Open Access Journal [doi: 10.54584/lms.2023.44] Research Article

Life and Medical Sciences

In the Diagnosis of Liver Fibrosis Developing in Nonalcoholic Fatty Liver Patients; Could Serum Fetuin A Be a Candidate Biomarker?

Nonalkolik Yağlı Karaciğer Hastalarında Gelişen Karaciğer Fibrozisinin Tanısında; Serum Fetuin A Biyomarker Olabilir mi?

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Article Info: Received; 02.09.2023. Accepted; 12.10.2023. Published; 18.10.2023.
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Cite as: Akyol T. In the Diagnosis of Liver Fibrosis Developing in Nonalcoholic Fatty Liver Patients; Could Serum Fetuin A Be a Candidate Biomarker? Life Med Sci 2023; 2(4): 171-178.

Abstract

Previous studies have reported in the literature that fetuin A, released from the liver, may be a useful serum biomarker in the diagnosis of nonalcoholic fatty liver disease (NAFLD). However, results are not consistent in predicting nonalcoholic steatohepatitis (NASH) and fibrosis, which are indicative of progressive NAFLD patients. The aim of this study was to investigate the importance of serum fetuin A in detecting NASH and fibrosis, which are determinants of morbidity, in patients with liver biopsy-proven NAFLD. This observational case-control study included 40 NAFLD patients diagnosed by liver biopsy and 25 healthy volunteer controls. The demographic characteristics of the patients and healthy volunteers were recorded, biochemical tests were done, and the serum fetuin A levels of the participants were determined by the enzyme-linked immunosorbent assay method. Serum fetuin A levels were significantly higher in both the nonalcoholic fatty liver (NAFL) (608.30 µg/mL; p<0.001) and NAFLD (536.58 µg/mL; p=0.008) groups compared to the control group (345.07 µg/mL). However, no significant difference was detected in the NASH group (417.06 µg/mL; p=0.740). When patients with and without fibrosis were compared, the mean serum fetuin A levels of patients with fibrosis were significantly lower (708 µg/mL and 380 µg/mL, respectively; p<0.001). When receiver operating characteristic (ROC) analysis was performed, the best cut-off point for patients with fibrosis was 510.98 µg/mL with 94.7% sensitivity and 66.7% specificity (p<0.001). Serum fetuin A may be a useful biomarker in the diagnosis of NAFLD patients and in predicting liver fibrosis.

Keywords: Fetuin A, Biomarker, Liver fibrosis, Metabolic-associated fatty liver disease, Nonalcoholic fatty liver disease.

Özet

Esas olarak karaciğerden salınan fetuin A'nın nonalkolik yağlı karaciğer hastalığının (*nonalcoholic fatty liver disease*, NAFLD) tanısında faydalı bir serum biyomarkerı olabileceği önceki çalışmalarda bildirilmiştir. Ancak ilerleyici NAFLD hastalarının göstergesi olan nonalkolik karaciğer yağlanması (*nonalcoholic steatohepatitis*, NASH) ve fibrozisin tahmininde sonuçlar tutarlı değildir. Bu çalışmanın amacı karaciğer biyopsisi ile kanıtlanmış NAFLD hastalarında, morbiditenin belirleyicisi olan NASH ve fibrozisin saptanmasında serum fetuin A'nın önemini araştırmaktır. Bu gözlemsel vaka kontrol çalışmasına karaciğer biyopsisi ile tanı almış 40 NAFLD hastası ve 25 sağlıklı gönüllüden oluşan kontrol grubu dahil edildi. Hastaların ve sağlıklı

gönüllülerin demografik özellikleri kaydedildi, biyokimyasal testler yapıldı ve katılımcıların serum fetuin A düzeyleri enzim-bağlı immünosorbent assay yöntemi ile belirlendi. Serum fetuin A düzeyleri kontrol grubu (345.07 µg/mL) ile kıyaslandığında hem nonalkolik yağlı karaciğer (*nonalcoholic fatty liver*, NAFL) (608.30 µg/mL; p<0.001) hem de NAFLD (536.58 µg/mL; p=0.008) grubunda anlamlı olarak yüksek idi. Ancak NASH grubunda (417.06 µg/mL; p=0.740) anlamlı bir fark saptanmadı. Fibrozisi olan ve olmayan hastalar kıyaslandığında, fibrozisi olan hastaların serum fetuin A ortalama düzeyleri anlamlı olarak düşük idi (sırasıyla 708 µg/mL ve 380 µg/mL; p<0.001). ROC analizi yapıldığında, Fibrozisi olan hastalar için en iyi kesim noktası; 94.7% sensitivite ve 66.7% spesifite ile 510.98 µg/mL idi (p<0.001). Serum fetuin A, NAFLD hastalarının tanısında ve karaciğer fibrozisini öngörmede yararlı bir biyomarker olabilir.

