Association of nonalcoholic fatty liver disease grades with the plasma cell antigen-1 (PC-1) gene polymorphism

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is described as a major public health issue worldwide. It affects about 20–30% of the general population. NAFLD includes a broad range of pathologies, from simple steatosis (SS) to nonalcoholic steatohepatitis (NASH), that can lead to cirrhosis and hepatocellular carcinoma (HCC) [1]. There is a number of mechanisms that are clearly involved in the pathogenesis of NAFLD. These included free fatty acid flux, insulin resistance, oxidative stress, endoplasmic reticulum stress, and inflammation [2]. Liver steatosis develops when the fatty acid supply to the liver exceeds the consumption of mitochondrial oxidation and synthesis of phospholipids, triacylglycerols, and cholesterol [3]. Triacylglycerol accumulation results from either uptake of lipids in the liver [4]. Insulin resistance has frequently been concerned as a significant reason for lipid accumulation in the liver [5].

Besides the environmental parameters that influence the development of NASH [6], previous studies showed that NAFLD could also be affected by genetic variants. Certainly, numerous single-nucleotide polymorphisms (SNPs) have been stated to influence the pathophysiology in NAFLD patients via a genome-wide association study (GWAS). Over the last decade, many reports have also shown the importance of genetic factors in the development of NAFLD [7]. Insulin resistance is the fundamental factor in NAFLD...
pathophysiology and is intensely involved with the progression of liver disease. The ectoenzyme nucleotide pyrophosphate phosphodiesterase (ENPP1), also known as plasma cell membrane glycoprotein 1 (PC-1), is a type II transmembrane glycoprotein which is associated with insulin receptor on the cell surface and inhibits insulin signaling. Polymorphisms of genes involved in insulin sensitivity might affect both the mechanism and extent of hepatic steatosis. The possible clinical relevance of polymorphism of the gene coding for PC-1 in NAFLD pathogenesis was pointed out [8].

2. Aim of the study

In this study, the K121Q polymorphism of PC-1 was investigated in NAFLD grades. The association between K121Q polymorphism and clinical parameters in obese patients with NAFLD was also investigated to see whether it could serve as a future diagnostic and therapeutic biomarker.

3. Subjects and methods

3.1. Subjects

A total of 87 non-diabetic (fasting blood sugar < 126 mg/dl) obese patients with NAFLD (BMI ≥ 30 kg/m²) who have never taken any medication for diabetes, along with 47 non-obese control subjects (BMI from 18 to 23 kg/m²), were recruited from the liver clinic, Medical Service Unit at The National Research Centre, Giza, Egypt. All patients and controls were examined ultrasonographically for NAFLD diagnosis. Written informed consent was obtained from all subjects and this study was approved by the Human Ethics Committee of National Research Center (Code No. 14075). The work has been carried out in accordance with The code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans.

Patients with hepatitis B, hepatitis C infection, diabetes, splenectomy, cholestasis, coronary artery disease or pregnancy were excluded. Patients who smoke, or those who use amiodarone, corticosteroids, tamoxifen or methotrexate were also excluded.

3.2. Methods

3.2.1. Diagnosis of NAFLD

All the subjects were examined by ultrasonography using SonoAce R5 (6 MHz; Samsung) for diagnosing and classifying grades of NAFLD, as follows: normal controls, and NAFLD patients subdivided into Grade-I, Grade-II and Grade-III according to Kakrani et al. [9].

3.2.2. Anthropometric measurements

Body mass index (BMI) was calculated by dividing weight by square height (kg/m²). Waist circumference (WC) was measured at the midpoint between the lower rib margin and the iliac crest using a conventional tape graduated in centimeters (cm). Hip circumference was measured as the greatest abdominal circumference at the level of the greater trochanters. Waist-to-hip ratio was calculated by dividing the waist circumference by the hip circumference.

