

# Combination of Aspartate Aminotransferase and Tumor Necrosis Factor- $\alpha$ as Non Invasive Diagnostic Tools for Non Alcoholic Steatohepatitis (NASH)

Hery D. Purnomo<sup>1</sup>, Farmaditya EP. Mundhofir<sup>2</sup>, Kasno<sup>3</sup>, Eddy Sudijanto<sup>4</sup>, Darmono<sup>1</sup>, Daldiyono<sup>5</sup>, R. Djokomoeljanto<sup>1</sup>, Sultana MH. Faradz<sup>2</sup>

<sup>1</sup> Department of Internal Medicine, Faculty of Medicine Universitas Diponegoro - dr. Kariadi Hospital, Semarang, Indonesia.

<sup>2</sup> Center for Biomedical Research, Faculty of Medicine Universitas Diponegoro, Semarang, Indonesia.

<sup>3</sup> Department of Pathology, Faculty of Medicine Universitas Diponegoro - dr. Kariadi Hospital, Semarang, Indonesia.

<sup>4</sup> Department of Radiology, Faculty of Medicine Universitas Diponegoro - dr. Kariadi Hospital, Semarang, Indonesia.

<sup>5</sup> Department of Internal Medicine, Faculty of Medicine Universitas Indonesia - dr. Cipto Mangunkusumo Hospital, Jakarta, Indonesia.

## Correspondence mail:

Hery Djagat Purnomo. Division of Gastroenterohepatology, Department of Internal Medicine, Faculty of Medicine Universitas Diponegoro - Dr. Kariadi Hospital. Jl. Dr. Sutomo 16, Semarang, Indonesia. email: herydjagat@yahoo.co.id

## ABSTRAK

**Tujuan:** untuk mengembangkan tes diagnostik non invasif untuk NASH. **Metode:** penelitian ini menggunakan desain potong lintang dengan pengambilan sampel secara konsekutif. Kriteria diagnosis NAFLD berdasarkan biopsi hati. Variabel klinis yang dianalisis terdiri atas sindrom metabolik, AST, ALT, adiponektin, TNF- $\alpha$ , insulin, HOMA-IR indeks dan biopsi hati. Pasien dibagi menjadi 2 kelompok berdasarkan biopsi hati yaitu non NASH, skor NAFLD  $\leq 3$  dan NASH, skor NAFLD  $\geq 4$ ) Analisis statistik dilakukan menggunakan tes Student-t, Mann Whitney U, chi-square, kurva ROC, dan sensitivitas, spesifisitas. **Hasil:** dari 50 pasien NAFLD (30 pria, 20 wanita) 12 orang menderita diabetes tipe 2 (24%), 36 orang memiliki sindroma metabolik (72%) dan 2 orang tidak memenuhi kriteria sindroma metabolik (4%). Biopsi hati pasien menunjukkan 21 (42%) non NASH dan 29 (58%) NASH. Terdapat perbedaan yang bermakna secara statistik antara dua kelompok AST, ALT, adiponektin dan TNF  $\alpha$ . Kombinasi AST  $\geq 25$  U/L dan TNF- $\alpha$   $\geq 3,28$  pg/cc mempunyai akurasi diagnostik yang baik untuk diagnosis NASH (Akurasi 82%, Sensitivitas 76%, Spesifisitas 90%, PPV 92%, dan NPV 73%. **Kesimpulan:** kombinasi AST dan kadar plasma TNF- $\alpha$  menunjukkan akurasi yang baik untuk mendeteksi NASH pada pasien NAFLD. Tes ini dapat digunakan sebagai metode non-invasif untuk mendiagnosis NASH.

