher than borderline NASH ($p = 0.026$) (Table III). Also, in another comparison we assessed simple steatosis and borderline NASH in one group versus definite NASH in another group. In this analysis we found mean FBS ($p = 0.040$), cholesterol ($p = 0.013$) and HDL ($p = 0.038$) levels in definite NASH were significantly higher than the first group (Table III). Other comparisons among these groups were not statistically significant.

In the patient group, mean level of hs-CRP (0.29 ± 0.21 mg/dl) was not significantly higher than controls (0.21 ± 0.27 mg/dl) ($p = 0.21$). Similarly, PTX-3 level in patients was nearly in the same range with controls; 1.75 ± 0.76 ng/dl and 1.59 ± 0.51 ng/dl, respectively ($p = 0.34$). There are no considerable differences between cases and controls in other biochemical variants (Table I).

Comparison of Serum Biomarkers Based on Fibrosis Stage

Evaluations of plasma hs-CRP and PTX-3 levels among patients in various stages of fibrosis are as following: differences between stage 0 and stage 1 fibrosis, stage 0 and 2 were not significant. Comparing stage 0 and 3, hs-CRP level was considerably higher in stage 3 fibrosis ($p = 0.017$) but PTX-3 level was not. Comparison between any degree of fibrosis and no fibrosis

Table III. Clinical and biochemical data of subgroups.

Variable	Kleiner criteria			NAS score		
	NASH N = 20 mean ± SD	non-NASH N = 12 mean ± SD	p value	Simple steatosis N = 10	Borderline NASH N = 10	Definite NASH N = 12
Age (year)	45.75 ± 10.42	39.92 ± 17.44	0.24	40.80 ± 9.62	46.30 ± 18.96	43.58 ± 11.46
Weight (kg)	77.65 ± 9.42	84.00 ± 18.06	0.20	82.40 ± 12.51	77.70 ± 7.55	80.00 ± 17.94
Height (cm)	164.80 ± 8.79	167.33 ± 9.44	0.44	170.3 ± 8.13	163.80 ± 10.44	163.58 ± 7.50
BMI (kg/m ²)	28.64 ± 3.22	30.10 ± 5.91	0.36	28.4 ± 3.98	29.05 ± 2.74	29.96 ± 5.83
ALT (U/L)	78.10 ± 33.33	70.50 ± 30.12	0.52	75.30 ± 25.31	77.10 ± 39.13	73.67 ± 32.90
AST (U/L)	47.90 ± 16.81	41.67 ± 16.75	0.31	39.50 ± 12.75	44.70 ± 20.76	51.33 ± 15.46
ALP	213.50 ± 93.11	193.92 ± 61.24	0.52	176.30 ± 47.59	240.80 ± 95.36	202.2 ± 88.2
Platelet (× 1000/ml)	226.38 ± 40.53	329.5 ± 194.3	0.076	243 ± 35.40	313.2 ± 199.2	226.1 ± 58.94
FBS (mg/dl)	107.95 ± 26.97	94.83 ± 11.51	0.12	96.10 ± 17.21	97.10 ± 11.38	113.8 ± 30.93
TG (mg/dl)	209.90 ± 98.28	156.08 ± 83.24	0.12	196.70 ± 103.4	162.00 ± 39.60	207.0 ± 120.4
Cholesterol (g/dl)	199.0 ± 38.97	180.83 ± 28.47	0.17	183.70 ± 31.66	176.80 ± 25.77	212.1 ± 39.78
HDL (mg/dl)	47.00 ± 10.49	46.83 ± 8.86	0.96	44.00 ± 8.27	44.40 ± 8.99	51.50 ± 10.49
LDL (mg/dl)	112.30 ± 30.35	121.92 ± 66.04	0.57	124.60 ± 72.28	107.1 ± 27.44	116.0 ± 31.42
CRP (mg/l)	0.031 ± 0.022	0.025 ± 0.020	0.46	0.021 ± 0.016	0.037 ± 0.030	0.030 ± 0.016
Pentraxine (ng/ml)	1.68 ± 0.85	1.86 ± 0.58	0.52	1.70 ± 0.711	1.72 ± 0.711	1.81 ± 0.892

showed no considerable difference among groups. Differences between significant (F3-F4) and mild fibrosis (F1-F2) was considerable for hs-CRP ($p = 0.014$) but not for PTX-3. Performing *t*-test for equality of variances demonstrated that hs-CRP and PTX-3 amounts did not differ significantly among NASH and Non-NASH cases based on Kleiner criteria. Data on the number of NASH and non-NASH patients based on fi-

bro sis stages is demonstrated in Table II. As we can see stage 0 fibrosis was the most common form among two subgroups.

