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Original Research

Serum levels of adiponutrin in differentiating non-alcoholic steatohepatitis from simple steatosis in non-alcoholic fatty liver disease patients

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Abstract

Background and aims: Non-alcoholic fatty liver disease is one of a chronic liver diseases, characterized by an unusual accumulation of fats in hepatocytes. It's a multifactorial disease and the pathogenesis is not completely known. The genetic association is seen, where the patatin-like phospholipase domain-containing protein 3 (PNPLA3) is the most relevant genetic association. PNPLA3 encodes for a protein called adiponutrin (ADPN), which has triglyceride hydrolase and acyltransferase activity. Hepatic fat accumulation results due to excess lipogenesis or decreased fat export. Liver biopsy is the only gold standard available for differentiating the stages of NAFLD, but it's an invasive procedure that may lead to potential infections. The study aimed to evaluate serum levels of ADPN in stages of NAFLD. **Materials and methods:** This is an observational study comprised of 60 NAFLD cases, among which 10 subjects were stage 1 (simple steatosis) and 50 subjects stage 2 (non-alcoholic steatohepatitis-NASH). Blood samples were collected from the subjects and serum ADPN levels were assessed by enzyme-linked immunosorbent assay. **Results:** The serum levels of ADPN were significantly decreased in NASH as compared to simple steatosis ($p<0.001$). **Conclusions:** ADPN serum level was inversely associated with the progression of NAFLD.

Keywords: non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, patatin-like phospholipase domain-containing protein 3, adiponutrin, simple steatosis.

Background and aims

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease seen in people with no history of alcohol consumption and is characterized by excess accumulation of fats in the liver. NAFLD includes the simple deposition of fats in hepatocytes, which is also called simple steatosis or nonalcoholic fatty liver (NAFL). If left untreated, it results in inflammation of hepatocytes that is nonalcoholic steatohepatitis (NASH) and then fibrosis and cirrhosis followed by hepatocellular carcinoma in some cases [1]. Hence, NAFLD is a broad spectrum of NAFL, NASH, fibrosis, and cirrhosis. The global prevalence of NAFLD in the general population is 24%. The prevalence of NASH is 1.5% to 6.45%, and 41% of NASH patients develop fibrosis [2]. The community prevalence of NAFLD in India varies from 5% to32% depending on age, sex, and area of residence. In South India, the overall prevalence is 24.7% [3], and in North India, it is 32% [4]. Multiple factors, such as obesity, diabetes mellitus, hypertension, consumption of a high-calorie diet

that consists of high fructose composition, lack of exercise, or a sedentary lifestyle, are the reasons for the progression of fatty liver to NASH [5]. Genetic and environmental factors are also found to be one of the causes of the development and progression of fatty liver [6].

Fat accumulates in hepatocytes in the form of triglycerides (TGs). Studies have reported that increased hepatic triglycerides are associated with single nucleotide polymorphisms (SNPs) in the patatin-like phospholipase domain-containing protein-3 (PNPLA3) gene [7]. The location of human PNPLA3 is on the long arm (q) of chromosome number 22 at the 13.31 position, which encodes 481 amino acids [8]. It belongs to the PNPLA family, which consists of 9 members. PNPLA1-PNPLA9. The PNPLA3 gene provides instructions for the formation of a protein called adiponutrin (ADPN). PNPLA3 is also termed adiponutrin, calcium-independent phospholipase A2 epsilon, acylglycerol O-acyltransferase or the ADPN gene. ADPN is a transmembrane protein with a molecular weight of 52.8 kDa. It belongs to a group of lipid metabolizing enzymes and is a multifunctional enzyme. It has triglyceride hydrolase activity that mediates the breakdown of triglycerides and transacylase activity, which mediates the conversion of lysophosphatidic acid to phosphatidic acid in lipid metabolism. Recombinant PNPLA3 protein showed effects on both lipid synthesis and lipid hydrolysis [9]. Therefore, it promotes either catabolism or anabolism of triglycerides.

