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*J.Biomed.Transl.Res*
*ISSN: 2503-2178*

JOURNAL OF BIOMEDICINE AND TRANSLATIONAL RESEARCH

**Available online at JBTR website: https://jbtr.fk.undip.ac.id**



Original Research Article

# Increased Level of the Plasminogen Activator Inhibitor Type-1 Is Associated with Severity of NAFLD

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**Article Info**
History
Received: 28 Oct 2022
Accepted: 17 May 2023
Available: 31 Aug 2023

**Abstract**

**Background**: Non-Alcoholic Fatty Liver Disease (NAFLD) has been the most common cause of chronic liver disease worldwide. In NAFLD, elevated Plasminogen Activator Inhibitor-1 (PAI-1) is associated with risk factors for thrombosis and a hypercoagulable state.

**Objective**: This study explored the relationship between NAFLD as an independent factor for increasing PAI-1 in the presence of metabolic syndrome and insulin resistance.

**Methods**: This observational study used a cross-sectional design with 80 subjects including 40 patients with NAFLD and 40 without NAFLD at dr. Kariadi General Hospital Semarang who met the inclusion and exclusion criteria. Measurement of plasma PAI-1 levels was done using ELISA method.

**Results**: A total of 80 patients were included. In the case group, based on abdominal ultrasound, NAFLD was mostly found in the mild category (86%), while the rest were moderate (10%), and severe (4%). Only 25% were found in the Simple Steatosis category, and 75% were suspected of having NASH (NAS = 3-4) and NASH (NAS > 5). There was a significant difference between plasma PAI-1 level and the incidence of NAFLD (p = 0.011). The mean PAI-1 level between the three NAFLD severity categories also showed a significant difference (p = 0.032).

**Conclusion**: There is an increase in PAI-1 levels in patients with NAFLD. PAI-1 levels have an independent effect on the degree of liver fibrosis in patients with NAFLD.

**Keywords**: *NAFLD; NASH; PAI-1; liver fibrosis*
**Permalink/ DOI:** https://doi.org/10.14710/jbtr.v9i2.16293

## INTRODUCTION

Non-Alcoholic Fatty Liver Disease (NAFLD) has been the most common cause of chronic liver disease worldwide. The global prevalence of NAFLD is estimated at 24%; the highest rates are reported from South America and the Middle East, followed by Asia, the USA, and Europe.[1,2,3] The prevalence of NAFLD worldwide increased from 391.2 million in 1990 to 882.1 million in 2017, with the prevalence rate increasing from 8.2% to 10.9% during this same period. In the general population, the prevalence of NASH is estimated at 1.5–6.45%.[4,5]

A study about the prevalence of NAFLD in Asia found that the prevalence of NAFLD cases was projected to increase by 6%-20% during 2019-2030, with related mortality projected to increase between 65% and 100% from 2019 to 2030.[6] Recent meta-analyses and systematic reviews have estimated that the global prevalence of NAFLD is between 25.2% and 29.8%.[7] The prevalence increases among people with obesity, diabetes, and metabolic syndrome, where these patients also experience an increased risk of developing advanced fibrosis and cirrhosis.[8]

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**Table 1.** General characteristics of research subjects

| Characteristics | Group | | P |
|---|---|---|---|
| | Case | Control | |
| Gender | | | |
| • Men | 20 (50%) | 20 (50%) | |
| • Women | 20 (50%) | 20 (50%) | 1.000 |
| Age | 43.30±8.68 ;43;29-59 | 46.3±8.35; 48;26-60 | 0.074 |
| Body Mass Index[£] | 29.71±5.06 ;29.17;22.49-47.45 | 22.99±2.55; 23.45;18.3-27.5 | **<0.0001** |
| Waist circumference (cm)[$] | 98.2±10.55 ;98;77-134 | 84.95±7.15; 86.5;64-98 | **<0.0001** |
| Blood pressure (n) [¥] | | | |
| • ≥ 130/85 / history of hypertension | 11 (27.5%) | 6 (15.0%) | 0.274 |
| • < 130/85 | 29 (72.5%) | 34 (85.0%) | |