Anahtar Kelimeler: Fetuin A, Biyomarker, Karaciğer fibrozu, Metabolik ilişkili yağlı karaciğer hastalığı, Nonalkolik yağlı karaciğer hastalığı.

Introduction

Nonalcoholic fatty liver disease (NAFLD) occurs due to excessive accumulation of lipids in the liver and is closely associated with obesity, type 2 diabetes, and metabolic syndrome (MS) [1,2]. Therefore, it has recently been renamed "metabolic-related fatty liver disease" (MAFLD) [3]. NAFLD, one of the most common causes of chronic liver diseases, continues to increase in prevalence in parallel with the obesity epidemic and affects a significant part of the world population [4].

NAFLD has the potential to progress from simple liver steatosis (*nonalcoholic fatty liver*, NAFL) to nonalcoholic steatohepatitis (NASH), hepatic fibrosis, cirrhosis, and liver cancer. This risk is highest in people with liver fibrosis, and fibrosis is a precursor to the risk of morbidity [5,6]. The main aim is to early detect and treat individuals with progressive disease among NAFLD patients, which are commonly observed in the society. Therefore, diagnosis of NASH and determination of liver fibrosis, which are the most important determinants of the prognosis and morbidity of the disease and can also be used to monitor response to treatment, are important goals of patient management [7]. Liver biopsy remains the gold standard for the diagnosis of NAFLD, NASH, and fibrosis [8]. However, due to reasons such as being invasive, differences between pathologists' assessments, sampling error, and difficulty in applying to large masses, it has necessitated the development of diagnostic noninvasive serum biomarkers and imaging methods and has paved the way for studies on this subject [7].

Fetuin A, also known as alpha 2-Heremans-Schmid glycoprotein (AHSG), is a plasma carrier protein (*phosphorylated glycoprotein*). It is expressed mainly in hepatocytes, but to a lesser extent in adipocytes and monocytes [9].

Fetuin A-induced disruption of mitochondrial bioenergetics and increased lipogenesis leads to hepatic steatosis and insulin resistance [9]. The progression of this process has been demonstrated by studies that fetuin A plays a key role in the development of various clinical conditions such as type 2 diabetes, metabolic disorders, NAFLD, cardiovascular diseases, and familial mediterranean fever [9,10]. Previous studies have reported a significant relationship between circulating fetuin A level and NAFLD [11], but the study results regarding circulating fetuin A in the detection of NASH and fibrosis are contradictory and a definitive conclusion has not been reached.

The aim of this study was to investigate the importance of fetuin A in detecting NASH and fibrosis, which are determinants of morbidity, in NAFLD patients diagnosed by liver biopsy.

Material and Method

The study protocol was approved by the institute's committee on human research. The local ethics committee of GATA Haydarpasa Training and Research Hospital (Istanbul, Türkiye) approved the study on 03.05.2016 with the decision number 6/277. This research comply with the guidelines for human studies and the research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all subjects.

Study design and patient selection

This observational case-control study included 40 NAFLD patients (36 men and 4 women, mean age 33 years) and 25 healthy volunteer controls (20 men and 5 women, mean age 30 years). Participants were recruited from people followed in the gastroenterology outpatient clinic at GATA Haydarpaşa Training and Research Hospital (Istanbul, Türkiye).

Inclusion criteria were patients who had elevated aminotransferase enzymes for at least 6 months, who had ultrasonographic evidence of hepatosteatosis, who were diagnosed with NAFLD as a result of a liver biopsy performed within the last year, without any other liver or biliary tract disease, and were included in the study.

