

# PNPLA3 PROTEIN EXPRESSION IN RELATION TO FIBROSIS SEVERITY MEASURED BY FIB-4 AND APRI IN NON-ALCOHOLIC FATTY LIVER DISEASE

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## ABSTRACT

**Background:** Non-alcoholic fatty liver disease (NAFLD) is a major cause of chronic liver disease, with hepatic fibrosis being an important determinant of disease progression and long-term outcomes. Patatin-like phospholipase domain-containing protein 3 (PNPLA3) has an established role in hepatic lipid metabolism and fibrogenesis; however, the relationship between circulating PNPLA3 protein expression and fibrosis severity remains uncertain.

**Objective:** To evaluate the relationship between serum PNPLA3 protein expression and liver fibrosis severity assessed using the Fibrosis-4 (FIB-4) index and Aspartate Aminotransferase to Platelet Ratio Index (APRI) among patients with NAFLD.

**Methods:** A cross-sectional analytical study was conducted among 120 adult patients with ultrasound-confirmed NAFLD at Asian Hospital, Hyderabad, Pakistan, from March to August 2025. Serum PNPLA3 protein concentrations were measured using enzyme-linked immunosorbent assay (ELISA). Participants were categorized into low, moderate, and high PNPLA3 expression groups. Liver fibrosis was assessed using FIB-4 and APRI scores. Associations between PNPLA3 expression categories and fibrosis categories were assessed using one-way analysis of variance, while correlations between serum PNPLA3 levels and fibrosis indices were evaluated using Spearman's rank correlation coefficient. A p-value <0.05 was considered statistically significant.

**Results:** Of the 120 participants, 74 (61.7%) were male and 46 (38.3%) were female. Low, moderate, and high PNPLA3 expression was observed in 52 (43.3%), 22 (18.3%), and 46 (38.3%) participants, respectively. According to FIB-4, 79 (65.8%) participants had scores <1.45, 34 (28.3%) had scores of 1.45–3.25, and 7 (5.8%) had scores >3.25. According to APRI, 73 (60.8%) had scores <0.5, 27 (22.5%) had scores of 0.5–1.0, 13 (10.8%) had scores of 1.0–2.0, and 7 (5.8%) had scores >2.0. PNPLA3 expression was not significantly associated with FIB-4 categories ( $p=0.098$ ) or APRI categories ( $p=0.24$ ). No significant correlation was observed between PNPLA3 and FIB-4 ( $r=-0.03$ ,  $p=0.735$ ) or APRI ( $r=0.06$ ,  $p=0.513$ ).

**Conclusion:** Serum PNPLA3 protein expression was not significantly associated with fibrosis severity assessed by FIB-4 or APRI in this cohort of patients with NAFLD. Circulating PNPLA3 protein expression alone may have limited utility as an independent marker of fibrosis severity when evaluated against conventional non-invasive fibrosis indices. Further longitudinal studies involving larger populations and complementary fibrosis assessments are warranted.

**KEYWORDS:** PNPLA3; non-alcoholic fatty liver disease; hepatic fibrosis; FIB-4; APRI; ELISA; liver fibrosis.

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most prevalent chronic liver disorders worldwide and has become a public-health challenge. A systematic review and meta-analysis of 78 million individuals from 38 countries estimated the global prevalence of NAFLD at 30.2%, including 30.9% in Asia, while prevalence among adults with obesity reached 57.5% {1}. A global meta-analysis estimated prevalence at 32.4% and demonstrated an increase from 25.5% in or before 2005 to 37.8% in 2016 or later, highlighting the expanding disease burden {2}. NAFLD encompasses a spectrum ranging from hepatic steatosis to steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. Hepatic fibrosis is particularly important because its severity is closely associated with liver-related and adverse outcomes {3}.

Accurate identification of fibrosis risk is central to management. Although liver biopsy remains a reference method for staging, its invasive nature, sampling variability, and feasibility have increased reliance on non-invasive fibrosis assessment. The Fibrosis-4 (FIB-4) index and Aspartate Aminotransferase to Platelet Ratio Index (APRI) use available

clinical and biochemical parameters and are widely evaluated for fibrosis risk stratification in NAFLD {4}. FIB-4 has also demonstrated prognostic associations with mortality and liver-related outcomes, supporting its relevance for risk assessment {5}.

