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Evaluating Serum Neuregulin 4 as a Noninvasive Biomarker in Patients with Non-alcoholic Fatty Liver Disease

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Abstract

BACKGROUND: Non-alcoholic fatty liver disease (NAFLD) spectrum ranges from simple hepatic steatosis to non-alcoholic steatohepatitis. Considering the restrictions of liver biopsy, various serological biomarkers have recently emerged for non-invasive diagnosis of NAFLD.

AIM: This study aimed to evaluate the association between serum neuregulin 4 (Nrg4) and NAFLD and the use of serum Nrg4 as a noninvasive marker for diagnosis of NAFLD.

METHODS: Sixty-three Egyptian NAFLD patients and 63 controls were enrolled and subjected to detailed history taking, thorough clinical examination including anthropometric measures (body mass index [BMI] and waist circumference). Laboratory investigations included complete blood count, lipid profile (total cholesterol, low-density lipoprotein cholesterol [LDL-C], high-density lipoprotein-cholesterol [HDL-C], triglycerides), serum albumin, transaminases, bilirubin levels, hepatitis markers (anti-HCV antibodies and hepatitis B surface antigen), anti-nuclear antibodies and anti-smooth muscle antibody. Measurement of serum Nrg4 by ELISA and non-invasive NAFLD scores such as NAFLD fibrosis score and FIB4 score were applied to all patients.

RESULTS: There was a statistically significant difference between cases and controls regarding the BMI, waist circumference, hemoglobin level, total leukocytic count, total cholesterol, LDL-C, HDL-C, and serum triglycerides levels. Nrg4 was significantly decreased in NAFLD patients as compared to controls. Moreover, Nrg4, total cholesterol, and LDL-C levels were statistically significant independent predictors of NAFLD. No significant differences were observed between Nrg4 level and the variable stages of hepatic fibrosis by NAFLD fibrosis score.

CONCLUSION: Decreased serum Nrg4 level is frequent in NAFLD patients and is an independent predictor of NAFLD, suggesting that Nrg4 might have a potential role in prevention and treatment of NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) comprises a broad scope of liver disease ranging from simple hepatic steatosis to severe hepatic necro-inflammation (non-alcoholic steatohepatitis or NASH), and can eventually evolve into cirrhosis and hepatocellular carcinoma, with a concomitant increase in morbidity and mortality [1], [2], [3], [4], [5]. There has been a pronounced rise in the incidence of NAFLD over the past 20 years worldwide [6].

Liver biopsy remains the gold standard for NAFLD diagnosis and for discriminating between simple steatosis and NASH. However, biopsy is an invasive method, with a probable risk of complications [7]. In addition, the diagnosis and staging of NAFLD could be influenced by sampling bias. Considering these liver biopsy drawbacks, various serological indices and modes of imaging have emerged in recent years for non-invasive diagnosis of simple steatosis and NASH [8].

Neuregulin 4 (Nrg4) is recognized as a brown adipose tissue (BAT)-enriched adipokine that has a positive impact on hepatic lipid metabolism and metabolic homeostasis, thus providing the link between BAT activation and improvement in insulin resistance and hepatic steatosis induced by a high-fat diet (HFD) [9], [10]. The chief target for Nrg4 is liver tissue with various effects on it including metabolic homeostasis and guarding against diet–induced hepatic injury [11]. Previous animal studies revealed that decreased Nrg4 function correlated significantly with a greater expression of genes involved in hepatic *de novo* lipogenesis, while overexpression of Nrg4 was associated with a reduction in the expression of lipogenic genes by the liver [9]. Furthermore, a study by Safa and Pollok expressed evidence for increased hepatic levels of cellular FLICE-like inhibitory protein (c-FLIP), an anti-apoptotic protein, in Nrg4-transgenic mice as compared to controls [12]. Nrg4 was found to have a cytoprotective impact on hepatocytes by inhibiting the ubiquitination and proteasome-mediated degradation of c-FLIP [11]. c-FLIP protects the hepatocytes by inhibiting apoptosis, necrosis, and necroptosis – which are pivotal in the initiation of NASH as well as its progression and are important triggers for chronic inflammation and

hepatic fibrosis [13], [14], [15]. c-FLIP overexpression attenuated NASH in mice and other animal studies, and this suggests the crucial protective role of this element in the pathogenesis of NASH [16]. In a study by Guo *et al.*, restoration of c-FLIP expression in hepatocytes improved diet-induced NASH in Nrg4-deficient mice without affecting steatosis [11], hence implying that Nrg4/c-FLIP signalling pathway has an independent impact on progression of steatosis to NASH.