Variation in the PNPLA3 gene changes the amino acid isoleucine to methionine at the 148 positions, which is represented by I148 M. Studies have associated this genetic variation of PNPLA3 with NAFLD and have an increased risk of progressing to further stages, such as NASH, fibrosis, cirrhosis, and hepatocellular carcinoma, in different populations. Genome-wide association studies (GWAS) have established the involvement of PNPLA3 single nucleotide polymorphisms (SNPs) in the development and progression of NAFLD, especially the I148 M variant (*rs*738409 C/G) [10]. Studies have also reported that hepatic lipid accumulation in PNPLA3 I148 M carriers is associated with decreased secretion of triglyceride-rich lipoproteins from the liver [11]. SNPs are single nucleotide substitutions in DNA that usually alter the expression of a specific gene or the function of the respective protein [12]. As this gene is responsible for the synthesis of ADPN, any defect in the functioning of this gene could affect ADPN formation and its function, leading to disrupted metabolism of triglycerides and their accumulation in hepatocytes.

Early diagnosis of NAFLD before its progression to NASH is essential to prevent further complications. Various noninvasive medical imaging techniques, including ultrasound, magnetic resonance imaging, and computerized tomography, are available but are unable to differentiate the stages of NAFLD [13]. Differentiating the stages of NAFLD is essential for monitoring disease progression and treatment. For the diagnosis of simple steatosis, imaging techniques such as ultrasonography have been clinically practiced [14]. Once simple steatosis is diagnosed, the actual challenge is to measure the severity and presence of NASH. Liver enzymes such as alanine transaminase (ALT) and aspartate transaminase (AST) are used but are not specific in predicting the severity of NAFLD.

Hence, liver biopsy remains the only precise test to assess the severity and progression of stages of NAFLD, but it is an invasive procedure and may lead to potential infections, excessive bleeding or accidental injury to nearby organs. This necessitates an add-on marker; thus, we propose ADPN to be a complementary molecule that can be helpful in differentiating simple steatosis and NASH in NAFLD patients.

Material and method

Study design and study participants

The present study is an observational study that was carried out in the Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka. Subjects were enrolled between November 2019 and January 2021 from the Department of Medicine of R. L. Jalappa Hospital and Research Centre, teaching hospital of Sri Devaraj Urs Medical College. The study was approved by the Institutional Ethics

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Committee of Sri Devaraj Urs Medical College, Kolar, Karnataka, India. Written informed consent was obtained prior to the recruitment of the subjects.

The study included 60 NAFLD subjects in the age group of 25–60 years who were divided into stage 1 and stage 2.

Stage 1 (n=10) included NAFLD cases with simple steatosis (increased fat accumulation without inflammation).

Stage 2 (n=50) included NAFLD cases with steatohepatitis or NASH (increased fat accumulation with inflammation).

Stages were differentiated according to the ATP III guidelines – National cholesterol education program [15] and ultrasonography. Cases with hepatomegaly impression in ultrasonography and high triglycerides (>150 mg/dl) were considered simple steatosis (stage 1). Cases with hepatomegaly with fatty infiltration, high serum levels of liver enzymes and high triglycerides (>150 mg/dl) were considered NASH (stage 2). In addition to the above, cases with high sensitivity C-reactive protein (hs-CRP) within the normal range (<10 mg/l) were considered stage 1, and hs-CRP >10 mg/L was considered stage 2 in this study.

Furthermore, subjects of each stage were subdivided into two groups: those with comorbidities and those without comorbidities.

Stage 1 (simple steatosis).

1. Group 1 (n=05): NAFLD cases with comorbidities such as obesity, hyperlipidemia, diabetes mellitus and cardiovascular disease.
2. Group 2 (n=05): NAFLD cases without comorbidities

Stage 2 (NASH)

1. Group 1 (n=25): NAFLD cases with comorbidities.
2. Group 2 (n=25): NAFLD cases without comorbidities.

Exclusion criteria

Subjects with other chronic liver diseases, such as hepatitis, chronic obstructive pulmonary disease (COPD), alcohol consumption, and smoking history, were excluded from the study.

Sample collection and storage

Five milliliters of venous blood were collected from all the subjects in tubes containing sodium fluoride for FBS estimation. For investigations such as hs-CRP and liver function tests, lipid profile blood was collected in tubes without anticoagulant. Blood samples were centrifuged at 3000 rpm for 10 minutes at room temperature within 2 hours of collection. Basic parameters were analyzed immediately, and serum for ADPN estimation was aliquoted and stored at –70°C until further analysis.