In parallel, increasing attention has been directed toward genetic and molecular determinants of NAFLD progression. Patatin-like phospholipase domain-containing protein 3 (PNPLA3), particularly the rs738409 variant, is strongly associated with NAFLD susceptibility and disease severity {6}. Recent evidence further indicates that PNPLA3 may influence fibrosis through pathways involving steatosis, inflammation, and fibrogenesis {7}. However, clinical evidence regarding circulating PNPLA3 protein expression and its relationship with used non-invasive fibrosis indices remains limited, particularly in South Asian populations. Therefore, this study aimed to evaluate the relationship between serum PNPLA3 protein expression and fibrosis severity assessed by FIB-4 and APRI among patients with NAFLD.

## METHODOLOGY

A cross-sectional analytical study was conducted at Asian Hospital, Hyderabad, Pakistan, a tertiary care center with established diagnostic facilities and a high patient turnover. The study was carried out over a six-month period from March 2025 to August 2025, following approval from the Ethical Review Committee of Isra University. The study population comprised adult patients with ultrasound-confirmed non-alcoholic fatty liver disease (NAFLD) who were recruited from outpatient clinics and inpatient medical units. A total of 120 patients of both sexes, aged 18–55 years and belonging to different ethnic backgrounds, were included.

The sample size was calculated using OpenEpi version 3.01, based on an accessible population of 180 patients, a 5% margin of error, and a 95% confidence level. The calculated sample size was 124 participants; however, 120 participants were ultimately included according to study feasibility. Non-probability convenience sampling was used for participant recruitment. Patients aged 18–55 years with ultrasound-confirmed fatty liver disease who provided written informed consent were included. Patients with significant alcohol intake (>20 g/day for females and >30 g/day for males), viral hepatitis including HBV, HCV, HEV, or HDV, known cirrhosis or hepatocellular carcinoma, autoimmune liver disease, metastatic liver disease, diabetes mellitus, pregnancy or lactation, or a history of hepatotoxic drug use were excluded.

After obtaining written informed consent, demographic and clinical information was recorded using a structured proforma. Data regarding age, sex, ethnicity, dietary habits, physical activity, smoking status, and alcohol consumption were documented. Anthropometric measurements were obtained under standardized conditions, and body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Following an overnight fast of at least 12 hours, venous blood samples were collected under aseptic conditions. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured according to standard laboratory protocols, while platelet counts were obtained from complete blood count analysis using automated analyzers.

Serum PNPLA3 protein expression was quantified using a commercial enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Optical density was measured at 450 nm using a microplate reader, and PNPLA3 concentrations were derived from a standard calibration curve. All samples were analyzed in duplicate to ensure analytical accuracy. Participants were categorized into low, moderate, and high PNPLA3 expression groups according to the predefined serum PNPLA3 concentration categories.

Liver fibrosis severity was assessed using the Fibrosis-4 (FIB-4) index and the Aspartate Aminotransferase to Platelet Ratio Index (APRI). The FIB-4 index was calculated using the formula  $FIB-4 = (Age \times AST) / (Platelet\ count \times \sqrt{ALT})$ . FIB-4 scores were categorized as <1.45, 1.45–3.25, and >3.25. APRI was calculated using the formula  $APRI = [(AST / Upper\ Limit\ of\ Normal\ AST) \times 100] / Platelet\ count\ (10^9/L)$ . APRI values were categorized according to the predefined categories used in the study.

The primary study variable was serum PNPLA3 protein expression, while the principal outcome variables were FIB-4 and APRI scores as non-invasive measures of liver fibrosis severity. Demographic and clinical variables included age, sex, ethnicity, BMI, dietary habits, physical activity, smoking status, and alcohol consumption, while laboratory variables included ALT, AST, and platelet count. Data were analyzed using SPSS version 26.0. Continuous variables were expressed as mean  $\pm$  standard deviation, whereas categorical variables were presented as frequencies and percentages. Comparisons of fibrosis indices across PNPLA3 expression groups were performed using one-way ANOVA, and correlations between serum PNPLA3 levels and fibrosis indices were assessed using Spearman's rank correlation coefficient. All statistical tests were two-tailed, and a p-value <0.05 was considered statistically significant. Ethical approval was obtained from the Ethical Review Committee of Isra University before commencement of the study. Written informed consent was obtained from all participants prior to enrollment. Confidentiality and anonymity of the participants were maintained throughout the study, and the research was conducted in accordance with the principles of the Declaration of Helsinki.

## RESULT

A total of 120 patients with ultrasound-confirmed non-alcoholic fatty liver disease were included in the analysis. The demographic and clinical characteristics of the study population are presented in Table 1. The mean age was  $42.3 \pm 9.4$  years, with 74 (61.7%) male and 46 (38.3%) female participants. The mean body mass index was  $31.2 \pm 9.0$  kg/m<sup>2</sup>. Diabetes mellitus was reported in 48 (40.0%) participants, hypertension in 39 (32.5%), and a family history of fatty liver disease in 34 (28.3%).