We sought to evaluate the possible association between serum Nrg4 and NAFLD and to investigate serum Nrg4 as a non-invasive marker for NAFLD diagnosis and to open possible doors of using Nrg4 in the prevention and treatment of NAFLD.

Methods

This prospective observational case–control study enrolled 126 subjects: 63 with NAFLD and 63 healthy non-obese control subjects. Participants were prospectively recruited from the Internal Medicine Outpatient Clinic of Kasr Al-Ainy Hospital and the inpatient wards of the Internal Medicine Department.

Informed consent was signed by all participants before being recruited to the study. The present study was conducted with adequate approval by the Ethics and Scientific Committee of the Internal Medicine Department, Cairo University, and adhering to the ethical guidelines of the Declaration of Helsinki [17].

The diagnosis of NAFLD was established on finding a bright liver on abdominal ultrasound. In all control subjects, the absence of any existing or previous liver disease was confirmed by normal liver function tests and normal abdominal sonographic findings.

NAFLD patients with age over 18 years and the presence of a bright liver on abdominal ultrasound with or without concomitant elevation of liver enzymes were included in the present study. Exclusion criteria included the presence of liver diseases due to causes other than NAFLD such as viral hepatitis, autoimmune hepatitis, or metabolic liver disease (e.g. Wilson's disease) and consumption of alcohol.

All participants were subjected to the following:

Comprehensive clinical evaluation with anthropometric evaluation (height, weight, waist circumference, and body mass index [BMI] was calculated).

Laboratory workup included a complete blood count, lipid profile (cholesterol, low-density lipoprotein-cholesterol [LDL-C], high-density lipoprotein-cholesterol [HDL-C], triglycerides), serum transaminases (aspartate aminotransferase [AST], ALT), albumin, bilirubin level, anti-HCV antibodies, and hepatitis B surface antigen to exclude viral hepatitis, anti-nuclear antibodies, and anti-smooth muscle antibody to exclude autoimmune hepatitis and serum levels of Nrg4.

Measurement of serum Nrg4 by sandwich ELISA: Samples were collected in serum separating tubes, centrifuged, and stored at −20°C until processing. Serum level of Nrg4 was assessed by sandwich enzyme-linked immunosorbent assay technology (sandwich ELISA) using Fine Test Human NRG-4 (Nrg4) ELISA Kit (Wuhan Fine Biotech Co., Ltd., China. Cat. No: EH3443). Captured antibody (anti-Nrg4) is precoated onto the supplied well plates. Standards and test samples were added, allowing Nrg4 to bind. Biotin-conjugated anti-Nrg4 was used as a detector antibody. Results were determined using standard curves.

Imaging

Abdominal ultrasound was performed for all participants to evaluate steatosis (bright liver) by only one operator to avoid variability between examiners, using a Hitachi device curved probe on 3.5 megahertz. Examination was performed following 8 h fast by scanning patients in the supine, right, and left lateral positions. The diagnosis of fatty liver was made when the liver echogenicity exceeded that of the renal cortex and spleen, with attenuation of the ultrasound wave, loss of definition of the diaphragm, and poor delineation of the intrahepatic architecture.

Noninvasive methods for diagnosis of hepatic fibrosis

Fib-4 score

FIB 4 = (Age × AST)/(platelet count × √ALT). Using a lower cutoff value of 1.45, a FIB-4 score < 1.45 had a negative predictive value of 90% for ruling out advanced fibrosis. On the contrary, a FIB-4 score >3.25 had a 97% specificity and a positive predictive value of 82.1% for advanced fibrosis [18].

NAFLD fibrosis score

1.675 + 0.037 × age (years) + 0.094 × BMI (kg/m^2) + 1.13 × IFG/diabetes (yes = 1, no = 0) + 0.99 × AST/ALT ratio – 0.013 × platelet (×109/l) – 0.66 × albumin (g/dl).

According to the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association, patients with a NAFLD fibrosis score above 0.676 were considered to have advanced liver fibrosis. In patients with a NAFLD fibrosis score below −1.455, advanced liver fibrosis was excluded. Scores between −1.455 and 0.676 were considered indeterminate [19], [20].