Patients with elevated aminotransferase levels or chronic liver disease other than NAFLD, those with alcohol or drug use, viral hepatitis, hemochromatosis, Wilson's disease, autoimmune hepatitis, alpha-1 antitrypsin deficiency, primary biliary cholangitis, sclerosing cholangitis, biliary obstruction, celiac disease were excluded from the study. Additionally, patients with renal dysfunction, ischemic heart disease, heart failure, cerebrovascular or rheumatological diseases, or malignancies were excluded from this study. Patients who drank any amount of alcohol regularly, even if their daily alcohol intake was below 20 g/day, were excluded also from the research. People over 55 years of age and body mass index (BMI) >35 kg/m^2 were also not included in the study.

All subjects included in the control group were healthy, had normal liver function tests, and were confirmed to have normal livers by ultrasound. Those with alcohol consumption >20 g/day or those using any medication were not included in the control group.

Serum fetuin-A test

Serum obtained from the venous blood of all volunteers and outpatient follow-up patients, taken for routine biochemistry tests after an overnight fast (samples were centrifuged at 2500 g for 10 minutes). The samples were stored at -80°C. Serum fetuin-A levels were tested using a commercially available ELISA (*enzyme-linked immunosorbent assay*) test kit according to the manufacturer's protocol (type; sandwich ELISA, HRP-labelled antibody. Biovendor, Modrice, Czech Republic). All measurements were made in duplicate in random order and the results were averaged.

Histological analysis

Liver biopsy samples were evaluated using the NAFLD histological scoring system by Kleiner et al. [12]. A definite diagnosis of NASH was made in cases whose histological NASH diagnosis was the sum of steatosis (0-3), lobular inflammation (0-3) and ballooning (0-2) scores of 5 or more. Cases with activity scores of 0-2 were considered as simple steatosis (NAFL), and cases with scores of 3 and 4 were considered as borderline NASH.

Fibrosis was staged according to the Nonalcoholic Steatohepatitis Clinical Research Network system [12]: Stage 0, Stage 1 (1A, 1B, 1C), Stage 2, Stage 3, and Stage 4.

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences, vs 20.0 program (SPSS Inc., NY, USA). Categorical data were defined as percentage. In statistical evaluation, descriptive values are presented as basic statistical measures such as mean, median, percentage and standard deviation. Variables that do not show normal distribution are expressed as median value.

Comparisons between groups were performed by chi-square test and Fisher's Exact test in case of low expected cell frequency. Comparison results were considered statistically significant at $p<0.05$ within the 95% CI.

Normality test for continuous variables in independent groups was performed with Kolmogorov-Smirnov test, and groups were compared with student's T or Mann-Whitney U test according to normality distribution.

Pearson correlation analysis was applied to analyze the relationships between serum fetuin A levels (continuous variable) and scoring used in liver diseases. Point biserial correlation coefficient was used to analyze the relationships between serum fetuin A levels and the presence of fibrosis, lobular inflammation, ballooning, NAFLD, and NASH.

Results

No differences were observed between the groups in terms of age and gender (Table 1). Many parameters such as mean body mass index, presence of metabolic syndrome, homeostasis model of insulin resistance (HOMA-IR), aminotransferases, low-density lipoprotein cholesterol, and uric acid levels were significantly higher in the patient groups than in the control groups (Table 1).

Serum fetuin-A levels were significantly higher in both the NAFL (608.30 µg/mL) and NAFLD (536.58 µg/mL) groups compared to the control group (345.07 µg/mL), p<0.001 and p=0.008, respectively. When receiver operating characteristic (ROC) analysis is performed, the best fetuin-A cut-off point for the detection of NAFLD patients was determined as 388.25 µg/mL with 70% sensitivity and 60% specificity (p=0.008) (Figure 1).

Table 1. General characteristics and laboratory findings of the groups.