Methods

Basic biochemical parameters were analyzed by standard methods using Vitros 5.1 FS clinical chemistry analyzer. Serum levels of ADPN were estimated by enzyme-linked immunosorbent assay (ELISA) using a commercial kit. (Catalog no. SEH868Hu, Cloud clone Corp., USA).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism V.9. Quantitative variables are represented as the mean, standard deviation and confidence interval. The Shapiro–Wilk test was used to assess the normality of the data. The mean was determined if the data showed normal distribution; otherwise, the median was calculated. The means of the two groups were compared using Student's t-test for parametric data and the Mann–Whitney-Whitney test for non-parametric data. A p-value <0.05 was considered statistically significant, and <0.001 was considered highly significant.

Results

The demographic and biochemical parameters of the study participants are

Table 1: Demographic and biochemical parameters of the study participants.

Parameters	Stage 1 (n=10)	Stage 2 (n=50)	p-Value
Age (years)	46.5±10.18	48.85±10.43	1.02
Gender(F/M)	50%/50%	64%/36%	
BMI	27.56±7.82	27.69±7.17	0.6457
hs-CRP	7.136±2.24	32.08±36.36	<0.0001***
FBS	184.5±105.022	169.67±80.86	0.2354
LFT			
ALT	50.4±35.38	43.71±38.41	0.848
AST	40.7±19.18	49.21±45.45	0.008*
ALKP	125.2±85.42	98.49±50.13	0.02*
Albumin	3.42±0.66	3.38±0.79	0.59
GGT	26.3±10.39	26.38±10.19	0.85
Lipid profile			
SC	128.5±58.62	158.67±57.71	0.86
TG	193.2 ±80.30	217.71±97.16	0.56
HDL	24.6±6.619	27.30±9.24	0.28
LDL	64.2±29.32	66.56±42.46	0.23
VLDL	38.64±16.06	43.54±19.43	0.56

Student's unpaired t test was used. Values are expressed as the mean ± SD. (*p<0.05, **p<0.001, ***p<0.0001). BMI: body mass index, hs-CRP: high-sensitivity C-reactive protein, FBS: fasting blood sugar, ALT: alanine transaminase, AST: aspartate transaminase, ALKP: alkaline phosphatase, GGT: gamma glutamyl transferase, SC: serum cholesterol, TG: triglycerides, HDL: high-density lipoprotein, LDL: low-density lipoprotein, VLDL: very-low-density lipoprotein.

presented in Table 1. Significantly increased hs-CRP levels were observed in stage 2 patients compared to stage 1 patients, indicating inflammation in stage 2 patients.

When the serum levels of ADPN were compared between the two stages, the levels were significantly decreased in stage 2 NAFLD cases (NASH) compared to stage 1 NAFLD cases (simple steatosis) (Figure 1).

Furthermore, when the levels of ADPN were compared between patients with comorbidities (group 1) and without comorbidities (group 2) of stage 1, group 1 patients had decreased levels compared to group 2 patients (Figure 2). However, the data were not statistically significant.

Comparing the levels of ADPN between groups 1 and 2 patients with stage 2 NAFLD, group 1 presented significantly decreased levels of ADPN (p<0.0001) compared to group 2 (Figure 3).

