Detection of patatin-like phospholipase domain-containing protein 3 in nonalcoholic fatty liver disease among egyptian patients
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Background and aim
Recently, studies have identified patatin-like phospholipase domain containing 3 (\textit{PNPLA3}), which is localized in the endoplasmic reticulum and at the surface of lipid droplets. The association of \textit{PNPLA3} polymorphisms with fatty liver and histological severity of NAFLD was shown in many studies. We aimed to investigate the association of \textit{PNPLA3} with the development and severity of NAFLD in an overweight and obese Egyptian population.

Patients and methods
Eighty overweight and obese patients with NAFLD were enrolled in the study. Patients were divided into 2 subgroups according to results of liver biopsy: group 1 included 30 patients with simple steatosis, and group 2 included 50 patients with non-alcoholic steatohepatitis (NASH). In addition to 10 age-matched healthy subjects served as a control group. All NAFLD patients underwent a confirmatory biopsy. Laboratory investigations included fasting glucose, liver enzymes and lipid profile were done. Abdominal ultrasound was performed and \textit{PNPLA3} was detected in each patient by Quantitative ELISA.

Results
Levels of \textit{PNPLA3} were higher in NAFLD patients compared with controls (85.70 ±76.42 vs 3.10±2.11 respectively) and levels were also higher in NASH than simple steatosis (125.09±71.78 vs 20.06±8.47 vs 3.10±2.11 respectively, \(P<0.001\)). There were highly significant positive correlations between the \textit{PNPLA3} and waist circumference, BMI, ALT, AST, total cholesterol and TGs in the NAFLD patients.

Conclusions
Our study confirmed the association of \textit{PNPLA3} with the incidence of NAFLD and progression to NASH in the Egyptian patients.

Keywords: NAFLD, NASH, \textit{PNPLA3}, simple steatosis

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Introduction
Nonalcoholic fatty liver disease (NAFLD) is increasingly recognized as the leading cause of chronic liver disease worldwide [1]. There is growing evidence that genetic as well as environmental factors play an important role in the development and progression of NAFLD [2].

In recent years, studies have identified patatin-like phospholipase domain-containing protein 3 (\textit{PNPLA3}), also called adiponutrin, which encodes a 481-amino acid membrane protein localized in the endoplasmic reticulum and at the surface of lipid droplets [3].

The association of \textit{PNPLA3} polymorphisms is not only with fatty liver and triglyceride content but also with histological severity of NAFLD as shown in many studies [4,5].

Aim
In the present study, we investigated the association of \textit{PNPLA3} with the development and severity of NAFLD in overweight and obese Egyptian population.

Patients and methods
Patients
A total of 80 overweight and obese patients (50 females and 30 males) with NAFLD were enrolled in the study. They were prospectively recruited from Gastroenterology and Hepatology outpatient clinic of Helwan University.
Hospital (Badr Hospital) and Ain Shams University Hospitals. Their age ranged from 30 to 58 years.

Inclusion criteria for NAFLD patients were as follows: age above 18 years, overweight or BMI more than 25 kg/m², and bright echo pattern of the liver on abdominal ultrasound with or without elevated liver enzymes.

Patients were excluded from the study if one of the following criteria were present: any liver disease other than NAFLD such as hepatitis B or C, autoimmune hepatitis, alpha-1 antitrypsin deficiency or Wilson’s disease, alcohol consumption, history of drug intake (such as use of amiodarone, corticosteroids, tamoxifen, methotrexate, and oral contraceptives), pregnancy, diabetes, hypertension, thyroid disease, malignancy, and decompensated liver disease.

Any participants with evidence of local or systemic infection on physical examination were excluded from the study. In all controls, the absence of any current or past liver disease was established based on the presence of normal liver function tests and the presence of a normal abdominal ultrasound finding.

The included patients were suspected to have NAFLD (bright echo on abdominal ultrasound±elevated liver enzymes). A confirmatory liver biopsy was done after a written informed consent was obtained.

The study was conducted with appropriate approval by the Ethics Committee of Helwan and Ain Shams Universities and in accordance with the ethical guidelines of the Declaration of Helsinki [6].