**Kata kunci:** AST, TNF- $\alpha$ , tes diagnostik, NASH.

## ABSTRACT

**Aim:** to develop a non-invasive diagnostic test for non-alcoholic steatohepatitis NASH. **Methods:** this is a cross-sectional study on non-alcoholic fatty liver disease (NAFLD) subjects. Sample was taken by consecutive sampling method. Diagnostic criteria of NAFLD were confirmed by liver biopsy. Clinical variables include metabolic syndrome, aspartate aminotransferase (AST), alanine aminotransferase (ALT), adiponectin, TNF- $\alpha$ , insulin, homeostatic model assessment insulin resistance (HOMA-IR) index and liver biopsy. Patients were

divided into two groups based on their liver biopsy, group 1: Non-NASH (NAFLD activity score <3) and group 2: NASH (NAFLD activity score of  $\geq 4$ ). Statistical analyses were performed using Student's *t*-test, Mann Whitney U, chi-square, the ROC curve, sensitivity and specificity test. **Results:** fifty NAFLD patients were recruited, 30 males and 20 females. Among these patients, 12 (24%) had type 2 diabetes, 36 (72%) had metabolic syndrome, the remaining 2 (4%) did not fulfilled metabolic syndrome. Liver biopsy confirmed 21 (42%) non- NASH and 29 (58%) NASH respectively. Level of AST and ALT, plasma level of adiponectine and TNF- $\alpha$  were statistically different between two groups. The AST level ( $\geq 25$  U/L) in combination with TNF- $\alpha$  ( $\geq 3.28$  pg/cc) demonstrated a good diagnostic accuracy for NASH (Accuracy 82%, Sensitivity 76%, Specificity 90%, PPV 92%, and NPV 73%). **Conclusion:** the combined diagnostic tests of AST and TNF- $\alpha$  plasma levels demonstrated a good accuracy for the detection of NASH among NAFLD patients. This combination test can be used as a noninvasive method to diagnose NASH.

**Key words:** AST, TNF- $\alpha$ , diagnostic test, NASH.

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has become an emerging problem worldwide as a result of increased incidence of obesity. The severity spectrum of liver damage is ranging from simple macrovesicular steatosis to steatohepatitis (NASH), cirrhosis or liver carcinoma.<sup>1</sup> In the general population, the estimated prevalence ranges between 20 and 30% in the Caucasian and 30% in Indonesian.<sup>2,3</sup>

Majority of NAFLD patients were asymptomatic, therefore, case finding usually begins after detection of abnormal liver enzymes on routine evaluation.<sup>4</sup> Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) quite often shows mild to moderate elevation, while the correlation with gammaglutaryl-transferase (GGT) remains uncertain. Therefore, there is no overall correlation between the degree of liver enzyme elevation and the level of damage observed on histopathological analysis.<sup>1,4</sup>

Non-alcoholic steatohepatitis is the severe histological form of NAFLD. It is emerging as the most common clinically important form of liver disease in obese patients, diabetes and metabolic syndrome.<sup>5-9</sup> Liver biopsy is invasive and expensive, however, it is the gold standard for the diagnosis of NASH to differentiate simple bland steatosis. Liver biopsy has several limitations and not easily performed, since, it is an invasive method with potentially important complications happening with a mortality and morbidity rate of 0.03 and 3% respectively.

Moreover, a percutaneous liver biopsy obtain only a very small piece of the liver (~1/50,000 of whole size of the liver), leading to major sampling variability (25%-40%) and bias of inter-observer as high as 20%.<sup>10</sup>

Ultrasound (US), computerized tomography (CT), magnetic resonance imaging (MRI), and H magnetic resonance spectroscopy (H MRS) are noninvasive methods. However, they do not provide reliable quantitative information.<sup>11</sup> Because of these limitations, it is important to develop new methods to asses NAFLD, particularly the presence or absence of NASH and the severity of any complicating fibrosis or cirrhosis. To achieve this aim, the tests should be non-invasive, accurate, reproducible and affordable.<sup>12</sup> Studies to determine non-invasive diagnostic tests of NASH in NAFLD patients in Indonesia were rarely conducted, therefore producing clinical laboratory tool that capable for identifying individual with most risk for NASH in NAFLD patients is important. This study was aimed at producing a clinical laboratory tool capable of identifying individual with most risk for NASH in NAFLD patients. In addition, this tool will predict NASH without the risks associated with hepatic biopsy and clinically important to prognosis and therapeutic intervention.