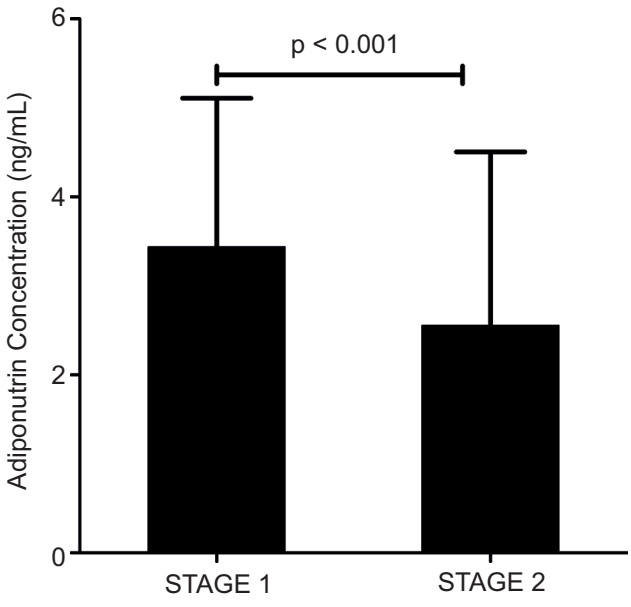

Figure 1: Serum levels of adiponutrin in stage 1(simple steatosis) and stage 2(NASH) of NAFLD subjects.

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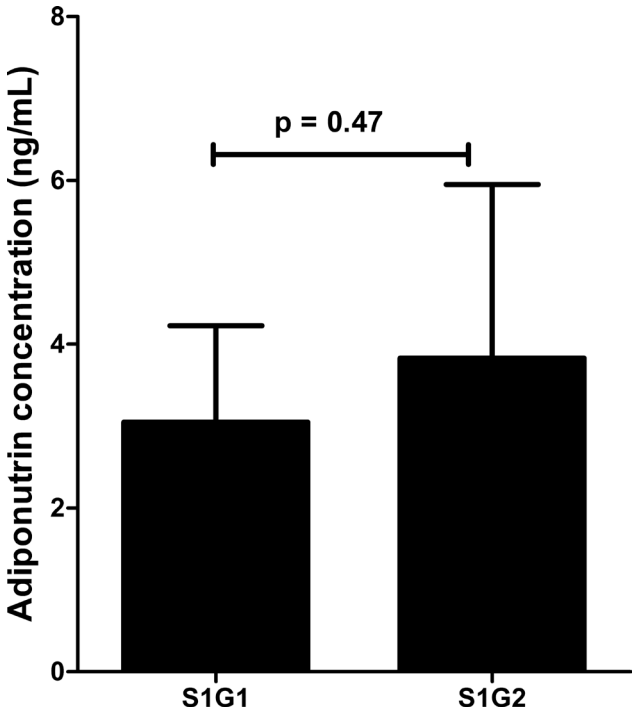

Figure 2: Serum levels of adiponutrin between groups 1 (with comorbidities) and 2 (without comorbidities) of stage 1 patients.

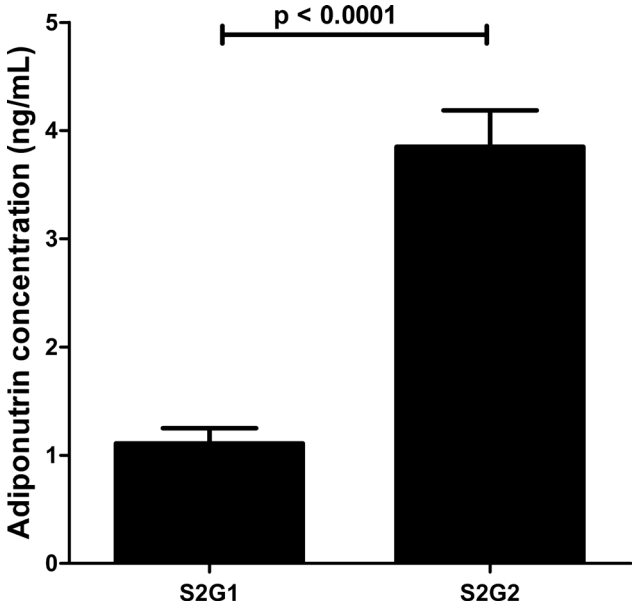

Figure 3: Serum levels of adiponutrin between groups 1 (with comorbidities) and 2 (without comorbidities) of stage 2 patients.

Discussion

The pathogenesis of NAFLD is a complex and multifactorial process. Factors such as a high fat/calorie diet, metabolic disorders such as obesity, insulin resistance, and genetic and environmental factors contribute to the progression of NAFLD. Several theories have been hypothesized, where the initial theory was termed the "two-hit model". In this, the first hit was a hepatic accumulation of lipids due to sedentary lifestyle, obesity, and insulin resistance; sensitizing the liver to further insults acts as the second hit. The second hit further progresses to inflammation, resulting in NASH and fibrosis. This theory was supported by animal models of obesity, where a second hit is necessary to initiate inflammation and fibrosis [16]. The most relevant genetic association of NAFLD was seen in patatin-like phospholipase domain-containing protein-3 (PNPLA3), which encodes ADPN [17].