The patients were divided into two subgroups according to results of liver biopsy: group 1 included 30 patients with simple steatosis, and group 2 included 50 patients with nonalcoholic steatohepatitis (NASH). In addition, 10 healthy participants with average weight served as a control group. An informed written consent was obtained from all participants before enrollment.

Methods

(1) Full medical history was taken from all participants.
(2) The weight and height of each participant were measured while the participant was clothed only in a light gown, and the BMI was calculated as body weight divided on height (kg/m²).

(3) The waist circumference was measured midway between last rib margin and the iliac crest in a standing position by the same examiner.
(4) Blood samples were obtained from each participant after a fasting period of at least 8 h for all laboratory investigations and another sample while they fasted 12–14 h for lipid profile.
(5) The blood glucose level was measured using the glucose oxidase method.
(6) Serum total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglyceride levels were measured on an auto-analyzer using enzymatic calorimetry.
(7) Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also measured.
(8) PNPLA3 was detected in each patient by Quantitative ELISA kit (Aviva Systems Biology Corporation, San Diego, CA, USA). A blood sample of 7 ml was withdrawn from each participant, and samples were centrifuged for ~15 min at 15 000g. The supernatants were collected carefully, and assay was performed immediately.
(9) Abdominal ultrasound using a Toshiba Apilo XV scanner (Toshiba, Osaka, Japan).
(10) Sonar-guided liver biopsy was done for the 80 included patients.

Abdominal ultrasonography
Abdominal ultrasonography was performed for all participants using a Toshiba Apilo XV scanner equipped with a broad-band 3.5 MHz curved array probe to assess the presence of liver steatosis (bright liver) and by a single operator to avoid interobserver variability. Patients were examined after at least 8 h fasting and were examined in the supine, right and left lateral positions.

Liver steatosis was defined and graded using this semiquantitative scale from 1 to 4 [7] as follows:

(1) A diffuse hyperechoic echo texture (bright liver).
(2) Increased liver echo texture compared with the kidney.
(3) Vascular blurring.
(4) Deep attenuation.

Liver biopsy
It was taken for all patients who were suspected of NAFLD guided by abdominal ultrasonography (according to guidelines of liver biopsy for patients with NAFLD in 2012 by the American Association for the Study of Liver Diseases) [8].
The procedure of liver biopsy (percutaneous technique)

Before the procedure

A written consent should be taken from the patients after informing them about the procedure details and risks of complications. Platelet count and platelet count-international normalized ratio were done for all patients before the procedure. The patient lies supine with his/her right side near to the bed. The right hand should be elevated behind head.

During the procedure

Ultrasound-guided insertion of the core biopsy needle (16 G, 4.5 inches) was done in the seventh or eighth right intercostal spaces in mid-axillary line after injection of a local anesthesia subcutaneously over the site of biopsy, and a half an inch incision was made in the site of injection with sterile surgical blade, using sterile gloves. Removal of the needle and local compression with a sterile gauze pad was performed to stop local bleeding.

After the procedure

Patient should lie on back at least for 2–4 h for monitoring and exclusion of recent complications followed by 24 h complete rest.

Liver biopsy was fixed in 10% neutral buffered formalin and then embedded in paraffin blocks. Five-micrometer thick sections were cut and stained with hematoxylin and eosin and examined under light microscope for histopathological diagnosis.

Statistical methods

Analysis of data was performed by Statistical Program for Social Science (SPSS) version 10 as follows: Quantitative parameters were described as mean, standard deviation and range

\[ \text{Mean (} X' \text{)} = \frac{\text{Sum } X}{n} \]

Whereas \( X' \) = individual values, \( n \) = number of values, \( X \) = mean.

Standard deviation (SD)\[ \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}} = \]

Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test in normally distributed quantitative variables while non-parametrical Kruskal-Wallis test and Mann-Whitney test were used for non-normally distributed quantitative variables. For comparing categorical data, Chi square (\( \chi^2 \)) test was performed. Exact test was used instead when the expected frequency is less than 5. Correlations between quantitative variables were done using Spearman correlation coefficient. Linear regression analysis was done to predict pnpla3 gene using different parameters.