## METHODS

This cross-sectional study was performed on NAFLD patients at the outpatient clinic of Dr. Kariadi Hospital, Semarang between August

2009 and July 2011. Recruitment was conducted using consecutive sampling method. Inclusion criteria for this study were patients with one or more symptoms of metabolic syndrome and feature of fatty liver by ultrasound. These patients should be confirmed for diagnostic of NAFLD and the severity of liver damage using histopathology biopsy without any other evidence of liver injury due to alcoholism or viral hepatitis. Patients with alcohol consumption more than 20 g per day or having regular intake of drugs which were known to produce steatosis or any other circumstance with associated liver disease were excluded. This study was approved by the human research ethics committee of The Faculty of Medicine Diponegoro University–Dr Kariadi Hospital Semarang and informed consent was obtained from each patient.

The following data were evaluated: gender, age, body mass index (BMI), waist circumference, presence of hypertension and diabetes mellitus, high density lipoprotein (HDL), fasting glucose (FG), triglycerides, homeostatic model assessment of insuline resistance (HOMA-IR) index, aspartate aminotransferase (AST), alanin aminotransferase (ALT), plasma levels of adiponectine and TNF- $\alpha$  from the 50 patients recruited. Plasma levels of Insuline, adiponectine and TNF- $\alpha$  were measured using ELISA method. The diagnosis of metabolic syndrome followed the criteria established by the International Diabetes Federation (IDF) 2005.<sup>13</sup>

NAFLD was diagnosed based on ultrasound by 2 radiologists (Kappa=0.8). The ultrasonography criteria that were used to diagnose and severity of fatty liver was described as previous study by Sherif Saadeh, et al.<sup>14</sup>

Liver biopsy was performed in all 50 patients with ultrasound-guided using a 16-gauge Menghini type needle (Hepafix® B. Braun Melsungen AG, Germany) under local anesthesia.<sup>15</sup> All liver biopsies were reviewed by two experienced pathologists (Kappa=0.87) who were blinded to the patient data. Diagnosis of NASH was based on Histological Scoring System for Non Alcoholic Fatty Liver Disease Score and Fibrosis staging by NASH clinical research network (CRN) scoring system (NAFLD activity score=NAS). Based on the scoring system, the

value of NAS  $\leq 2$  = Simple Fatty, NAS 3-4 = Possible NAS and NAS  $\geq 5$  = NASH.<sup>16</sup>

Patients were classified by modified NAS in two groups: Non-NASH group (patients without NAFLD if NAS  $\leq 3$  and NASH group if NAS  $\geq 4$ . Demographic, laboratory and imaging data were compared between both groups).

Statistical analyses used were student T-test, Mann Whitney U test, chi-square test and ROC curve analysis. To evaluate the sensitivity and specificity of cut off levels, computer software SPSS for Windows PC version 21 (SPSS Inc, Chicago, IL) and a significant level of 5% were used.

## RESULTS

We evaluated 50 patients with NAFLD, 30 (60%) male, with mean (SD) of 45.5 (10.4) years of age and median of BMI of 29.5 kg/m (22.0-47.5), mean (SD) of waist circumference 100.2 cm (10.8), Metabolic syndrome was present in 36 patients (72%) and type 2 DM in 12 subjects (8 male). The chief complaints of these patients consist of 10% asymptomatic, 30% fatigue, 60% abdominal discomfort. Clinical manifestation of the patients were obesity/BMI > 25 kg/m<sup>2</sup> (82%), hypertriglyceridemia (78%), fasting hyperglycemia in 56% patients and level of ALT (72%) and AST (62%) patients within normal range, diabetes (24%) and hypertension in 50% patients. (**Table 1**)

Female presented higher BMI 30.2 (24-47) kg/m than male 28.5 (22-40) p=0.03 Mann-Whitney U test), and it similar age (males 42.5 y (29-74); female: 49.0 y (23-61), p=0.27, Mann-Whitney U test). The proportion of cases with metabolic syndrome was also similar (males 24/30 cases; women 12/20 cases, p=0.12, chi-square test). Metabolic syndrome occurrence was similar in the NASH group (22/29 cases) when compared to Non NASH group (14/21; p=0.47, chi-square test).