[¥] Statistical test with Chi square
[£] Statistical test with Mann Whitney test
[$] Statistical test with T test
Mean±SD;median;min-max

**Table 2.** Differences in plasma PAI-1 levels with the incidence of NAFLD

| Variable | n | Mean ±SD | Median | Min | Max | p |
|---|---|---|---|---|---|---|
| Case | 40 | 112.47±47.55 | 114.04 | 93.92 | 123.67 | **0.011*** |
| Control | 40 | 107.22±94.89 | 108.96 | 83.75 | 119.85 | |

* Statistical test with Mann-Whitney Test

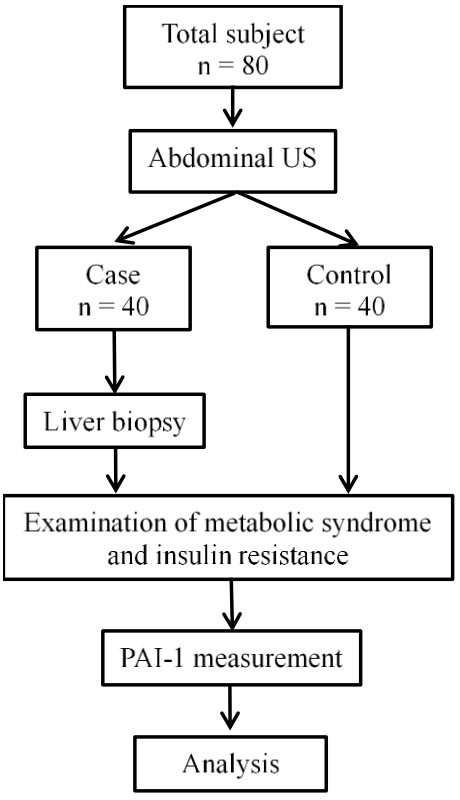


**Figure 1.** Study Flow Chart

NAFLD is strongly associated with an increased risk of cardiovascular disease (CVD). NAFLD and CVD share several risk factors for metabolic syndrome, including central obesity, hypertension, dyslipidemia, insulin resistance, metabolic syndrome, impaired glucose tolerance, type 2 diabetes, endothelial dysfunction, low plasma insulin-like growth factor-1 (IGF-1) and high plasma hemostatic.[9,10] In NAFLD, inflammation occurs when several pro-inflammatory and oxidative stress cytokines are released, such as CRP, IL-6, TNF- and other reactive oxygen species. Proatherogenic and hypercoagulable-hypofibrinolytic factors were also increased such as fibrinogen, factor VIII, tissue factor and Plasminogen Activator Inhibitor-1 (PAI-1).[11,12]

Elevated PAI-1 is associated with risk factors for thrombosis and liver fibrosis. Thus, there is a relationship between the development of liver fibrosis in chronic liver disease and hypercoagulable conditions, especially triggered by prothrombotic conditions in NAFLD.[12,13] Various studies have attempted to evaluate the role of PAI-1 in the pathogenesis of NAFLD, but they demonstrated conflicting results. Animal studies demonstrate that inhibition of PAI-1 prevents fatty liver.[14] Therefore, we conducted the association between PAI-1 and NAFLD in different severity biopsies proven

**Table 3**. Differences in Mean plasma PAI-1 levels according to NAFLD severity by NAS

| Variable | n | PAI-1 level (ng/ml) | | | | p* |
|---|---|---|---|---|---|---|
| | | Mean ±SD | Median | Min | Max | |
| NASH | 30 | 112,54±7.58 | 114.74 | 93.92 | 123.67 | 0.032 |
| Simple Steatosis | 10 | 112,28±4.54 | 111,66 | 105.62 | 121.82 | |
| Control | 40 | 107.22±9.48 | 108.96 | 83.75 | 11.85 | |

* Statistical test with Kruskal-Wallis Test
Statistical Mann-Whitney Test : NASH vs Simple steatosis p = 0,417 , NASH vs control p= 0.013 , Simple Steatosis vs control p= 0.174