	NAFL n=25	NASH n=15	NAFLD n=40	Control n=25	p value (NAFL-Control)	p value (NASH-Control)	p value (NAFLD-Control)
Age (years)	36	32	33	30	0.059	0.637	0.129
Gender (M/W)	23/2	13/2	36/4	20/5	0.221	0.591	0.256
BMI (kg/m^2)	29.38	27.77	29.38	24.35	<0.001	<0.001	<0.001
HT (yes/no)	9/11	2/9	11/20	0	<0.001	0.028	<0.001
Diabetes mellitus (yes/no)	9/15	5/10	14/25	0	<0.001	0.002	<0.001
MS (yes/no)	14/11	7/8	21/19	0	<0.001	<0.001	<0.001
HOMA-IR	4.05	6.3	4.91	1.45	<0.001	<0.001	<0.001
AST (IU/mL)	46	53	49.5	23	<0.001	<0.001	<0.001
ALT (IU/mL)	81	115	86.5	21	<0.001	<0.001	<0.001
GGT (IU/mL)	48	40	47	12	<0.001	<0.001	<0.001
Total cholesterol (mg/dL)	215	200	212	173	<0.001	0.239	0.004
HDL-C (mg/dL)	39	36	39	40	0.846	0.361	0.563
LDL-C (mg/dL)	142	113.5	136	108	0.006	0.361	0.016
Triglycerides (mg/dL)	152	141	145	104	0.116	0.14	0.024
Uric acid (mg/dL)	5.2	5.2	5.2	4.1	<0.001	0.003	<0.001
Ferritin (µg/L)	181	164	173	74	<0.001	<0.001	<0.001
Hs-CRP (mg/dL)	2.25	2.66	2.36	0.87	0.006	0.023	0.002
Fetuin A (µg/mL)	608.30	417.06	536.58	345.07	<0.001	0.740	0.008

Appropriate data are presented as median values.
ALT; alanine aminotransferase. AST; aspartate aminotransferase. BMI; Body mass index. GGT; gamma-glutamyltransferase. HDL; high-density lipoprotein. HT; hypertension. HOMA-IR; homeostasis model of insulin resistance. hs-CRP; high sensitivity C-reactive protein. LDL; low-density lipoprotein. MS, Metabolic syndrome. NAFL; nonalcoholic fatty liver. NAFLD; nonalcoholic fatty liver disease. NASH; non-alcoholic steatohepatitis.

There was no significant difference in serum fetuin A levels when comparing the NASH (417.06 µg/mL) and the control group (345.07 µg/mL) (0.740) (Table 1). No significant relationship was detected between serum fetuin A levels and histopathological scores (Table 2).

Table 2. Correlation analysis of fetuin A levels and histopathological scores.

		*NAS	**SAF activity score	Steatosis score	Lobular inflammation score
Fetuin A	r	-0.196	-0.118	-0,175	-0.133
	p	0.225	0.469	0.281	0.414

*NAS (NASH Clinical Research Network scoring system), **SAF (Steatosis, activity, fibrosis score).

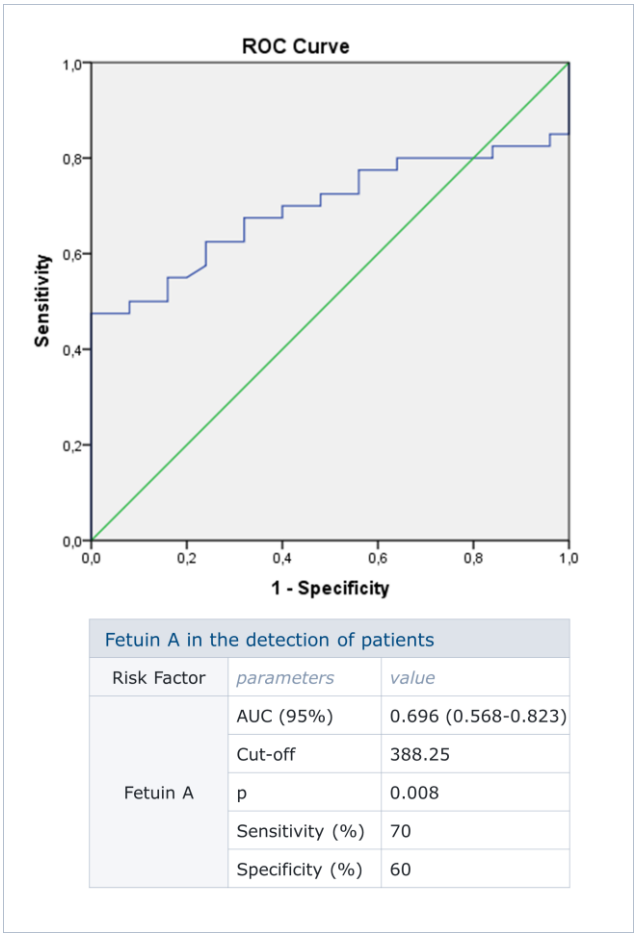
There was no significant difference in fetuin A levels between the NAFL and NASH groups. However, when patients with and without fibrosis were compared, the mean serum fetuin A levels of patients with fibrosis were significantly lower (708 µg/mL, 380 µg/mL, p<0.001) (Table 3).