Statistical analysis

Data were encoded and inserted utilizing the Statistical Package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). Data were presented using mean and standard deviation for quantitative variables and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. Comparisons between groups were done using unpaired *t*-test when comparing two groups and analysis of variance (ANOVA) with multiple comparisons *post hoc* test when comparing more than two groups [21]. Chi-square (χ^2) test was performed when comparing categorical data, and Fisher's exact test was performed alternatively when the expected frequency was <5 [22]. Pearson's correlation coefficient was applied to correlate between quantitative variables. Logistic regression was performed to identify independent predictors of fatty liver [23]. Receiver operating characteristic (ROC) curve was created with analysis of the area under the curve to define the optimum cutoff value of Nrg4 for the detection of different stages of fatty liver. $p < 0.05$ were considered statistically significant.

Results

Clinical and biochemical characteristics of NAFLD patients and control subjects

The study population included 63 NAFLD patients and 63 healthy control subjects. The age ranged from 19 to 72 years, with a mean age of 40.89 ± 13.94 years for patients and a mean age of 36.68 ± 11.30 years for control group. The NAFLD group included 42 females (63.7%) and 21 males (33.3%). The control group included 19 females (30.2%) and 44 males (69.8%). Only 34.9% (22 out of 63) NAFLD patients were found to be diabetic and all control subjects were not diabetic.

There were statistically significant differences between cases and control as regards BMI, waist circumference, hemoglobin level, total leukocytic count, total cholesterol, serum LDL-C, HDL-C, and triglycerides with $p < 0.001$, < 0.001, 0.003, 0.045, < 0.001, < 0.001, 0.001, and < 0.001, respectively. However, no statistically significant differences were detected between cases and controls as regards platelet count, serum transaminases, and albumin level (Table 1).

Evaluation of hepatic fibrosis in NAFLD patients using the NAFLD fibrosis score and the Fib-4 score

9.5% (6 out of 63) of NAFLD patients had low fibrosis stage by NAFLD fibrosis score, 50.8% (32 out of 63) had advanced fibrosis, and 39.7% (25 out of 63) were indeterminate. None of the NAFLD cases showed fibrosis using the Fib-4 score.

Table 1: Clinical and biochemical characteristics of non-alcoholic fatty liver disease cases and control subjects

Parameter	Mean ± SD		P value
	NAFLD cases (total = 63)	Controls (total = 63)	
BMI (kg/m^2)	35.89 ± 3.90	25.01 ± 1.03	< 0.001*
Waist circumference (cm)	110.40 ± 13.68	84.27 ± 3.47	< 0.001*
Hemoglobin (g/dL)	12.54 ± 2.94	13.93 ± 2.08	0.003*
TLC (10^3/cmm)	7.13 ± 2.42	6.31 ± 2.15	0.045*
Platelets (10^{3}cmm)	297.98 ± 82.33	299.22 ± 72.96	0.929**
AST (U/L)	20.35 ± 8.97	16.92 ± 12.24	0.075**
ALT (U/L)	20.87 ± 10.11	25.00 ± 41.37	0.443**
Albumin (g/dL)	3.52 ± 0.40	3.67 ± 0.45	0.053**
Cholesterol (mg/dL)	249.84 ± 56.16	186.60 ± 27.22	< 0.001*
LDL-C (mg/dL)	139.19 ± 43.64	103.02 ± 12.81	< 0.001*
HDL-C (mg/dL)	42.30 ± 9.18	46.76 ± 5.56	0.001*
TG (mg/dL)	159.98 ± 56.32	104.51 ± 36.06	< 0.001*
Nrg4 (ng/ml)	40.39 ± 8.84	46.48 ± 5.45	< 0.001*

*Statistically significant, **Statistically non-significant. BMI: Body mass index, TLC: Total leukocytic count, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, LDL-C: Low-density lipoprotein-cholesterol, HDL-C: High-density lipoprotein-cholesterol, TG: Triglycerides, Nrg4: Neuregulin 4, SD: Standard deviation, NAFLD: Non-alcoholic fatty liver disease.

Evaluation of neuregulin 4 levels in NAFLD cases and control

NAFLD patients had significant lower mean levels of Nrg4 (mean; 40.39 ng/ml ± 8.84 SD) as compared to control group, which demonstrated higher mean Nrg4 levels (mean 46.48 ng/ml ± 5.45 SD) with $p < 0.001$ (Table 1).