**Table 4.** Differences in plasma PAI-1 levels with fatty liver status based on US

| Degree | n | PAI-1 level (ng/ml) | | | | p* |
|---|---|---|---|---|---|---|
| | | Mean ±SD | Median | Min | Max | |
| Severe | 4 | 114.64±7.47 | 115.40 | 104.79 | 122.97 | 0.046 |
| Moderate | 11 | 113.27±3.76 | 113.56 | 105.87 | 119.47 | |
| Mild | 25 | 111.78±7.92 | 111.78 | 93.92 | 123.67 | |
| Control | 40 | 107.22±9.48 | 108.96 | 83.75 | 119.85 | |

* Statistical test with Uji ANOVA
Post Hoc test: Severe vs Moderate p = 0.78 ; Severe vs Mild p= 0.527 ; Severe vs Control p= 0.095 ; Moderate vs Mild p=0.623 ; Moderate vs Control p=0.037 ; Mild vs Control p=0.036

in humans. Whether the NAFLD acts as independent risk factor for increasing PAI-1 in the presence of metabolic syndrome and insulin resistance or not, needs a further study.

## MATERIALS AND METHODS

This observational study used a cross-sectional design with 80 subjects including 40 patients with NAFLD and 40 without NAFLD at dr. Kariadi General Hospital Semarang who met the inclusion and exclusion criteria. This study was approved by the Health Research Ethics Committee of Dr. Kariadi General Hospital Semarang (number 696/EC/KEPK-RSDK/XII/2017). The case group contained subjects who had fatty liver based on abdominal ultrasound (US) and had undergone liver biopsy to determine the severity of liver fibrosis. The control group contains healthy subjects without fatty liver as evidenced by abdominal US.

Diagnosis of NAFLD was confirmed by the abdominal US GE Logiq S4 done by 2 experienced radiologists, with or without a liver biopsy performed by 1 experienced hepatologist. The inclusion criteria in this study included: adult age more than 14 years old, met the NAFLD criteria from abdominal US examination results which showed fatty liver and without history of alcohol consumption or alcohol consumption did not exceed 70 g/week for women and 140 g/week for men. The exclusion criteria were patients with hepatitis B and C, patients with alcoholic liver disease, taking drugs that can cause fatty liver, have a disorder or history of disease that can cause fatty liver, patients with other liver disorders, arteritis, connective tissue disorders, kidney disease, or thyroid disorders, and patients with weight loss more than 3 kg in the last three months.

The blood samples used in this study were venous blood of patients with NAFLD cases and non-NAFLD controls that had been stored (stored sample). The blood taken was stored as serum/plasma at a temperature of -$80^0$C. Measurement of plasma PAI-1 levels was done using ELISA method with the Human PAI-1 (Elabscience, United States). Examination of plasma PAI-1 levels was carried out in the GAKI laboratory Faculty of Medicine Universitas Diponegoro which is licensed and standardized. The PAI-1 levels were measured using the following steps : 50 µL of standard and sample incubated for 90 minutes at 37 °C, then mixed with 100 µL Biotinylated detection Ab, incubated again for 1 hour at 37 °C. Aspirate and wash 3 times, add 100 ml HRP and incubate for another 30 minutes at 37°C. Aspirate and wash 5 times then add 90 µL of substrate reagent, incubate for 15 minutes at 37 °C, add 50 µL of stop solution then read immediately at 450 nm. Results are recorded in ng/ml units. The flow of the study is explained in Figure 1