Based on the results of liver biopsy; 21 (42%) were non NASH and 29 (58%) patients were NASH and non fibrosis (62%). (**Table 2**) The degree of fatty liver (FL) by ultrasound were as follow; 29 patients (58%) FL grade I, 13 (26%) FL grade II, and 8 patients FL grade III. Abdominal US with degree/grade II/III

**Table 1. Clinical and laboratory characteristics of the groups**

| Variables                 | Non-NASH<br>Mean (SD),<br>Median (Range) | NASH<br>Mean (SD),<br>Median (Range) | p      |
|---------------------------|--|--------------------------------------|--------|
| Ages (years)              | 44.7 (11.4)                              | 46.3 (10)                            | 0.50   |
| BMI (kg/m <sup>2</sup> )  | 30.1 (23-38)                             | 29.0 (22-47)                         | 0.79   |
| Waist Circumference (cm)  | 100.8 (8.5)                              | 100.1(12.3)                          | 0.24   |
| Cholesterol total (gr/dl) | 205.2 (50)                               | 196 (32.3)                           | 0.11   |
| Triglyceride (gr/dl)      | 202 (54-643)                             | 205 (48-467)                         | 0.24   |
| Fasting glucose (gr/dl)   | 101 (87-202)                             | 102 (68-208)                         | 0.48   |
| AST (IU/L)                | 25 (17-77)                               | 40 (10-120)                          | 0.019* |
| ALT (IU/l)                | 50 (14-177)                              | 60 (19-264)                          | 0.045* |
| Adiponectine (ng/ml)      | 4222 (493-9766)                          | 2116 (679-4500)                      | 0.005* |
| TNF- $\alpha$ (pg/ml)     | 3.35 (1.8-87)                            | 11.55(1.6-332)                       | 0.001* |
| Insuline ( $\mu$ IU/ml)   | 10.02 (1.8-87)                           | 14.8 (2.0-117)                       | 0.163  |
| Homa IR-index             | 3.3 (0.6-20)                             | 4.5 (0.4-29)                         | 0.258  |

\*P&lt;0.05

**Table 2.** Distribution of NAFLD activity score according to the liver biopsy; NASH or Non-NASH

|                            | NASH     | non-NASH |
|----------------------------|----------|----------|
| Fatty Liver (NAS $\leq$ 2) |          | 21 cases |
| Possible NASH (NAS 3-4)    | 13 cases |          |
| NASH (NAS $\geq$ 5)        | 16 cases |          |
| Fibrosis positive          | 8 cases  | 5 cases  |
| Fibrosis negative          | 21 case  | 16 cases |

associated with presence of NASH p=0.027) sensitivity and specificity of 52.2% and 76.2%, respectively to identify NASH.

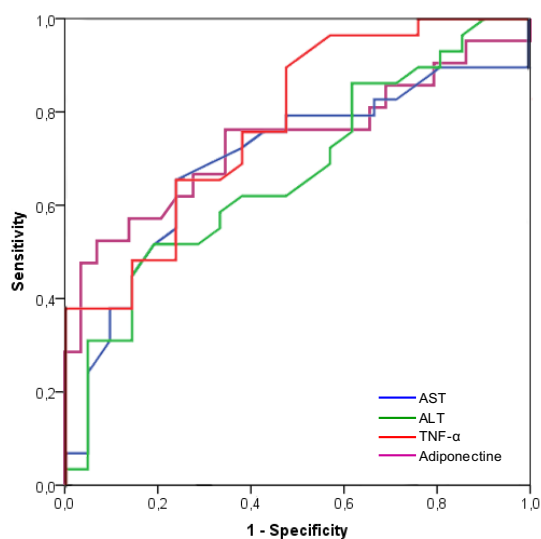
There were no statistically differences in the variables of age, BMI, waist circumference, HDL cholesterol, triglyceride, fasting blood sugar, insuline and HOMA-IR between two groups. There were statistically differences in variables of AST, ALT, adiponectine, and plasma levels of TNF- $\alpha$  between the two groups. (**Table 1**)