Serum PNPLA3 protein expression was categorized into low, moderate, and high expression groups. As shown in Table 2, 52 (43.3%) participants had low PNPLA3 expression, 22 (18.3%) had moderate expression, and 46 (38.3%) had high expression. The overall mean serum PNPLA3 concentration was  $412 \pm 214$  ng/mL.

Fibrosis severity according to the FIB-4 index is presented in Table 3. A total of 79 (65.8%) participants had FIB-4 scores below 1.45, 34 (28.3%) had scores between 1.45 and 3.25, and 7 (5.8%) had scores above 3.25. The overall mean FIB-4 score was  $1.12 \pm 0.61$ .

The distribution of APRI categories is shown in Table 4. An APRI score below 0.5 was observed in 73 (60.8%) participants, 27 (22.5%) had scores between 0.5 and 1.0, 13 (10.8%) had scores between 1.0 and 2.0, and 7 (5.8%) had scores above 2.0. The overall mean APRI score was  $0.61 \pm 0.54$ .

The distribution of PNPLA3 expression across FIB-4 categories is presented in Table 5. Among participants with FIB-4 scores below 1.45 (n=79), 38 had low PNPLA3 expression, 9 had moderate expression, and 32 had high expression. Among those with FIB-4 scores of 1.45–3.25 (n=34), 11 had low, 11 had moderate, and 12 had high PNPLA3 expression. Among participants with FIB-4 scores above 3.25 (n=7), 3 had low, 2 had moderate, and 2 had high PNPLA3 expression. The association between PNPLA3 expression category and FIB-4 category was not statistically significant (p=0.098).

The distribution of PNPLA3 expression across APRI categories is presented in Table 6. Among participants with APRI scores below 0.5 (n=73), 37 had low PNPLA3 expression, 10 had moderate expression, and 26 had high expression. Among participants with APRI scores of 0.5–1.0 (n=27), 8 had low, 7 had moderate, and 12 had high expression. Among those with APRI scores of 1.0–2.0 (n=13), 6 had low, 2 had moderate, and 5 had high expression. Among participants with APRI scores above 2.0 (n=7), 1 had low, 3 had moderate, and 3 had high PNPLA3 expression. The association between PNPLA3 expression category and APRI category was not statistically significant (p=0.24).

Correlation analyses are summarized in Table 7. Serum PNPLA3 expression showed a Spearman correlation coefficient of  $-0.03$  with FIB-4 (p=0.735) and  $0.06$  with APRI (p=0.513).

**Table 1. Demographic Characteristics of Study Participants (n = 120)**

| Characteristic           | Mean $\pm$ SD / n (%) | Range |
|--------------------------|-----------------------|-------|
| Age (years)              | $42.3 \pm 9.4$        | 20–70 |
| Male                     | 74 (61.7%)            | -     |
| Female                   | 46 (38.3%)            | -     |
| BMI (kg/m <sup>2</sup> ) | $31.2 \pm 9.0$        | 18–68 |
| Diabetes Mellitus        | 48 (40.0%)            | -     |
| Hypertension             | 39 (32.5%)            | -     |
| Family history of FLD    | 34 (28.3%)            | -     |

**Table 2. PNPLA3 Protein Expression in NAFLD Patients**

| PNPLA3 Expression (ng/mL) | n (%)         |
|---------------------------|---------------|
| Low ( $\leq 250$ )        | 52 (43.3%)    |
| Moderate (251–480)        | 22 (18.3%)    |
| High ( $> 480$ )          | 46 (38.3%)    |
| Overall Mean $\pm$ SD     | $412 \pm 214$ |

**Table 3. Fibrosis Severity by FIB-4 Score**

| FIB-4 Category        | n (%)           |
|-----------------------|-----------------|
| $< 1.45$              | 79 (65.8%)      |
| 1.45–3.25             | 34 (28.3%)      |
| $> 3.25$              | 7 (5.8%)        |
| Overall Mean $\pm$ SD | $1.12 \pm 0.61$ |

**Table 4. Fibrosis Severity by APRI Score**

| APRI Category | n (%)      |
|---------------|------------|
| $< 0.5$       | 73 (60.8%) |

|                          |                    |
|--------------------------|--------------------|
| <b>0.5–1.0</b>           | <b>27 (22.5%)</b>  |
| <b>1.0–2.0</b>           | <b>13 (10.8%)</b>  |
| <b>&gt;2.0</b>           | <b>7 (5.8%)</b>    |
| <b>Overall Mean ± SD</b> | <b>0.61 ± 0.54</b> |