Comparison of Serum Biomarkers Based on Steatosis Grade

Analysis of the hs-CRP and PTX-3 levels according to steatosis grade showed no significant difference between NASH and non-NASH cases

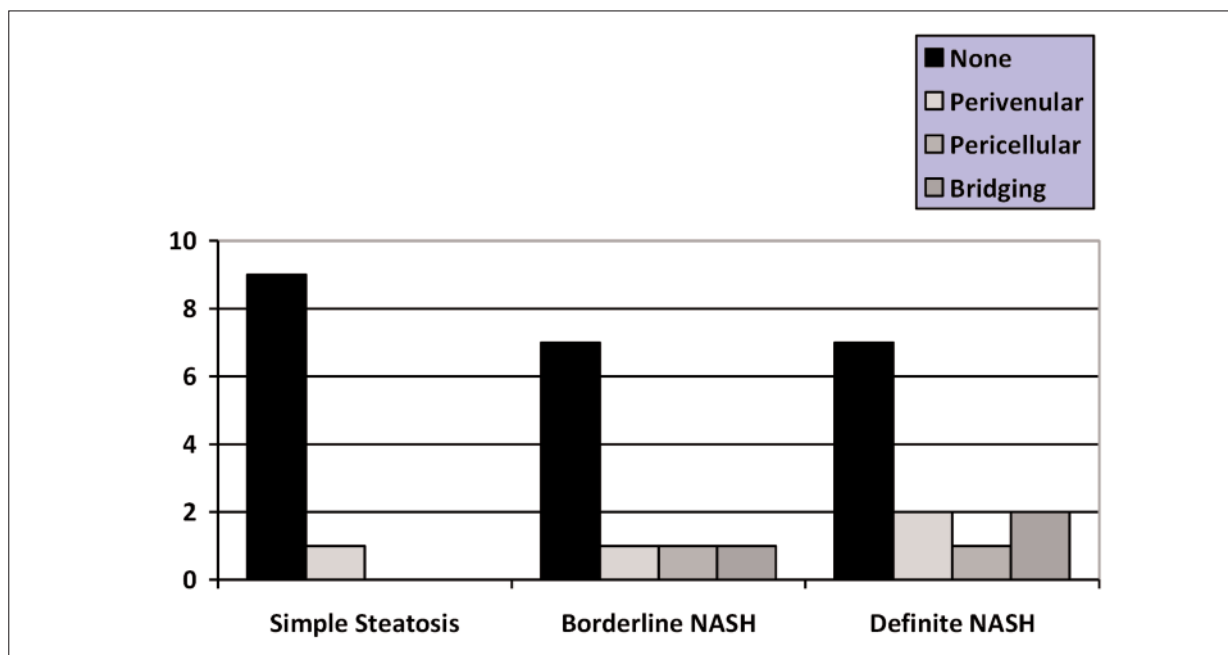


Figure 1. Distribution of fibrosis in cases based on NAS score subtypes.

based on Kleiner et al, and either no difference between the three groups based on NAS score (Table II, Figure 2). As shown in Table II, in the NASH and non-NASH group, steatosis grade 2 and grade 1 were the most common forms of steatosis, in order.

Discussion

NAFLD is a continuum of a chronic liver disease starting from simple and benign steatosis to steatohepatitis with or without fibrosis and cirrhosis. Hepatocellular carcinoma (HCC) can complicate the course of the disease after significant fibrosis has been deposited in the liver. Diagnosis of significant steatosis (more than 30% of hepatocytes) is usually made by ultrasound. In the course of the disease two milestones can be recognized, namely infiltration of inflammatory

cells (steatohepatitis) and deposition of fibrosis, those dictate the prognosis and are very important in the management of the patients.

Percutaneous liver biopsy can detect the above mentioned milestones with a reasonable sensitivity and specificity, but has limitations, that have been reviewed earlier. Multiple biomarkers have been studied in an effort to substitute invasive liver biopsy. Various study designs and the large number of biomarkers studied in the literature has not been able to give definite guidelines, though some authorities have proposed some potential guidelines to reduce the need for liver biopsy using a combination of serologic and imaging techniques^{21,32}.

Previously an association between hs-CRP and NAFLD has been reported; its hepatocyte level was elevated in cases of steatohepatitis compared to simple steatosis²³. A correlation of hs-CRP level was shown with the severity of fibrosis.