3.2.3. Samples collection

Five milliliters of blood were drawn in the morning after 12 h fasting, then divided into four portions; the first portion (2 ml) was left to clot for 30 min at room temperature then centrifuged at 3000 rpm for serum separation to determine the levels of insulin and lipid profile parameters. The second portion (1 ml) was collected in EDTA-containing tubes and centrifuged at 3000 rpm for plasma separation to determine the level of PC-1. The third portion (1 ml) was collected on a mixture of EDTA and fluoride for plasma separation to determine glucose concentration. The fourth portion (1 ml) was collected in an EDTA-containing tube for investigation of PC-1 polymorphism.

3.2.4. Biochemical analyses

Fasting plasma glucose was performed according to Heinz and Beushausen [10] using the kit supplied by STANBIO laboratory, USA. Plasma PC-1 level was determined quantitatively by an ELISA kit from Glory Science Company, USA. Serum insulin concentration was determined by an ELISA kit purchased from Monobind Company, USA. Serum total cholesterol was determined by an enzymatic colorimetric method [11]. Serum triacylglycerols were determined by an enzymatic colorimetric method according to Fredrickson et al. [12]. High density lipoprotein cholesterol (HDL-cholesterol) was determined according to Finley et al. [13]. Low density lipoprotein cholesterol (LDL-Cholesterol) was calculated according to Friedewald et al. [14] equation. Lipid profile (total cholesterol, triacylglycerols and HDL-cholesterol) kits were provided from Stanbio laboratory Company, USA.

The insulin resistance index was assessed by the homeostasis model assessment insulin resistance (HOMA-IR), calculated with Matthews et al. [15] equation.

3.2.5. Genotyping of PC-1 gene polymorphism rs1044498 (K/Q)

Genomic DNA was extracted and purified from whole peripheral blood samples using Qia-amplification DNA extraction kit (Qiagen Inc., Valencia, CA, USA). The K121Q variant in the glycoprotein PC-1 gene was detected using PCR-restriction fragment length polymorphism [16]. The PCR was performed using T-Gradient thermal cycle (Biometra, Germany). A 238-bp amplified PCR product was digested with AvaI restriction enzyme (New England Biolabs, Hitchin, UK). PCR products were analyzed by electrophoresis using 2% high-strength agarose gel and were stained with Red-Safe, then the electrophoretic products were visualized using ultra-violet light transillumination. The wild K alleles are displayed as a single uncut band of 238 bp, while the mutant Q alleles are shown as a doublet of 148 and 90 bp bands (Fig. 1).

3.3. Statistical analyses

The Statistical Package for the Social Science Software [SPSS 17.0, Chicago, IL, USA] was used. Normally distributed continuous variables were expressed as mean (±SE); qualitative variables were
presented as proportions. Quantitative variables were compared using one-way ANOVA. On the other hand, qualitative variables were compared using Chi square [$X^2$] test or Fischer’s exact test.

4. Results

Significant differences were found comparing the demographic characteristics of patients and controls. The same was true for FPG, insulin, HOMA index and lipid profile parameters (Table 1).

Comparing the distribution frequency of different PC-1 K/Q genotypes in NAFLD group revealed predominance of the KK genotype (56.3%), followed by the KQ genotype (43.7%), with complete absence of the QQ genotype. (Table 2).

The same was true for the control group, with a frequency of (66%) for the KK genotype followed by the KQ genotype (34%), with complete absence of the QQ genotype.

However, comparing the distribution frequency of PC-1 KQ genotype between NAFLD and control groups revealed higher prevalence in the NAFLD group (43.7%) compared to the control group (34%) with a $p$-value of .003. (Table 2).

In contrast, comparing the distribution frequency of PC-1 KK genotype between the studied groups revealed higher prevalence in the control group (66%) compared to NAFLD group (56.3%) with a $p$-value of .04. (Table 2).

Comparing the distribution frequency of different PC-1 alleles in the NAFLD group revealed predominance of the K allele (78.2%), followed by the Q allele (only 21.8%). The same was true for the control group, with a frequency of 84% for the K allele followed by a frequency of only 16% for the Q allele. (Table 2).