The mean Nrg4 level did not differ significantly regarding gender, presence of diabetes, or consumption of alcohol. No significant differences in Nrg4 level were observed in NAFLD patients with variable grades of NAFLD fibrosis score.

Correlation between serum Nrg4 levels and different demographic and laboratory parameters revealed a statistically significant positive correlation between serum Nrg4 and platelets and serum LDL-C levels with $p = 0.047$ and 0.030, respectively. However, it did not correlate significantly with other parameters.

On multivariate logistic regression analysis, total cholesterol level, LDL-C, and Nrg4 were found to be statistically significant independent predictors of NAFLD with $p = 0.002$, <0.001, and 0.001, respectively (Table 2).

Table 2: Multivariate logistic regression analysis to detect independent predictors of non-alcoholic fatty liver disease

Fatty liver	OR	95% CI		
		Lower	Upper	p
Cholesterol	1.024	1.008	1.040	0.002*
LDL-C	1.067	1.033	1.102	< 0.001*
Nrg-4	0.869	0.798	0.946	0.001*

*Statistically significant. CI: Confidence interval, OR: Odds ratio, LDL-C: Low-density lipoprotein-cholesterol, Nrg4: Neuregulin 4.

ROC curve analysis identified a serum Nrg4 level of 40.55 ng/ml (AUC: 0.706, 95% confidence interval [CI]: 0.615–0.797, 52.4% sensitivity, 85.7% specificity and $p < 0.001$) as the best cutoff value for the detection of fatty liver (Table 3 and Figure 1).

Table 3: Receiver operating characteristic curve analysis to explore the best cutoff value of Nrg4 in the detection of fatty liver

AUC	p	95% CI		Cutoff value	Sensitivity (%)	Specificity (%)	PPV	NPV	Accuracy
		Lower boundary	Upper boundary						
0.706	<0.001	0.615	0.797	40.55	52.4	85.7	78.57	64.29	69.05

PPV: Positive predictive value NPV: Negative predicted value, AUC: Area under curve, CI: Confidence interval.

Discussion

The present study revealed that NAFLD patients had significantly lower mean levels of Nrg4 as compared to control subjects who demonstrated higher mean Nrg4 levels with p < 0.001. This is in accordance with a study by Wang *et al*. [24], which revealed significantly lower levels of serum Nrg4 in the NAFLD group (2.24 [1.20, 3.22] ng/ml) when compared with control group (5.50 [2.45, 10.85] ng/ml) (p < 0.001) [24]. Our results were also similar to a study by Dai et al conducted on 87 NAFLD subjects and 87 controls. They demonstrated reduced level of serum Nrg4 in NAFLD patients compared to control subjects (median level of 0.40 ng/ml versus 0.50 ng/ml respectively; p = 0.029). The levels did not, however, differ significantly between obese and non-obese subjects (p = 0.932) [25]. Furthermore, in the latter study, the serum Nrg4 levels did not vary significantly in subjects with varying grades of fatty liver by abdominal ultrasound (p = 0.08). By multivariate logistic regression analysis, the latter study also showed that reduced serum levels of Nrg4 were independently associated with higher risk for NAFLD (odd ratio = 0.251, 95% CI = 0.081–0.779, p = 0.017) [25].