**Table 5. PNPLA3 Expression Across FIB-4 Categories**

| <b>FIB-4 Category</b> | <b>Low PNPLA3</b> | <b>Moderate PNPLA3</b> | <b>High PNPLA3</b> | <b>Total</b> | <b>p-value</b> |
|-----------------------|-------------------|------------------------|--------------------|--------------|----------------|
| <b>&lt;1.45</b>       | <b>38</b>         | <b>9</b>               | <b>32</b>          | <b>79</b>    |                |
| <b>1.45–3.25</b>      | <b>11</b>         | <b>11</b>              | <b>12</b>          | <b>34</b>    |                |
| <b>&gt;3.25</b>       | <b>3</b>          | <b>2</b>               | <b>2</b>           | <b>7</b>     |                |
| <b>Total</b>          | <b>52</b>         | <b>22</b>              | <b>46</b>          | <b>120</b>   | <b>0.098</b>   |

**Table 6. PNPLA3 Expression Across APRI Categories**

| <b>APRI Category</b> | <b>Low PNPLA3</b> | <b>Moderate PNPLA3</b> | <b>High PNPLA3</b> | <b>Total</b> | <b>p-value</b> |
|----------------------|-------------------|------------------------|--------------------|--------------|----------------|
| <b>&lt;0.5</b>       | <b>37</b>         | <b>10</b>              | <b>26</b>          | <b>73</b>    |                |
| <b>0.5–1.0</b>       | <b>8</b>          | <b>7</b>               | <b>12</b>          | <b>27</b>    |                |
| <b>1.0–2.0</b>       | <b>6</b>          | <b>2</b>               | <b>5</b>           | <b>13</b>    |                |
| <b>&gt;2.0</b>       | <b>1</b>          | <b>3</b>               | <b>3</b>           | <b>7</b>     |                |
| <b>Total</b>         | <b>52</b>         | <b>22</b>              | <b>46</b>          | <b>120</b>   | <b>0.24</b>    |

**Table 7. Correlation Between PNPLA3 Expression and Fibrosis Scores**

| <b>Parameter</b>       | <b>R</b>     | <b>p-value</b> |
|------------------------|--------------|----------------|
| <b>PNPLA3 vs FIB-4</b> | <b>-0.03</b> | <b>0.735</b>   |
| <b>PNPLA3 vs APRI</b>  | <b>0.06</b>  | <b>0.513</b>   |

## DISCUSSION

The present study evaluated the relationship between serum PNPLA3 protein expression and liver fibrosis assessed using FIB-4 and APRI in patients with NAFLD. The principal finding was that circulating PNPLA3 protein expression was not significantly associated with either fibrosis index. This finding differs from the substantial body of recent literature linking the PNPLA3 rs738409 genetic variant with hepatic fibrosis, but the distinction between genetic variation and circulating protein concentration is important when interpreting the present findings. Recent biopsy-based studies have demonstrated that the PNPLA3 rs738409 G allele is associated with significant fibrosis and may modify the relationship between visceral adiposity and fibrosis, while longitudinal evidence has also linked PNPLA3 genotype with progression of NAFLD and liver-related outcomes {8,9}.

The absence of a significant relationship between serum PNPLA3 protein expression and FIB-4 or APRI therefore does not necessarily contradict the established biological importance of PNPLA3. Most recent clinical evidence has focused on PNPLA3 genotype rather than circulating PNPLA3 protein levels. A 2023 meta-analysis involving 3,240 patients with NAFLD and 5,210 controls demonstrated a significant association between the rs738409 polymorphism and NAFLD susceptibility across several genetic models {10}. Similarly, longitudinal biopsy-based research has shown that PNPLA3 genetic variation can influence progression and long-term aggravation of NAFLD {11}. These findings suggest that the pathogenic effect of PNPLA3 may depend more strongly on genotype, tissue-specific expression, protein localization, and interaction with metabolic factors than on a single circulating protein measurement.

Several biological mechanisms may explain why PNPLA3 remains mechanistically relevant despite the absence of a measurable association with circulating protein expression in this study. PNPLA3 is involved in hepatic lipid-droplet biology and lipid remodeling, while the I148M variant has been associated with intracellular lipid accumulation and fibrogenic signaling. Experimental work has demonstrated that PNPLA3-I148M can promote free-cholesterol accumulation in hepatic stellate cells, mitochondrial dysfunction, oxidative stress, and altered mitochondrial respiration, providing a potential pathway linking PNPLA3 dysfunction to fibrosis {12}. Recent reviews have further emphasized interactions between hepatocytes, hepatic stellate cells, macrophages, and inflammatory mediators in PNPLA3-associated fibrogenesis {13}. Thus, serum PNPLA3 concentration may not adequately capture intracellular or hepatic stellate-cell-specific PNPLA3 activity.