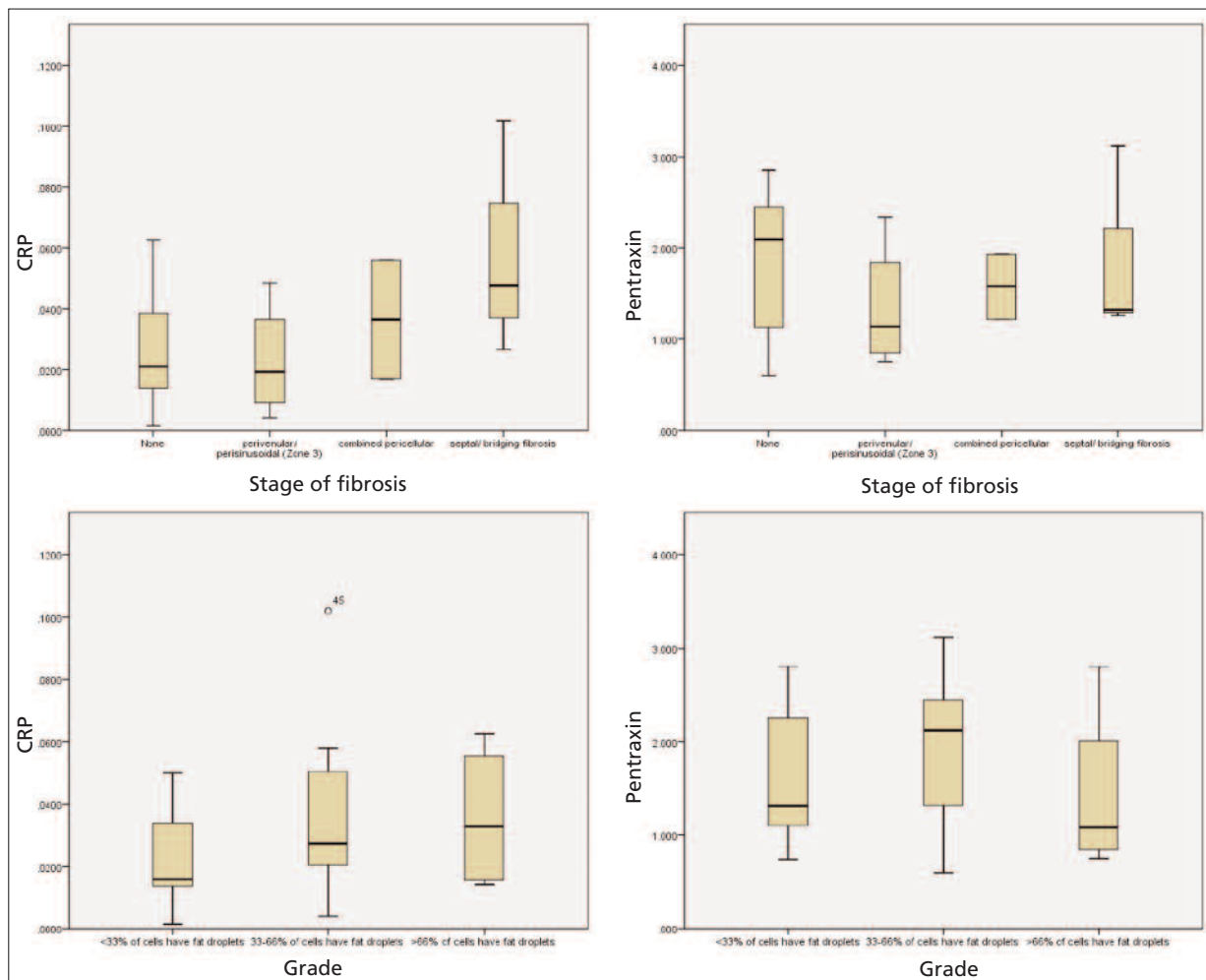


Figure 2. Boxplots of relation between severity of fibrosis and steatosis with serum biomarkers.

Hs-CRP was higher in controls than cases, though not significant, so it cannot be as an indicator of NAFLD as whole. But it was higher in NASH population versus non-NASH group (Table III). Rises of this biomarker was also proportional to the stage of fibrosis and semi-linear increase was detected in the higher degrees of fibrosis.

Pentraxine 3 was shown in one study to be valuable in differentiation of NASH from non-NASH and also was higher in higher degrees of fibrosis²⁴. Our study is the second experiment of this biomarker in NAFLD cases. We could not find any significant difference in comparison of cases/control, NASH/non-NASH, lower/higher degrees of fibrosis and steatosis in our study population. Pentraxine 3 is nonspecific marker of inflammation and has many confounding factors, so its use in the setting of NAFLD seems to be shaky.

Considering available data from this study and previous literature, it seems that no single biomarker will be able solely to differentiate multiple stages of NAFLD; so, a wise combination of biomarkers should be sought and used in this setting.

Pathologic evaluation of liver in NAFLD is the gold standard and should be reviewed cautiously. Many studies in this field have used NAS score for the diagnosis and staging of the disease. But as the developers of NAS score have clarified lately³⁰ this score was not developed for this reason and the Kleiner et al criteria should be used for the diagnosis of NAFLD instead.

We do recommend this type of studies on proposed biomarkers of NAFLD to be performed in different population around the world to find universal effective biomarkers in the setting of non-invasive evaluation of NAFLD.

Conclusions

Hs-CRP unlike pentraxine 3 can be used in combination with other serologic biomarkers in noninvasive diagnosis of NAFLD.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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