To determine the relation between two variables, the correlation coefficient “r” was used.

\[ \text{Correl (} X, Y \text{)} = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}} \]

\( X \) & \( y \) are 2 variables.\( r \) is the sample correlation coefficient. Probability levels (\( P \)) were as follows:

\( P > 0.05 \) insignificant.
\( P < 0.05 \) significant.
\( P < 0.001 \) highly significant.

Results

The demographic, anthropometric, and laboratory data of patients with NAFLD and their controls are shown in Table 1.

The NAFLD group included 50 (62.5%) female patients and 30 (37.5%) male patients, whereas the control group consisted of seven (70%) female and
three (30%) male patients. The mean age in NAFLD group was 42.74±10.35 years, whereas the mean age in the control group was 28.30±2.83 years.

Older age, higher BMI, larger waist circumference, and higher serum levels of AST and ALT, total cholesterol, triglycerides, and LDL were detected in patients with NAFLD compared with those in the control group (P<0.001).

According to the results of the liver biopsy, the NAFLD group was divided into two subgroups: simple steatosis group (30 patients) and NASH group (50 patients).

Comparison of anthropometric and laboratory characteristics between the two subgroups of patients with NAFLD and the controls is shown in Table 2. Older age, higher values of BMI, and higher levels of ALT, AST, total cholesterol, and triglycerides were seen in patients with NASH compared with the patients of simple steatosis, with the least values detected in the control group (P<0.001).

Levels of PNPLA3 were higher in the patients with NAFLD, with a mean±SD value of 85.70±76.42, compared with 3.10±2.11 in the control participants.

Table 3 showed higher level of PNPLA3 in the patients with NASH compared with that in the patients with simple steatosis or controls (125.09±71.78 vs. 20.06±8.47 vs. 3.10±2.11, respectively, P<0.001).

As shown in Table 4 and Fig. 1, there were highly significant positive correlations between the PNPLA3 and waist circumference, BMI, ALT, AST, total cholesterol, and triglycerides (TGs) in the patients with NAFLD.

**Discussion**

NAFLD is a silent disease influencing the Egyptian population. Numerous risk factors have been suggested in NAFLD pathogenesis, including advanced age, obesity, insulin resistance, and hyperlipidemia, besides the roles of proinflammatory and anti-inflammatory cytokines [9].

Table 3 Comparison between patatin-like phospholipase domain-containing protein 3 in the studied groups

|       | NASH group | Simple steatosis group | Control group | P value |
|-------|------------|-----------------------|---------------|---------|
| PNPLA3| 125.09±71.78 | 20.06±8.47            | 3.10±2.11     | <0.001  |

NASH, nonalcoholic steatohepatitis; PNPLA3, patatin-like phospholipase domain-containing protein 3.

Table 4 The correlation coefficient between the patatin-like phospholipase domain-containing protein 3 and patients with nonalcoholic fatty liver disease

|                          | Correlation coefficient (r) | Significance |
|--------------------------|-----------------------------|-------------|
| Age (years)              | 0.398                       | NS          |
| Waist (cm)               | 0.321                       | S           |
| BMI (kg/m²)              | 0.602                       | HS          |
| ALT (U/l)                | 0.978                       | HS          |
| AST (U/l)                | 0.927                       | HS          |
| T-CHOL (mg/dl)           | 0.709                       | HS          |
| TG (mg/dl)               | 0.768                       | HS          |
| LDL (mg/dl)              | 0.125                       | NS          |
| BIL (mg/dl)              | 0.025                       | NS          |
| ALB (g/dl)               | 0.200                       | NS          |
| INR                      | 0.304                       | NS          |

ALB, serum albumin; ALT, alanine aminotransferase; AST, aspartate transaminase; BIL, bilirubin; HS, highly significant; INR, international normalized ratio; LDL, low-density lipoprotein; S, significant; T-CHOL, total cholesterol; TG, triglycerides.
In recent years, there has been increasing evidence that genetic [3], as well as environmental factors, are implicated in the progression of NAFLD [10]. In the present study, we evaluated the clinical and metabolic parameters, the liver histology, and the presence of PNPLA3 in Egyptian patients with

Correlation between PNPLA3 and BMI (a), ALT (b), AST (c), total cholesterol (d), and triglycerides (e). ALT, alanine aminotransferase; AST, aspartate aminotransferase; PNPLA3, patatin-like phospholipase domain-containing protein 3.
nonalcoholic fatty liver (steatosis and NASH) and its relation to disease severity.