TNF- $\alpha$  and AST cut-off values were determine separately from smallest distance value that computed from the value of sensitivity and specificity from ROC curve analysis. Using receiver operating characteristic (ROC) analysis, we established the following best cut –off levels (equal or above being indicative of NASH: ALT >53.5 IU/L, AST >25 IU/L, TNF- $\alpha$  >3.28 pg/ml, adiponectine  $\leq$ 2174 ng/ml. (**Table 3, Figure 1**). Each test alone produced low accuracy; therefore, we decided to evaluate combination between two markers to obtain the best accuracy.

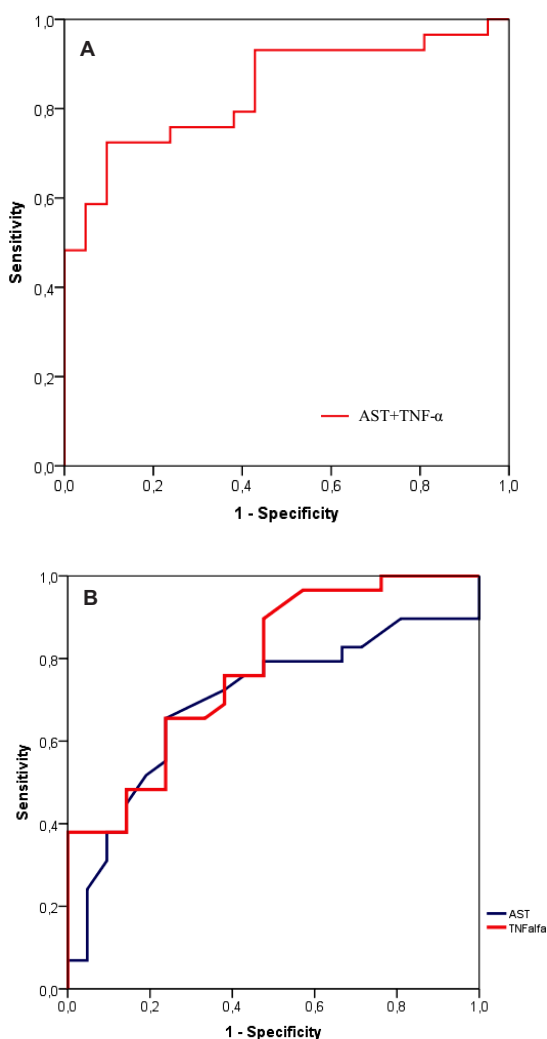
ROC curve of combination TNF- $\alpha$  and AST were generated from predictive value of TNF- $\alpha$  and AST for NASH prediction from logistic regression analysis output. Combination of serum AST (>25U/L) and plasma levels of TNF- $\alpha$  (3.28 pg/cc) has a moderate accuracy as a non-invasive diagnostic test for detection of NASH. (Accuracy 82%, Sensitivity 76%, Specificity 90%, PPV 92%, and NPV 73%). (**Figure 2**)

**Table 3.** Cut-off and ROC curve analysis of each marker for NASH

| Test                  | Cut-Off    | AUC (95% CI)       | p     | Sensitivity | Specificity |
|-----------------------|------------|--------------------|-------|-------------|-------------|
| AST (IU/L)            | 25 IU/L    | 66.7% (51.6-81.9)  | 0.019 | 79          | 52          |
| ALT (IU/L)            | 53.5 IU/L  | 69.6% (54.7-84.0)  | 0.045 | 79          | 62          |
| TNF- $\alpha$ (pg/ml) | 3.28 pg/ml | 77.8% (64.9-90)    | 0.001 | 93          | 48          |
| Adiponectine (ng/ml)  | 2174 g/ml  | 73.6% (58.4- 88.8) | 0.005 | 66          | 71          |