Table 3. Fetuin A averages and histopathological diagnosis and analysis of findings .

		n	Fetuin A, mean	p
Disease	NAFL	25	608	0.096
	NASH	15	417	
Fibrosis	yes	21	380	<0.001
	no	19	708	
Lobular inflammation	yes	34	516	0.256
	no	6	648	
Ballooning	yes	18	512	0.807
	no	22	556	

*NAS (NASH Clinical Research Network scoring system), **SAF (Steatosis, activity, fibrosis score).

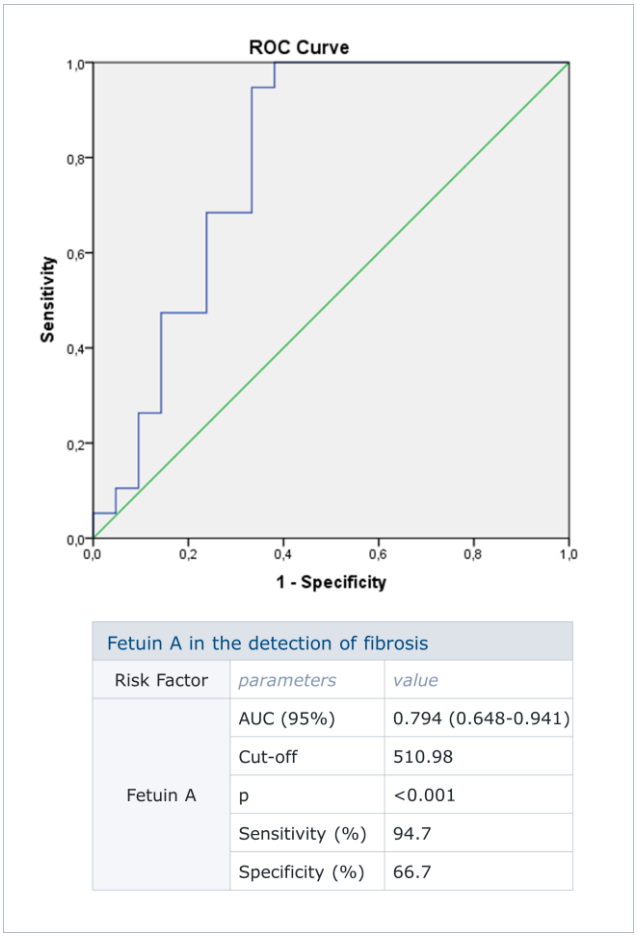
When ROC analysis is performed, the best cut-off point for patients with fibrosis is; it was 510.98 µg/mL with 94.7% sensitivity and 66.7% specificity (p<0.001) (Figure 2). Of the patients with fibrosis (n=21), 17 were stage 1, and 4 were stage 2. The average age of the patient was young (33 years) and there was no advanced fibrosis in these patients.



Fetuin A in the detection of patients

Risk Factor	*parameters*	*value*
Fetuin A	AUC (95%)	0.696 (0.568-0.823)
	Cut-off	388.25
	p	0.008
	Sensitivity (%)	70
	Specificity (%)	60

Figure 1. ROC analysis; fetuin A in the detection of NAFLD patients.



Fetuin A in the detection of fibrosis

Risk Factor	*parameters*	*value*
Fetuin A	AUC (95%)	0.794 (0.648-0.941)
	Cut-off	510.98
	p	<0.001
	Sensitivity (%)	94.7
	Specificity (%)	66.7

Figure 2. ROC analysis; fetuin A in the detection of liver fibrosis.

Discussion

Fetuin A has diagnostic potential as a biomarker for NAFLD, cardiovascular diseases, and metabolic syndrome-related disorders [13,14]. However, the relationship of serum fetuin A with fibrosis in NAFLD patients remains unclear [15]. Our study results showed that serum fetuin A levels were significantly associated with both liver fibrosis and NAFLD patients diagnosed by liver biopsy. NAFLD prevalence is increasing worldwide and can progress to end-stage liver disease and liver cancer. Moreover, NAFLD is also an important risk factor for cardiovascular diseases [16,17].