**Table 5.** Differences in plasma PAI-1 levels with clinical and laboratory parameters

| Variable | Plasma PAI-1 level (ng/ml) | | | p |
|---|---|---|---|---|
| | Simple Steatosis | Probable & definite NASH | Controls | |
| *Waist circumference* category | | | | |
| Central obesity | 112.28±7.25 | 112.44±8.17 | 106.61±1.04 | 0.066*[1] |
| Without central obesity | - | 113.00±3.95 | 107.83±8.67 | 0.227[#] |
| BMI category | | | | |
| Obesity | 111.38±4.24 | 115.07±6.11 | - | 0.121[£] |
| Without obesity | 112.89±5.03 | 110±8.24 | 107.22±9.48 | 0.266* |
| Blood pressure | | | | |
| ≥ 130/85 /history of anti-hypertension drug | - | 110.76±9.05 | 108.66±5.78 | 0.506[#] |
| < 130/85 | 112.02±4.74 | 113.43±6.81 | 106.97±10.04 | **0.040[¥]** |
| ALT level[¥] | | | | |
| Increased (>65 IU/L) | - | 111.64±6.32 | 103.68±1.94 | 0.751[£] |
| Normal | 112.04±4.74 | 113.32±8.67 | 107.41±9.16 | **0.039[¥]** |
| AST level[¥] | | | | |
| Increased (>37IU/L) | 116.03±26.94 | 111.57±9.14 | 107.74±12.61 | 0.537* |
| Normal | 110.67±3.45 | 113.09±6.73 | 107.16±9.30 | **0.040*** |
| Ratio AST/ALT | | | | |
| AST/ALT > 1 | 111.24±3.75 | 112.76±6.90 | 108.54±8.68 | 0.129[¥] |
| AST/ALT < 1 | 114.70±6.17 | 110.48±14.40 | 103.75±11.03 | 0.290* |
| Blood fasting glucose (mg/dL) | | | | |
| > 100 / history DM type 2 | - | 109.92±9.59 | 105.40±10.09 | 0.355[#] |
| ≤ 100 | 112.03±4.74 | 114.05±5.91 | 107.67±9.44 | **0.022*** |
| HDL cholesterol level (mg/dL) | | | | |
| < 40 (male) or < 50 (female) or history of lipid profile disturbance | 110.75±3.83 | 111.45±7.81 | 107.85±9.22 | 0.476[¥] |
| ≥ 40 (male) or ≥ 50 (female) | 113.30±5,02 | 113.07±7,61 | 104.23±10.89 | **0.048*** |
| Fasting TG level (mg/dl) | | | | |
| ≥ 150 / history of lipid profile disturbance | 111.98±2.86 | 112.75±8,85 | 110.53±5.44 | 0.328[¥] |
| < 150 | 112.99±8.19 | 112.09±4.41 | 106.63±9.97 | 0.257[¥] |

* Statistic test with uji ANOVA ; [1] post hoc test variables Central obesity NASH vs control p=0.028
[#] Statistic test with T-test
[£] Statistic test with dengan Mann-Whitney Test
[¥] Statistic test with Kruskal-Wallis Test

The data are described in frequency, percentage, and mean ± SD for categorical data, and presented in tabular form. Patient characteristics were analyzed descriptively, then continued with bivariate analysis to find the

significancy of the relationship between each variable with cross tabs. A correlation test was done to analyze the relationship between the independent variable and the dependent variable.

## RESULTS

This study involved 40 subjects with NAFLD as a case group and 40 healthy subjects as a control group, with the same sex ratio between the two groups. Table 1 represents the general characteristics of these 80 patients. Age and blood pressure status in the two groups were not significantly different. Body mass index and waist circumference were significantly higher in the case group than in the control group ($p = <0.0001$). Biochemical parameters such as ALT, AST, triglycerides, cholesterol, insulin, and HOMA-IR scores were significantly different between the two groups ($p<0.001$), while the fasting blood sugar levels of the two groups were not significantly different.

In the case group, there was an incidence of metabolic syndrome (58%) and insulin resistance (60%); compared to the control group, metabolic syndrome was found in only 8%, while the insulin resistance was found in 13%. There was a significant relationship between the incidence of metabolic syndrome and insulin resistance with the incidence of NAFLD ($p < 0.001$).

The distribution of fatty liver status was based on abdominal US examination. US of the liver in all control group subjects showed normal results. In the case group, it was mostly found in the mild category (86%), followed by moderate (10%), and severe (4%). The degree of fatty liver (NAFLD) is based on the Histological Scoring System for Non-Alcoholic Fatty Liver Disease Score and Fibrosis Staging (NASH activity score = NAS). It appeared that only 25% were found in the Simple Steatosis category, and among the group suspected of having NASH (NAS = 3-4) and NASH (NAS > 5), as much as 75%.