**Figure 1.** ROC analysis result of AST, ALT, TNF- $\alpha$  and adiponectin to identify presentation of NASH. TNF- $\alpha$  and adiponectin showed highest AUC value >70



**Figure 2.** ROC curve of combination AST and TNF- $\alpha$  for NASH prediction (n=50). Area under the curve was 0,841 (95% CI= 0,73 to 0,95) p<0,001

**DISCUSSION**

NAFLD is often not diagnosed properly in clinical practice because it is usually asymptomatic. Previous study showed that the majority of patient’s complaints are discomfort in the abdomen and fatigue which is similar with our study.<sup>17</sup> These symptoms are not specific to NAFLD but vague discomfort over the liver with hepatomegaly is common among patients with NAFLD in hospital setting. However, in the general population it is most often asymptomatic.

This study demonstrated that 82% of patients with obesity (BMI>25 kg/m<sup>2</sup>) and 72% with metabolic syndrome. Some studies showed that obesity is a metabolic illness closely associated with both steatosis and NASH. In addition, the severity of the hepatic illness is related to the increase of BMI.<sup>18,19</sup>

Another study found difference with this statement, that BMI did not differ between groups with and without NASH in morbidly obese patients, and that insuline resistance, hypertension, and elevated ALT were independently predicted the presence of NASH.<sup>20,21</sup> Our study showed that BMI and waist circumference, metabolic syndrome, ages, cholesterol total, triglyceride, fasting glucose, insulin resistance did not differ between groups with and without NASH.

The major abnormalities of serum liver are elevations of ALT and AST level. The majority of elevations are mild (<5x the upper normal limit), and exist in all degrees of NAFLD. However, there is no correlation between the degree of liver enzyme elevation and the level of damage observed in histopathological analysis. This study showed that the level of ALT and AST in majority of NAFLD patients were in normal laboratory range. However, there was a significant difference in group of NASH and without NASH (**Table 1**). Alanine aminotransferase (ALT) has been used as a substitute indicator for liver lesion. Some studies recommended that ALT is not an ideal biomarker for the diagnosis of NAFLD or distinguish steatosis from NASH. In a cross-sectional population study, based on hepatic triglyceride content by magnetic resonance spectroscopy for diagnose hepatic steatosis, nearly one-third (31%) of the patients

had hepatic steatosis. The majority of the patients with steatosis (79%) had normal serum ALT values. In another study, 58 out of 80 (72.5%) subjects who had ALT within normal value had varying degree of NASH during underwent surgery irrelevant to liver diseases. Twenty six patients had fibrosis and 8 had silent cirrhosis. The third study reported that the whole histologic continuum of NAFLD could be observed in the individuals within normal limit of ALT. The histological continuum of NAFLD in subjects with NAFLD and ALT within normal limit was not significantly different from those with NAFLD and elevated ALT. A low normal ALT value, therefore, does not guarantee to rule out of NASH and advanced fibrosis.<sup>22</sup>

Metabolic syndrome and insuline resistance in this study were not significantly different between NASH and non-NASH patients. This is probably due to proportion of samples as well as no significant difference in the frequency of central obesity in both groups. Various studies have shown that the basic components of the insulin resistance/metabolic syndrome are the presence of central obesity.