Considering its prevalence in society, it is not possible to perform liver biopsy, which is the gold standard, to detect NAFLD and patients with NASH and fibrosis, which are the progressive groups within NAFLD. For this reason, the development of serum markers that are non-invasive, readily available, and easy to apply has become a necessity. Haukeland et al. found that the serum fetuin A levels in 111 histologically-proven NAFLD patients) were significantly higher than the control group, but it was not significant in terms of NASH or fibrosis [18]. Yılmaz et al. [19] found serum fetuin A levels were significantly higher in 99 biopsy-proven NAFLD patients and those with fibrosis. There was no significant difference in terms of NASH [19]. In another study, the serum fetuin A level of 79 ultrasonography-proven NAFLD patients was found to be significantly lower compared to 79 controls [20].

On the other hand, in a meta-analysis that covering a total of 17 studies including 1,755 NAFLD patients and 2,010 healthy controls, circulating fetuin A level was found to be significantly higher in NAFLD patients than in the control group. However, it was not associated with NAFL and NASH classification in subgroup analysis, and association with the development of fibrosis remains controversial [21].

In another meta-analysis including 30 articles (3800 NAFLD patients and 3614 healthy participants) [22], serum fetuin-A levels were found higher in NAFLD patients than control group ($p<0.001$). In addition, the effect value of fetuin A in the NASH subgroup was found significantly higher than the NAFLD subgroup ($p=0.036$). However, the researchers emphasized that further research is needed to confirm these results [22]. Our results were consistent with meta-analyses in identifying NAFLD patients. When ROC analysis was performed to detect NAFLD patients, sensitivity was 70% and specificity was 60% ($p=0.008$) (Figure 1). According to our subgroup analysis, there was no significant difference between serum fetuin A levels in determining NAFL and NASH patients.

The strongest factor predicting mortality in NASH patients is hepatic fibrosis [23,24]. The most important finding of our study was that serum fetuin A could detect NAFLD patients with fibrosis. When patients with and without fibrosis were compared, serum fetuin A levels were significantly lower in the fibrosis patient group ($p<0.001$). When ROC analysis was performed, fetuin A was at the best cut-off point to detect patients with fibrosis; it had 94.7% sensitivity and 66.7% specificity ($p<0.001$) (Figure 2). On the other hand, Sato et al.'s study was also compatible with our current results. They showed that fetuin A levels were negatively associated with liver and vascular fibrosis [15].

Fetuin A inhibits transforming growth factor-b1 signaling, which increases in liver fibrosis and fibrosis of many other tissues; therefore, it has been suggested that fetuin A may prevent fibrotic changes in different organs [25,26]. This pathophysiological finding may explain the negative relationship between NAFLD patients with fibrosis and low fetuin A levels. Additionally, epidemiological studies have shown that circulating fetuin-A levels are associated with obesity, hyperglycemia, hypertriglyceridemia, metabolic syndrome, type 2. They revealed that it is associated with chronic diseases such as diabetes mellitus [14,27,28]. Our results are consistent with the literature, and fetuin A levels were associated with MS and its parameters.

This study has some limitations. First of all, our study is a case control study. In addition, there are many more confounding factors that may affect the serum concentrations of fetuin A, such as metabolic syndrome, insulin resistance, hyperlipidemia, cardiovascular diseases, age, and

smoking, and an isolated NAFLD group from these confounding factors was not created in the study. In addition, the number of participants in the study and control groups is small and does not represent all NAFLD patients and the control group population, as they consist of young age and early mild fibrosis cases.

Conclusion

In conclusion, this current study revealed that serum fetuin A may be a potential biomarker for the early diagnosis of NAFLD patients with fibrosis. In addition, it emphasizes that the serum fetuin A test, which is noninvasive, easy to obtain and apply, may be an important indicator for the detection of NAFLD patients in the community. These results showed that fetuin A has an important role in the pathophysiological process that develops from simple steatosis to fibrosis in NAFLD patients. In order to elucidate this issue prospective, homogeneous, and large population studies are needed.

Conflict of interest: The author declares that there is no conflict of interest with regard to the authorship and/or publication of this article. The author alone is responsible for the content and writing of the paper.
Financial disclosure: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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