The difference in plasma PAI-1 levels with the incidence of NAFLD can be seen in Table 2, which show that there were significant differences between plasma PAI-1 levels and the incidence of NAFLD ($p = 0.011$). Table 3 shows the relationship between plasma PAI-1 levels and the severity of NAFLD based on NAS, there were significant differences between the three NAFLD severity categories ($p = 0.032$). The highest mean value was obtained in the NASH category (112.54 ng/ml), and the highest median value was also in the NASH category (114.74 ng/ml). In the Mann-Whitney test, only the NASH variable and the control showed a significant difference ($p = 0.013$).

Table 4 shows a significant difference between the mean plasma PAI-1 levels and fatty liver status based on abdominal US ($p=0.046$). The PAI-1 value increases along with the fatty liver status, although in post hoc analysis, there were no significant differences between the case groups, but significant differences were found when case groups compared to controls.

Table 5 describes the differences in the PAI-1 levels based on clinical parameters, including the obesity component, the liver function component, and the component in the metabolic syndrome. All variables are divided into two major categories. In the category of blood pressure status in the category with hypertension, there was no significant difference ($p=0.506$) but a significant difference ($p=0.04$) in the non-hypertensive group. In the category of laboratory parameters, there were also significant differences in the categories of normal AST ($p=0.039$), normal ALT ($p=0.04$), normal fasting blood sugar ($p=0.022$), and high HDL cholesterol ($p=0.048$). There was no significant difference between patients with simple steatosis, NASH, and controls in the group with abnormal laboratory parameters.

There were significant differences in the categories of patients without hypertension ($p=0.04$), normal AST ($p=0.039$), normal ALT ($p=0.04$), normal fasting blood sugar ($p=0.022$), and high HDL cholesterol. ($p=0.048$). In the group with abnormal laboratory parameters, there was no significant difference between patients with simple steatosis, NASH, and controls. We also analyzed the mean PAI-1 levels between the case and control groups based on the category of metabolic syndrome status and insulin resistance, but there was no significant difference ($p>0.05$).

## DISCUSSION

The characteristics of the subjects in this study illustrate that patient with NAFLD have higher BMI and abdominal circumference than the control group. According to recent consensus, BMI and waist circumference are important measures in determining central obesity and metabolic risk.[15] Fat distribution is a major pathophysiological mechanism for metabolic disease and central obesity. Patients with NAFLD experienced a significant increase in body weight compared to those without.[16] This is also in line with previous studies that the prevalence of NAFLD increased in parallel with the increase in obesity.[17] Tilman Kühn examined anthropometric parameters in NAFLD patients with obesity. The presence of fatty liver showed the strongest correlation with waist circumference.[18]

This study also found significant differences in the components of the metabolic syndrome (fasting blood sugar levels, triglycerides, and serum HDL cholesterol) and components of insulin resistance (serum insulin levels and HOMA-IR index) between the case group and the control group. Insulin resistance is based on the HOMA-IR if the value is >2. An increase in the HOMA-IR indicates impaired insulin sensitivity.[19] NAFLD-induced changes in the secretion of liver proteins, lipids, and other metabolites alter metabolism in the liver, muscle, adipose tissue, and pancreas to induce insulin resistance.[20]

Based on abdominal US, NAFLD was mostly found 86% in the mild category, however, based on histology, only 25% were found in the Simple Steatosis category, and 75% suspected of having NASH (NAS = 3-4) and NASH (NAS > 5). Abdominal US is reported to have a sensitivity of 60-94% in detecting fat, so it can be used to determine the presence of fatty liver. However Abdominal US has the disadvantage of being subjective and poor in sensitivity and specificity in obese patients.[21] Therefore liver biopsy remains the reference standard to determine the severity of NAFLD.[22]

We found that PAI-1 levels were significantly higher in advanced NAFLD, assessed using NAS. Significantly increased PAI-1 levels were mainly found in patients with NASH compared to the control group. PAI-1 is an

important regulator of fibrinolysis, with a known profibrotic function in various tissue types. PAI-1 inhibits the activation of plasminogen to plasmin, which will have an impact on decreasing levels of matrix metalloproteinases (MMP) and increasing extracellular matrix (ECM) deposits so that the process of liver fibrogenesis occurs. Elevated serum PAI-1 has been shown to correlate with the degree of fibrosis in liver biopsies in patients with NASH.[12] In a previous study in children, it was found that circulating PAI-1 levels were closely correlated with the amount of hepatic steatosis as measured by magnetic resonance spectroscopy, regardless of adiposity and insulin resistance.[13]