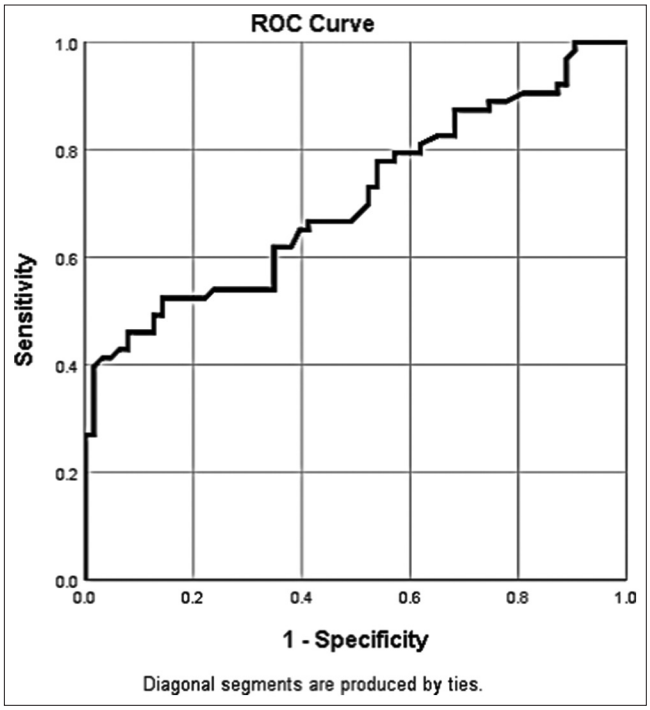

Figure 1: ROC curve analysis to explore the best cutoff value of Nrg4 in detection of fatty liver. ROC: Receiver operating characteristic. Nrg4: Neuregulin 4

A significant positive correlation between serum Nrg4 and platelet count and serum LDL-C levels was demonstrated in the present study with p = 0.047 and 0.030, respectively. However, it did not correlate significantly with any of the clinical parameters (age, BMI, and waist circumference) or other biochemical parameters (transaminases, total cholesterol, HDL-C, and serum TG levels). Moreover, Nrg4 levels did not correlate with NAFLD fibrosis score or the Fib-4 score.

Various studies have shown conflicting data when exploring the association between Nrg4 and parameters of adiposity and impaired lipid metabolism. A study by Yan *et al*. investigating the relation of Nrg4 with metabolic syndrome in newly diagnosed type II diabetes mellitus, who were divided up according to serum Nrg4 levels into four quartiles, revealed that subjects in the highest quartile displayed significantly lower levels of BMI and triglycerides and higher HDL-C and apo-A levels as compared to patients in the lowest quartile of Nrg4 concentration. Moreover, Nrg4 showed a negative correlation with serum triglyceride levels and positive correlation with HDL-C and apo-A levels [26].

Likewise, a study by Wang *et al*. demonstrated that serum Nrg4 was negatively associated with obesity indices such as BMI, waist circumference, and waist-to-hip ratio, thus implying the potential of Nrg4 in regulating lipid metabolism and body weight. Taking into consideration the biochemical parameters, serum Nrg4 levels were negatively associated with serum TG, fasting blood insulin, and HOMA-IR but positively correlated with serum HDL-C (p ≤ 0.05) in the latter study [24]. Nrg4 levels were, however, not associated with ALT or AST even after adjusting for age and sex, and this was in accordance with the results of our study. Previous studies by Kang *et al*. and Chen *et al*., conversely, showed a positive correlation between serum Nrg4 levels and parameters of adiposity and a negative correlation with serum HDL-C levels [27], [28]. The discrepancy in findings between studies may be attributable to the difference in study design and methodologies.

Insulin resistance plays a fundamental role in NAFLD and metabolic syndrome development [29]. Previous animal studies revealed that Nrg4 deficiency aggravates hepatic steatosis and insulin resistance and that overexpression of Nrg4 could reduce obesity-related insulin resistance and lower plasma triglycerides levels in transgenic mice [30]. Furthermore, Nrg4 has beneficial influence on energy balance and metabolic homeostasis. Collectively, these findings imply that Nrg4 may function as a novel adipokine linked to obesity and obesity-related metabolic disorders [31].

Our study showed that the mean Nrg4 level did not differ significantly regarding gender, alcohol consumption, or presence of diabetes. This agreed

with a study by Yao *et al*. evaluating the association between Nrg4 levels and DM, which also demonstrated no significant statistical difference between diabetic patients and normal controls (95% CI = −0.06–0.42, p = 0.143) [32].

To further quantify the association between Nrg4 levels and the risk of NAFLD in adults with obesity, a multivariate logistic regression analysis was conducted. It was confirmed in our study that serum Nrg4 levels, total cholesterol, and LDL-C were statistically significant independent predictors of NAFLD with p = 0.002, <0.001, and 0.001, respectively. This agreed with a study by Wang *et al*. which also confirmed that serum Nrg4 levels were the most important predictors of NAFLD in children with obesity and that each standard deviation increase in circulating Nrg4 levels was associated with an 85% decrease in the risk of NAFLD even after adjustment for other biochemical parameters [24].

In addition, a study by Singh *et al*. conducted on 71 patients with a histological diagnosis of NASH also demonstrated that cholesterol levels (p = 0.048) and LDL levels (p = 0.025) were independent predictors of disease severity in patients with NASH and may, hence, affect the decision to biopsy [33].