The present findings may also be related to the characteristics of the fibrosis assessment methods used. FIB-4 and APRI are inexpensive and readily available blood-based indices, but they are indirect measures of fibrosis and incorporate routinely measured laboratory parameters rather than directly measuring extracellular matrix deposition or liver stiffness. Contemporary guidelines recommend FIB-4 as an initial risk-stratification tool, followed where

appropriate by secondary assessment using techniques such as vibration-controlled transient elastography or other non-invasive tests {14}. Individual-participant meta-analysis has demonstrated that FIB-4 provides clinically useful prognostic information, although imaging-based measurements such as transient elastography may provide additional information regarding advanced fibrosis {15}. Consequently, the absence of a relationship between PNPLA3 protein concentration and these indices should be interpreted as an absence of association with these particular non-invasive fibrosis measures, rather than evidence that PNPLA3 has no role in hepatic fibrogenesis.

The results also need to be considered in the context of the relatively limited clinical spectrum captured by a cross-sectional study. PNPLA3-associated effects may become more apparent during progressive steatohepatitis and advanced fibrosis. Recent genetic studies have shown that PNPLA3-related risk is influenced by other genetic and metabolic factors. For example, a 2024 study evaluating multiple MASLD-associated genetic variants found that PNPLA3 and TM6SF2 were associated with liver-related outcomes, with combined genetic-risk assessment providing additional information beyond individual variants {16}. Other recent work has demonstrated an association between PNPLA3 and fibrosis severity in conjunction with additional genetic factors, including SIRT5, further supporting the concept that fibrosis susceptibility is multifactorial rather than determined by PNPLA3 alone {17}.

The use of two fibrosis indices was an important strength because it allowed the relationship between PNPLA3 expression and fibrosis to be examined using complementary routinely available measures. The study also used direct measurement of serum PNPLA3 protein by ELISA rather than relying exclusively on genotype, providing a different biological perspective from many previous studies. Furthermore, the study generated data from a Pakistani clinical population, for which evidence regarding circulating PNPLA3 protein expression remains limited. The use of a standardized blood-sampling procedure and duplicate ELISA measurements also supported analytical consistency.

Several limitations should be acknowledged. First, the cross-sectional design prevented assessment of temporal relationships between PNPLA3 expression and fibrosis progression. Second, fibrosis was assessed using FIB-4 and APRI rather than liver biopsy or elastography; therefore, the findings cannot establish whether PNPLA3 protein expression is related to histological or directly measured liver fibrosis. Third, the sample size was relatively modest and participants were recruited using non-probability convenience sampling at a single tertiary-care center, which may limit generalizability. Fourth, serum PNPLA3 concentration may not reflect hepatic or hepatic stellate-cell PNPLA3 activity. Finally, the study did not evaluate PNPLA3 rs738409 genotype, other fibrosis-associated genetic variants, or longitudinal changes in PNPLA3 expression. These limitations are particularly relevant because current evidence indicates that genetic and non-genetic determinants interact in determining MASLD progression {18}.

The clinical implication is that serum PNPLA3 protein expression should not currently be considered a substitute for established non-invasive fibrosis assessment based solely on the findings of this study. Current international guidance supports sequential use of blood-based scores and, when indicated, imaging-based fibrosis assessment for clinical risk stratification {14}. From a research perspective, future studies should investigate PNPLA3 protein expression together with PNPLA3 genotype, liver stiffness measurement, histological fibrosis, and other molecular biomarkers. Longitudinal studies involving larger and ethnically diverse populations would be particularly valuable for determining whether circulating PNPLA3 expression has prognostic significance or whether its clinical relevance depends on specific genetic and metabolic contexts.

## CONCLUSION

In this cohort of patients with NAFLD, serum PNPLA3 protein expression was not significantly associated with fibrosis severity assessed by FIB-4 or APRI. These findings indicate that circulating PNPLA3 protein concentration, when considered alone, may not correspond closely with fibrosis risk estimated by non-invasive indices. The study adds local data to limited evidence concerning PNPLA3 protein expression and non-invasive fibrosis assessment. Given the established genetic and biological contribution of PNPLA3 to fibrogenesis, future studies should evaluate PNPLA3 expression alongside genotype, elastography, histological fibrosis, and molecular markers in larger longitudinal cohorts to clarify its potential role in individualized fibrosis risk assessment.

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