Comparing PC-1 genotype distribution among different NAFLD grades revealed a significant difference of the KK genotype ($p = .031$), but not of the KQ genotype ($p = .881$) among the different grades. (Table 3).

Similarly, comparing PC-1 allele frequency among different NAFLD grades revealed a significant difference of the K allele ($p = .001$), but not of the Q allele ($p = .881$) among the different grades. (Table 3).

Logistic regression for lipid profile in NAFLD patients revealed serum cholesterol and triacylglycerol to be strong risk factors for NAFLD with odd ratios of 1.173 and 2.887, respectively, and $p$-value of .001. (Table 4).

Logistic regression results for PC-1 gene polymorphism in NAFLD patients revealed a significant direct correlation between the KQ genotype and high levels of triacylglycerol along with VLDL. (Table 5).

In addition, a significant direct correlation was found, only in grade 3, between PC-1 plasma levels along with TGs and VLDL ($r = 0.588$, $p = .001$).

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### Table 1
Baseline Characteristics of NAFLD Patients and Controls.

<table>
<thead>
<tr>
<th></th>
<th>NAFLD (n = 87)</th>
<th>Controls (n = 47)</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEX (M/F)</td>
<td>29/58</td>
<td>27/20</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>41.54 ± 1.13</td>
<td>35.81 ± 1.45</td>
<td>.012</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>38.39 ± 0.77</td>
<td>22.7 ± 0.25</td>
<td>.000</td>
</tr>
<tr>
<td>Waist/Hip Ratio</td>
<td>0.89 ± 0.007</td>
<td>0.85 ± 0.013</td>
<td>.000</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>126.4 ± 1.5</td>
<td>111.5 ± 1.8</td>
<td>.000</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>83.4 ± 1.16</td>
<td>73 ± 1.7</td>
<td>.000</td>
</tr>
<tr>
<td>FPG (mg/dL)</td>
<td>99 ± 2.4</td>
<td>84 ± 1.14</td>
<td>.000</td>
</tr>
<tr>
<td>Insulin (µU/mL)</td>
<td>10.1 ± 0.52</td>
<td>6.6 ± 0.25</td>
<td>.000</td>
</tr>
<tr>
<td>HOMA Index</td>
<td>2.4 ± 0.14</td>
<td>1.3 ± 0.05</td>
<td>.000</td>
</tr>
<tr>
<td>Serum cholesterol (mg/dL)</td>
<td>267.1 ± 6.1</td>
<td>179.4 ± 4.3</td>
<td>.000</td>
</tr>
<tr>
<td>Serum triacylglycerols (mg/dL)</td>
<td>218.4 ± 7.5</td>
<td>106.8 ± 3.1</td>
<td>.000</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>48.2 ± 1.8</td>
<td>72.1 ± 3.3</td>
<td>.000</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>175.2 ± 6.04</td>
<td>85.9 ± 5.4</td>
<td>.000</td>
</tr>
<tr>
<td>VLDL (mg/dL)</td>
<td>43.7 ± 1.5</td>
<td>214 ± 0.61</td>
<td>.000</td>
</tr>
</tbody>
</table>

NAFLD: non-alcoholic fatty liver disease; VLDL: very low density lipoproteins.

Data are mean ± SE; ANOVA test was used for comparison between studied groups.

$*$ P Values indicate significant difference $P \leq .05$ was considered significant.

### Table 2
Genotype distributions and allele frequencies of the PC-1 (K/Q) gene polymorphism in non-alcoholic fatty liver disease (NAFLD) and normal controls (C).