The results from the current study revealed that the risk of NAFLD development rises with increasing age. These results confirm the finding of Mahmoud et al. [11] who reported that age is an independent risk factor for developing more severe NAFLD. This finding may be attributed to increased fat accumulation that occurs in liver with advancing age.

In our study, BMI of patients in the NASH group was higher than that in patients of simple steatosis and control groups (33.42±4.11, 29.59±4.67, and 23.60±2.17, respectively, with $P\leq0.001$). Similarly, waist circumference was significantly higher in NAFLD group compared with the control group (106.66±15.22 vs. 72.53±4.53, respectively, $P=0.001$).

Similar results were obtained by Hegazy et al. [12], who found significantly higher BMI levels in patients with NAFLD compared with the controls.

Our finding is also consistent with the study by Marchesini et al. [13] which confirmed that the presence of metabolic syndrome carried a high risk of NASH among NAFLD patients. Moreover, Clark and Diehl [14] found that two thirds of patients with a BMI above 30 and more than 90% of patients with a BMI greater than 39 have steatosis.

Although a study by Harnois et al. [15] concluded that BMI was the only predictive factor for NASH, other studies reported that NAFLD can occur in nonobese patients who are physically inactive [16].

Kim et al. [17] have also reported a significant association between the occurrence of fatty liver and its severity with an increase in BMI and waist circumference. Multivariate analysis in the Egyptian study, by Borai et al. [18] showed that waist circumference, hip circumference, and waist to hip ratio were significantly associated with grades 1, 2, and 3, but surprisingly BMI was not associated ($P>0.05$) with all NAFLD grades.

Although previous studies have concluded that NAFLD and its complications are more prevalent in women [19,20], two Indian studies have reported an increased prevalence of NAFLD among men [21,22]. In the current study, approximately two thirds of the studied patients with NAFLD were females.

Similarly, in another Egyptian study by Hegazy et al. [12], their NAFLD group of patients included 94% females and 4% were males. On the contrary, a previous study [23] found no difference in sex between NASH and non-NASH group.

In our study, age was higher in patients of NAFLD group compared with age of the control group (42.74±10.35 vs. 28.30±2.83, respectively, $P\leq0.001$). This result was supported by Borai et al. [18] whose study revealed that the risk of NAFLD development rises with increasing age. Similarly, Mahmoud et al. [11] who reported that age is an independent risk factor for developing more severe NAFLD. This finding may be attributed to increased fat accumulation that occurs in the liver with advancing age.

ALT and AST levels can be elevated in a variety of hepatic disorders. Of the two, ALT is thought to be more specific for hepatic injury because it is present mainly in the cytosol of the liver and in low concentrations elsewhere. AST has cytosolic and mitochondrial forms and is present in tissues of the liver, heart, skeletal muscle, kidneys, brain, pancreas, lungs, white blood cells, and red blood cells [24].

In our study, ALT was statistically higher in patients with NAFLD group compared with controls (56.02±18.89 vs. 23.30±7.18, respectively, $P\leq0.001$) and also ALT levels were higher in patients with NASH group than those in patients with simple steatosis (68.46±11.30 vs. 35.30±6.76, respectively).

Shi et al. [24] and Fracanzani et al. [25] found that liver enzymes are higher in patients with steatohepatitis when compared with steatosis. This result was supported by Pulzi et al. [26] who found that the cutoff value of 30 IU/L for ALT was used to differentiate between NASH and non-NASH groups with a sensitivity of 70% and a specificity of 88.6%.

In the present study, AST had been proved to be significantly higher in patients with NAFLD cases compared with normal control participants (52.43±17.16, 20.40±7.56, respectively, $P\leq0.001$), and also AST levels were higher in NASH group compared with simple steatosis group (63.66±10.39 vs. 33.70±6.25, respectively).
This was in agreement with the studies showing that AST was found to be independently associated with NASH [27] and fair for predicting NASH among patients with NAFLD [28].