Thus, findings from our study and previous studies suggest that low serum Nrg4 levels increase the risk of NAFLD independent of abdominal adiposity and HOMA- IR and that Nrg4 levels may guard against NAFLD through mechanisms independent of insulin resistance and obesity.

In several gain- and loss-of-function studies in mice, circulating Nrg4 was found to safeguard against diet-induced obesity and hepatic steatosis by activating ErbB3/4 signaling in hepatocytes and mitigating hepatic *de novo* lipogenic signaling mediated by liver X receptor and sterol regulatory element-binding protein-1c (SREBP1c) in a cell-autonomous way [9], [30]. These data denote that Nrg4 may be involved in an interplay between BAT and hepatic lipogenesis [34].

A new study by Hu and Yang [35] recognized a mutation of NRG4 when performing whole-exome sequencing in 151 markedly obese subjects and exome array in 2388 community-based participants. An increase in visceral fat, liver dysfunction, and dyslipidemia was all reported in Nrg4 R44H mutation carriers. In a subsequent study to further assess the effect of mutation on the function of Nrg4 and on the enhanced the development of NAFLD, they found that Nrg4 R44H mice demonstrated more weight gain and substantially increased fat mass and hepatic lipid deposition when being fed on a HFD as compared to Nrg4 WT mice [35].

Nrg4 WT stimulated epidermal growth factor receptor 4 (ErbB4) *in vitro*, potentiated the STAT5 signaling pathway, and reduced the transcriptional activation of SREBP1c; however, this was not similarly observed in hepatocytes treated with Nrg4 R44H purified protein with failure to bind to the ErbB4 receptor and a subsequent loss in its physiological role. Nrg4 WT, thus, prevented NAFLD provoked by HFD and improved insulin resistance [35].

Furthermore, ROC curve analysis in our study identified an Nrg4 level of 40.55 (AUC: 0.706, 95% CI: 0.615–0.797, 52.4% sensitivity, 85.7% specificity, and p < 0.001) as the best cutoff value for detection of fatty liver and for distinguishing NAFLD patients from controls.

In the study by Wang *et al.* [24], serum Nrg4 level was evaluated in an attempt to distinguish between obese children with or without NAFLD. The AUC of serum Nrg4 to diagnose children with NAFLD was 0.723 (95% CI: 0.633, 0.814, p < 0.001); the optimal cutoff for serum Nrg4 levels for the diagnosis of NAFLD was 3.39 ng/ml (specificity = 0.708, sensitivity = 0.776) [24].

There are various points of strength in our current study. To the best of our knowledge, this is the first study to confirm an association between serum Nrg4 levels and NAFLD in Egyptian patients with obesity. Nrg4 was found to be a significant independent predictor of NAFLD, even after adjusting for other clinical and biochemical parameters, with the best cutoff value of 40.55 ng/ml and a high specificity for detection of fatty liver. Furthermore, our study comprehensively evaluated the association between serum Nrg4 and diabetes and other markers of adiposity and impaired lipid metabolism. Another point of strength is that the study design is a case–control study and, therefore, adds to the reliability of the results. The present study also highlights the significance of this novel adipokine as a link between activation of BAT and protection against hepatic steatosis which may therefore suggest its potential to become a diagnostic marker or target for therapy of NAFLD and obesity-related disorders.

Some of the limitations of the current study were the relatively small sample size and diagnosing NAFLD based on abdominal ultrasound, which may have led to diagnostic inaccuracy. The results could have been more reliable if the diagnosis of NAFLD was based on a liver biopsy, but unfortunately, many participants refused to perform a biopsy for safety concerns. The exact mechanisms by which Nrg4 contributes to NAFLD development could not be established owing to the study design as it was an observational cross-sectional study. The relation between serum Nrg4 and insulin resistance was not investigated as we aimed to find the association between serum Nrg4 levels and NAFLD independent of insulin resistance in the current study, which could have been a potential confounder. The relationship between serum Nrg4 levels and the grade of severity of steatosis was not studied, as abdominal ultrasound is not an accurate method of quantification of hepatic lipid content.

Conclusion

The current study revealed that reduced serum Nrg4 level is common in subjects with NAFLD and is a significant independent predictor of NAFLD. Future prospective studies are required to extensively explore the relationship between Nrg4 and NAFLD and to investigate its potential therapeutic benefits in the prevention and treatment of NAFLD.

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