<table>
<thead>
<tr>
<th>PC1 (K/Q)</th>
<th>Diagnosis</th>
<th>Chi-Square test for Diagnosis frequency</th>
<th>Chi-Square test for OR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>NAFLD</td>
<td>$X^2$</td>
</tr>
<tr>
<td>KK</td>
<td>31 (66%)</td>
<td>49 (56.3%)</td>
<td>4.05</td>
</tr>
<tr>
<td>KQ</td>
<td>16 (34%)</td>
<td>38 (43.7%)</td>
<td>8.963</td>
</tr>
<tr>
<td>K</td>
<td>79 (84%)</td>
<td>136 (78.2%)</td>
<td>15.72</td>
</tr>
<tr>
<td>Q</td>
<td>15 (16%)</td>
<td>38 (21.8%)</td>
<td>8.963</td>
</tr>
</tbody>
</table>

Data are number (%); variables were compared using chi square ($X^2$) test or Fischer’s exact test.

$P$ values for the frequency of each genotype within the studied groups.

$P$ values for comparison between NAFLD and Controls; OR: odd ratio; CI: confidence interval.

$^*$ Bold values indicate significant difference $P$ value $\leq .05$ was considered significant. PC-1: Membrane glycoprotein plasma cell antigen-1.

### Table 3
Genotype distributions and allele frequencies of the PC-1-K/Q gene polymorphism in NAFLD grades.

<table>
<thead>
<tr>
<th>PC1 gene</th>
<th>Normal (C) N (%)</th>
<th>Grades of NAFLD (G)</th>
<th>$X^2$</th>
<th>$P$ value</th>
<th>$X^2$</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1 n (%)</td>
<td>G2 n (%)</td>
<td>G3 n (%)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Genotypes

| KK       | 31 (66%) | 18 (58.1%) | 13 (50%) | 18 (60%) | 8.90 | 0.031$^*$ |
| KQ       | 16 (34%) | 13 (41.9%) | 13 (50%) | 12 (40%) | 0.667 | 0.881 |

Alleles

| K        | 79 (84%) | 49 (79%) | 39 (75%) | 48 (80%) | 16.093 | 0.001$^*$ |
| Q        | 15 (16%) | 13 (21%) | 13 (25%) | 12 (20%) | 0.667 | 0.881 |

Data are number (%); variables were compared using chi square ($X^2$) test or Fischer’s exact test.

$P$ value: Significant difference between KK & KQ genotypes or K & Q alleles within grades.

$P$1 frequency value: Significant difference of individual genotype or allele within grades.

$^*$ Bold values indicate significant difference $P$ value $\leq .05$ was considered significant. PC-1: Membrane glycoprotein plasma cell antigen-1.
The best cut-off value considering grade 2 as moderate-NAFLD group for PC-1 was 3.8 pg/ml, at which the sensitivity was 92.3% and the specificity was 27.7%, and the area under the curve was 0.58, indicating a validity of PC-1 level for distinguishing grade 2 from controls (Fig. 2a).

The best cut-off value considering grade 2 as moderate-NAFLD group for PC-1 was 7 pg/ml, at which the sensitivity was 92.3% and the specificity was 29.03%, and the area under the curve was 0.51, indicating a great validity of PC-1 level for distinguishing grade 2 from grade 1 group (Fig. 2b).

The best cut-off value considering grade 3 as severe-NAFLD group for PC-1 was 3.8 pg/ml, at which the sensitivity was 90% and the specificity was 27.7%, and the area under the curve was 0.53, indicating a greater validity of PC-1 level for distinguishing between grade 3 and controls (Fig. 2c).

The best cut-off value considering grade 3 as severe-NAFLD group for PC-1 was 7 pg/ml, at which the sensitivity was 96.7% and the specificity was 29.03%, and the area under the curve was 0.56, indicating a greater validity of PC-1 level for distinguishing grade 3 from grade 1 group (Fig. 2d).

The best cut-off value considering grade 3 as severe-NAFLD group for PC-1 was 6 pg/ml, at which the sensitivity was 90% and the specificity was 23.08%, and the area under the curve was 0.57, indicating a greater validity of PC-1 level for distinguishing grade 3 from grade 2 group (Fig. 2e).