The aim of this study was to measure the serum levels of ADPN and assess whether it can be helpful in differentiating the stages of NAFLD, specifically simple steatosis (stage 1) and NASH (stage 2). To the best of our knowledge, this is the first study to evaluate the serum levels of ADPN in humans with NAFLD, as existing studies have associated I148 M in the ADPN gene with NAFLD. Genetics plays a vital role in NAFLD, and the variations affect disease progression in patients with NAFLD. Romeo et al. [7] were the first to report SNPs in the ADPN gene, which encodes the I148 M variant. This variant was strongly associated with increased liver fat content. I148 M variant is associated with the degree of liver injury and all the histopathological characteristics of NAFLD, including NASH, fibrosis, cirrhosis and the development of hepatocellular carcinoma [18, 19]. The mechanisms through which the ADPN gene variant contributes to the progression and severity of NAFLD have been broadly studied, and the latest evidence suggests that the I148 M substitution induces a loss of function of ADPN gene hydrolase activity toward triglycerides and retinyl esters, which leads to their accumulation in lipid droplets of hepatocytes and hepatic stellate cells [20]. A study reported that the ADPN gene has lysophosphatidic acid acyl transferase (LPAAT) activity, which increased when I148 M was overexpressed, suggesting a gain-of-function mutation [21]. There is no consensus on whether ADPN I148 M is the gain of function of lipid synthesis or loss of function of lipid hydrolysis [21, 22].

However, ADPN gene variants increase the risk of NAFLD progression [23]. Bruschi and his coworkers [24] reported the contribution of ADPN gene polymorphisms to an increased risk of steatosis and fibrosis. Studies have also investigated the gene expression-based staging of NAFLD to understand its progression and identify effective treatments [25]. In the present study, significantly decreased levels of ADPN were observed in stage 2 (NASH) compared to stage 1 (simple steatosis) (Figure 1). This suggests that ADPN can be helpful in differentiating and assessing the progression of NAFLD.

Simple steatosis is the only stage that occurs in the absence of other stages of NAFLD [26]. It is a dormant condition and accounts for the first stage of NAFLD [27]. Simple steatosis is defined as the accumulation of lipid droplets in more than 5% of hepatocytes [28]. Perpetuation of a sedentary lifestyle leads to fat accumulation, resulting in other metabolic disorders and progression of NAFLD, including inflammation and scarring of tissues that account for steatohepatitis or NASH. Variation in the ADPN gene is reported to be associated with obesity and insulin resistance. Obesity and hypertriglyceridemia are known to be major risk factors for simple steatosis and NAFLD [29]. Insulin resistance is more prevalent in NASH than in simple steatosis. Insulin resistance is one of the "multiple hits" predisposing to the development of NAFLD and progression, critical for the establishment of lipotoxicity, oxidative stress and inflammatory cascade activation [16]. An association between ADPN gene polymorphisms and insulin resistance and NAFLD has been reported [30]. NAFLD is associated with comorbidities like obesity, diabetes mellitus, cardiovascular disease, hyperlipidemia and this carries an increased risk of liver-related morbidity and mortality. Therefore, we investigated the ADPN levels in cases with and without comorbidities and it was observed that ADPN levels were decreased in NAFLD subjects with comorbidities. Though the difference was not statistically significant in stage 1 (steatosis) subjects with and without comorbidities (Figure 2) as the disease advances to stage 2 (steatohepatitis) the difference was statistically significant between cases with and without comorbidities (Figure 3). This implies the protective role of ADPN against the progression of NAFLD. For further prospects, studies involving all stages of NAFLD in a larger population are needed.

Conclusions

The findings of the current study showed an inverse relationship between adiponutrin and NAFLD progression, signifying the use of adiponutrin as a complementary marker for differentiating simple steatosis and NASH in NAFLD patients.

Conflict of Interest

The authors declare no conflict of interest.

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