Adiponectine is plentiful adipocyte-derived hormone with well recognized anti-inflammatory and insulin sensitizing activity. Hypoadiponectinemia along with insulin resistance proved to be an independent risk factors for the metabolic syndrome.<sup>23</sup> The importance of adiponectine in protecting obesity-related NAFLD has been gradually more established. Even though the advances made in current years, the comprehensive underlying mechanism of hepato-protective functions remains largely undetermined.<sup>22</sup> Adiponectine usually predicts degree of steatosis and the severity of NAFLD; nevertheless, to what level this is a direct consequence or linked to the existence of more severe insulin resistance or obesity remains to be handled. Our study showed that plasma level adiponectine is significantly higher in non-NASH group compared to NASH group. Rinaldi (2009) also reported similar results with our study.<sup>17,24</sup>

Several data support the pathophysiological role of tumor necrosis factor (TNF) in non-alcoholic fatty liver disease (NAFLD).<sup>25,26</sup> Obese

and NASH patients have high plasma level of TNF because TNF gene expression is also increased in adipose and liver tissue of obese patients. NASH patients with hepatic fibrosis have higher TNF mRNA expression. Prevalence of TNF polymorphism was increased in NAFLD patients. NASH have similar liver histology with alcoholic steatohepatitis (ASH), a disease in which TNF is recognized to be an essential pathophysiological roles.<sup>25</sup> Our study found that plasma level of TNF- $\alpha$  is more higher in NASH compared to non-NASH group.

TNF- $\alpha$  plays a significant role in insulin resistance, the main characteristic of metabolic syndrome through inhibition of tyrosine kinase activity of the insuline receptor. Abiru et al. (2006) reported that NASH patients had significantly higher serum TNF- $\alpha$  and its soluble receptor (sTNFR1) than those with simple fatty liver, even though they did not give a cut-off value of the cytokine for clinical use. Current study reported that NASH patients had higher levels of TNF- $\alpha$  messenger ribonucleic acid (mRNA) than healthy controls. TNF- $\alpha$  mRNA cut-off value of 100 ng/mL for prediction of NASH [area under receiver operating characteristic curves (AUROC) 0.685, sensitivity 66.7%, specificity 74.1%].<sup>22</sup>

In clinical practice, the ideal biomarkers or panels of biomarkers are urgently needed which are cheaper, reliable, and reproducible for patients with NASH. It is needed to be able to assist in diagnosis, providing risk information, and monitoring disease progression and treatment response. Our study suggests that serum AST >25 IU/l and plasma level of TNF- $\alpha$  of 3.28 pg/ml had the best diagnostic value for the presence of NASH. To our knowledge this is the first study using combination of serum AST plus TNF- $\alpha$  plasma level for diagnosis NASH in NAFLD patients.

Various non-invasive diagnostic tests for NASH that have been studied include contrast enhanced ultrasound, NASH test and CK-18. Contrast enhanced ultrasound demonstrated AUROC of 100% for diagnosis of NASH. NASH test has Specificity, Sensitivity, PPV and NPV of 94%, 33%, 66% and 81% respectively for diagnosis of NASH. CK -18 was AUROC,

sensitivity and specificity for NASH of 0.82, 0.78 and 0.87 respectively.<sup>19</sup> Recent study concluded that serum fragmented CK-18 levels can be used to distinguish NASH with NAFL.<sup>18,27</sup>

Our study proves that the combination of level AST serum and TNF- $\alpha$  plasma can be used in clinical practice to diagnose the presence of NASH. At least it can be utilized in predicting the presence of NASH.

It should be considered that this study involved a small number (single center) of patients. Therefore, larger or multicenter studies are required to obtain more consistent results in order to be applied in clinical practice. Furthermore, another limitation is that healthy controls were not included in this study. As a consequence, we cannot observe the spectrum of abnormalities from normal to some extent of conditions, so that the implementation of this study is of limited only in patients with NAFLD, does not include a healthy population/without risk factors.

## CONCLUSION

The combination between AST serum and plasma levels of TNF- $\alpha$  tests showed a moderate accuracy for the detection of NASH among patients with NAFLD. This combination can be utilized as a screening tool for NASH in NAFLD patients, and can be alternative of liver biopsy for diagnosis of NASH. Further studies are still needed using large number of samples or using multicenter study in effort to examine this tool as a non-invasive diagnostic test for NASH in clinical practice.

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