5. Discussion

Non-alcoholic fatty liver disease (NAFLD) is characterized by the presence of extra lipid in the liver exceeding 5–10% of liver weight. NAFLD includes simple fatty liver (FL) and non-alcoholic steatohepatitis (NASH) in its spectrum; NASH can progress to liver cirrhosis [17]. NAFLD is a complex disease associated with dietary habitats, obesity, and a range of comorbidities correlated with

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**Table 4**

<table>
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<tr>
<th>Parameters</th>
<th>Coefficient</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>P value</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum cholesterol</td>
<td>0.159</td>
<td>1.173</td>
<td>1.1725–1.1725</td>
<td>.001</td>
<td>0.980</td>
</tr>
<tr>
<td>Serum triacylglycerols</td>
<td>1.060</td>
<td>2.887</td>
<td>0.0064–1294.43</td>
<td>.001</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>−0.156</td>
<td>0.856</td>
<td>0.8559–0.8559</td>
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<td></td>
</tr>
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**Table 5**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>B</th>
<th>Odd Ratio</th>
<th>95.0% CI</th>
<th>P value</th>
<th>Lower</th>
<th>Upper</th>
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<tbody>
<tr>
<td>TDG/C0</td>
<td>−4.907</td>
<td>0.007</td>
<td>0</td>
<td>.011</td>
<td>0.327</td>
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<tr>
<td>VLDL</td>
<td>24.540</td>
<td>4.54E + 10</td>
<td>266.326–7.75E + 18</td>
<td>.011</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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![Fig. 2.](image)

Fig. 2. Sensitivity (Se.) and Specificity (Sp.) of PC-1 (pg/ml) for differentiating between NAFLD grades.
insulin resistance. Although environmental parameters are essential in deciding risk of the disease, proofs from previous reports sustain the hypothesis that genetics provide a crucial modifier of NAFLD development and disease progression [5].

NAFLD is related to central obesity indices, one of which is waist–hip ratio [20]. In our study, waist–hip ratio and body mass index (BMI) showed significant increase when comparing NAFLD patients to normal controls. This is in agreement with the report by Tominaga et al. [21] which stated that BMI and WC were independent risk factors for NAFLD.

The current results showed high significant difference in age when comparing NAFLD patients with normal controls. These results support the finding of Mahmoud et al. [18], who stated that age is an independent risk factor for emerging of more severe NAFLD. Non-alcoholic fatty liver disease may be an independent risk factor for hypertension development [19]. This is in agreement with the present results where SBP and DBP were increased in NAFLD against controls. Insulin resistance is a well-known risk factor for NAFLD development [20]. The HOMA-IR index has been approved as an indicator of the insulin-resistant condition [21]. An upper boundary of normal HOMA-IR index is 1.5 [22]. HOMA-IR index and insulin level showed significant differences in NAFLD compared to normal controls; NAFLD patients showed HOMA-IR index value >1.5, indicating that they are in an IR state.

When comparing NAFLD patients to normal controls concerning lipid profile parameters, there were significant increases in all lipid profile parameters except for HDL-C which is decreased. In addition, the results of logistic regression showed a direct positive correlation between the severity of NAFLD and serum cholesterol along with triacylglycerol levels. This is in agreement with the report by Mahmoud et al. [18] which stated that hyperlipidemia was an independent predictor of NAFLD development.

Polymorphisms of genes involved in insulin sensitivity might impact both the mechanism and extent of hepatic steatosis. Numerous data in the literature emphasize the probable clinical significance of polymorphism of the gene coding for plasma cell differentiation antigen-1 (PC-1) or ectonucleotide pyrophosphatase/phosphodiesterase (ENPP-1) in the pathogenesis of NAFLD [23]. In 1999, Pizzuti and Cols [24] reported a polymorphism in the PC-1 gene exon 4, which causes the alteration of the amino acid lysine (K) to glutamine (Q) in codon 121 (K121Q; rs1044498). Pizzuti and Cols [24] also showed that the Q variant of this polymorphism interacts more powerfully with the insulin receptor than the K variant, decreasing the autophosphorylation of this receptor leading to the inhibition of insulin signaling pathway which causes insulin resistance.