Regarding lipid profile, our results revealed higher total cholesterol levels in patients with NAFLD compared with control (215.39±52.74 vs. 132.20±30.78, respectively) and higher levels in patients with NASH compared with patients with simple steatosis (219.82±9.20 vs. 208.00±8.86, respectively).

Accordingly, triglycerides levels were higher in patients with NAFLD compared with controls (169.86±34.66 vs. 110.20±16.93, respectively) and also were higher in NASH group versus simple steatosis (183.16±18.19 vs. 147.70±43.60, respectively).

In agreement with our results, Kashyap et al. [29] demonstrated that triglyceride levels are related to the severity of NAFLD, and also Assy et al. [30] found a strong correlation between hyperlipidemia, especially hypertriglyceridemia, and the incidence of hepatic fatty infiltration.

Moreover, Chen et al. [31] found that there is a significant association between hypercholesterolemia and hypertriglyceridemia with hepatic steatosis.

This is in agreement with the report by Mahmoud et al. [11] which stated that hyperlipidemia was an independent predictor of NAFLD development. In contrast, Paredes-Turrubiarte et al. [32] reported no significant differences in the values of lipid profile when comparing all different NAFLD grades.

Levels of PNPLA3 were higher in patients with NAFLD than those in controls (85.70±76.42 vs. 3.10±2.11, respectively), and also PNPLA3 was significantly higher in patients with NASH compared with patients with simple steatosis, and the difference was statistically significant (125.09 ±71.78 vs. 20.06±8.47, respectively, P<0.001). This was in agreement with Stickel et al. [33] who demonstrated that PNPLA3 level was significantly higher in patients with severe NAFLD (NASH) than patients with mild steatosis.

Moreover, Aragonès et al. [34] found that PNPLA3 is related to lipid accumulation in the liver, mainly in the development and progression of simple steatosis to NASH.

Our results showed significant positive correlations between the PNPLA3 in patients with NAFLD and BMI as well as waist circumference.

This result was supported by the study by Kotrones et al. [35], which showed that PNPLA3 was positively related to BMI and to liver fat content.

The current study showed positive correlations between PNPLA3 and ALT (r=0.978) as well as AST (r=0.927).

Our finding goes in agreement with Trépo et al. [36] who found a great relation between PNPLA3 variants and elevated ALT in the general population. Moreover, Kollerits et al. [37] found that PNPLA3 has a role as a susceptibility gene for hepatic dysfunction that leads to elevation of the liver enzymes in patients with NAFLD.

The current study also revealed a positive correlation between PNPLA3 and total cholesterol (r=0.709) and triglycerides (r=0.768).

This result was supported by the results of Ruhanen et al. [38] which showed that PNPLA3 increases lipid profile that leads to accumulation of TG and slows down TG hydrolysis upon cellular lipid depletion.

Finally, the present study had several strength points: first, we can rely on the PNPLA3 level for the prediction of the more aggressive form of the NAFLD, which is NASH; second, NASH is a cornerstone of metabolic syndrome; and third, the progression to NASH from simple steatosis did not take a long time duration as was assumed before so it must be searched for in the middle age group as shown in our study.

We should point out the following drawbacks of our study. The main limitation of this work is an adjusted sample size, and we recommend increasing the number of patients in future studies. Additionally, the study is cross-sectional. We could not prove a causal link between PNPLA3 expression and NALFD development. However, our study cohort of morbidly obese women has revealed clear relationships between the expression of PNPLA3 and NAFLD, without the interference of sex or age. Thus, our findings cannot be extrapolated to men or other obesity groups such as normal-weight or over-weight women.
We concluded that there is an association between PNPLA3 and the incidence of NAFLD as well as progression to NASH in the Egyptian patients. We also recommend considering the use of PNPLA3 as a part of the workup in diagnosing the progression of simple steatosis into NASH and subsequent fibrosis.

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Conflicts of interest There are no conflicts of interest.

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