Previous studies revealed that PAI-1 expression is influenced by various pro-inflammatory conditions and is associated with cardiovascular risk. Metabolic syndrome, obesity, and insulin resistance are associated with elevated PAI-1 levels.[23,24] Therefore, in our study, we developed an analysis to determine whether other factors influence PAI levels in patients with NAFLD. Then it was found that obesity, hypertension, elevated liver enzymes, diabetes mellitus, and HDL levels did not affect PAI-1 levels in patients with simple steatosis, NASH, or controls. It can be concluded that PAI-1 levels have an independent effect on the degree of liver fibrosis in patients with NAFLD.

Elevated PAI-1 can be used as a predictive factor for the occurrence of thrombosis associated with an increased risk of cardiovascular disease (CVD). The active form of PAI-1 specifically inhibits both tissue-type and urokinase-type plasminogen activators. As a result, fibrin breakdown is inhibited, which makes it easier for a thrombus to form. Study about the predictive value of PAI-1 in deep venous thrombosis (DVT) showed that level of PAI-1 in the DVT group was significantly higher than the control group ($P<0.05$).[25] Frischmuth et al. concluded that PAI-1 is associated with increased risk of future incident VTE.[26] Recent studies imply that local thrombotic events play a role in the progression of liver disease since patients with cirrhosis who received low-molecular-weight heparins saw a lower rate of complications.[27]

Significant differences in PAI-1 levels were actually found in patients who did not have hypertension, normal AST and ALT levels, and normal blood sugar levels. It can be caused by several factors that may still be a limitation of our study, we did not further investigate the drugs consumed by the patient. In hypertensive patients, using losartan – an angiotensin II type 1 receptor antagonist (AT1R) for 12 weeks can reduce PAI-1 gene expression, improve fibrosis, reduce liver inflammation, and improve insulin sensitivity. Sodium-glucose co-transporter-2 (SGLT2) inhibitors such as empagliflozin are known to reduce plasma PAI-1 concentrations in patients with type 2 diabetes. [28]

From our results, PAI-1 levels were not affected by the presence of metabolic syndrome and insulin resistance. Although the association between NAFLD and insulin resistance is generally evident in most patients, this is not always the case for genetically determined forms of fatty liver. For example, frequent sequence variation (I148M) in a protein containing the patatin-like phospholipase domain (PNPLA3) is strongly associated with fatty liver disease in the absence of insulin resistance or dyslipidemia, and similar dissociation was reported in individuals with single-nucleotide polymorphisms for acyl-coenzyme A (CoA), diacylglycerol acyltransferase (DGAT) and Lys167 allele in the transmembrane superfamily (TM6SF2).[20]

Our study has several strengths. This is the first study in Indonesia on PAI-1 levels involving patients with and without NAFLD. The findings from our study support the hypothesis that PAI-1 plays an important role in the pathophysiology of NAFLD. Diagnosis and grading of NAFLD in our study used 2 modalities, namely liver biopsy and ultrasound. This study also further analyzed other factors that might influence PAI-1 levels in patients with NAFLD.

This study has several limitations, including the small number of subjects. Therefore, larger studies need to be conducted to confirm our findings. A second limitation is its correlation analysis which does not allow evidence of a causal relationship between plasma PAI-1 and clinical variables. In this study, analysis of thrombosis status and coagulation studies were not performed.

## CONCLUSION

There is an increase in PAI-1 levels in patients with NAFLD. PAI-1 levels have an independent effect on the degree of liver fibrosis in patients with NAFLD. PAI-1 levels were not affected by the presence of metabolic syndrome and insulin resistance. The level of PAI-1 linked to thrombotic events rises with the severity of NAFLD.

## ACKNOWLEDGMENTS

Thank you to dr. Cecilia Oktaria, Sp.PD and dr. Debby Vania who helped this editing and publishing process, Dr. dr. Suhartono, M. Kes who helped statistical analysis process, and dr. Nani Maharani, M. Si. Med., Ph.D who have given many constructive comments in the preparation of this paper.

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