In the present study, it was surprising that the mutated homozygous genotype (QQ) of PC-1 gene polymorphism did not appear in any of the studied NAFLD patients although they were all insulin resistant. However, this is in agreement with a study done in a Danish population which did not find any correlation between this polymorphism and insulin resistance [25]. Discordant results in genotype-phenotype association studies are prevalent in complex disorders evaluation. The outcomes of any study depend on the differences in the genetic and/or environmental background of the sampled population, as well as in the subject recruitment manner; thus, conflicting findings among studies do not necessarily indicate false results [26].

Comparing PC-1 genotype distribution among different NAFLD grades revealed a significant difference of the KK genotype (p = .031), but not of the QQ genotype (p = .881). Similarly, Comparing PC-1 allele frequency among different NAFLD grades revealed a significant difference of the K allele (p = .001), but not of the Q allele (p = .881). However, comparing the KK and QQ genotypes among different NAFLD groups did not reveal any significant difference. The same was true for the K and Q alleles (p > .05).

On the other hand, there were significant differences in the distribution of the KK genotype in controls (66%) compared to NAFLD patients (56.3%) with a p-value of .04. The same was observed concerning the distribution of the KQ genotype in NAFLD patients (43.7%) compared to that in controls (34%) with a p-value of .003. These results suggest the KQ genotype to be one of the possible risk factors for NAFLD [26]. Carulli et al. [16] reported that there is no difference in the PC-1 K121Q polymorphism incidence between NAFLD and the control groups. However, Dongiovanni et al. [8] showed that the PC-1 K121Q was significantly linked with increased prevalence of fibrosis stage >1 and >2 and Li et al. [27] reported no significant associations between K121Q polymorphism and clinical factors in either metabolic syndrome (MS) patients or normal controls.

Despite the absence of the 121Q allele variant of PC-1 gene polymorphism in NAFLD patients, logistic regression results in all NAFLD patients showed a direct positive correlation of PC-1 gene and KQ genotype polymorphism with triacylglycerol and VLDL (OR = 0.007, p value = .011); this association was aligned with the positive significant correlation found in grade3 patients between PC-1 plasma level and triacylglycerol & VLDL. This correlation might be attributed to the effects of PC-1 on insulin resistance, which could be explained through causing triacylglycerol (TAG) accumulation in the liver which eventually leads to insulin resistance [28]. Interestingly, the accumulation of lipids, mainly TAG, in hepatocytes is the hallmark feature of the pathogenesis of NAFLD [29].

The similarity found in results of PC-1 association with TAG&VLDL, both at the genetic and protein levels, the insulin – resistant state present in NAFLD patients and high percentage of NAFLD patients that have heterozygous KQ genotype (risk Q allele) strongly suggests that NAFLD patients are at a high risk and will probably convert to the QQ mutant genotype. However, in previous studies, Tanyolac et al. [26] and Kubaszek et al. [30] reported the presence of 121Q allele variant of PC-1 gene polymorphism in studied NAFLD patients, but with no association with triacylglycerols.

Furthermore, the receiver operating characteristic (ROC) curve results showed that PC-1 plasma level has high sensitivity and specificity in differentiating between different NAFLD grades. Kouis et al. [31] stated that PC-1 is significantly associated with the progression of NAFLD. These results suggest PC-1 to be a promising biomarker in the pathogenesis of NAFLD.

6. Conclusion

- It can be concluded that patients with NAFLD risk factors (i.e., obesity, hyperlipidemia and advanced age) are highly recommended to undergo ultrasound examination for NAFLD diagnosis.
- Since triacylglycerol (TAG) is significantly correlated with PC-1 level in patients with severe NAFLD, and since there is a significant association between TG and PC-1 gene polymorphism in NAFLD patients, it can be concluded that TG is the hallmark in NAFLD pathogenesis and in the predisposition of PC-1 gene polymorphism.

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References