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Association of Pro-Ghrelin Leu72Met Polymorphism with Acylated Ghrelin Level and Alcohol Use Disorder: A Preliminary Study

Pro-Ghrelin Leu72Met Polimorfizmi ile Açil Ghrelin Düzeyi ve Alkol Kullanım Bozukluğu Arasındaki İlişki: Bir Ön Çalışma

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Abstract

Objective: Multiple environmental and genetic factors contribute to the progression of alcohol use disorder (AUD). Ghrelin is one of the important elements of the brain-gut axis that has been believed to involve in the pathophysiology of addiction. This study aimed to determine whether the *GHRL* Leu72Met gene polymorphism has an effect on the plasma acylated ghrelin levels in alcohol addicts for the first time.

Method: A sample of 50 alcohol-dependent men and 50 controls were enrolled in this study. Acylated ghrelin levels were detected by ELISA kit. The *GHRL* Leu72Met polymorphism was analyzed by the standard PCR-RFLP method.

Results: Acylated ghrelin levels were significantly higher in AUD patients than in controls, and were lower in AUD patients with Leu72Leu than those with Leu72Met and Met72Met. After detoxification, a dramatic decrease was seen in AUD patients having Leu72Met+Met72Met. The presence of 72Met allele was also found to be associated with an increased risk of AUD in Turkish men.

Conclusion: It was indicated for the first time that the *GHRL* Leu72Met variant was associated with higher plasma acylated levels in patients with AUD. The *GHRL* Leu72 allele compared to the Met72 allele seemed to be protective against AUD in Turkish men. Taken together, despite the small number of subjects evaluated, the findings in this study suggested the effect of the *GHRL* Leu72Met polymorphism on plasma acylated ghrelin levels and alcohol addiction.

Keywords: Alcohol use disorder, *GHRL* Leu72Met polymorphism, acylated ghrelin

Öz

Amaç: Birçok çevresel ve genetik faktör, alkol kullanım bozukluğunun (AKB) gelişimine ve ilerlemesine neden olmaktadır. Beyin-bağırsak ekseninin önemli unsurlarından biri olan ghrelinin, bağımlılığın patofizyolojisinde yer aldığı düşünülmektedir. Bu çalışmanın amacı, *GHRL* Leu72Met gen polimorfizminin plazma açil ghrelin düzeylerine etkisinin olup olmadığını ilk kez alkol bağımlılarında belirlemektir.

Yöntem: Bu çalışmaya 50 alkol bağımlısı erkek ve 50 kontrol dahil edilmiştir. Açil ghrelin düzeyleri ELISA kiti ile ölçülmüştür. *GHRL* Leu72Met polimorfizmi ise, standart PCR-RFLP yöntemiyle analiz edilmiştir.

Bulgular: Açil ghrelin seviyeleri, AKB tanılı hastalarda kontrollere göre anlamlı bir şekilde daha yüksek, Leu72Leu genotipli AKB hastalarında Leu72Met ve Met72Met genotipli hastalara göre daha düşük bulunmuştur. Detoksifikasyondan sonra Leu72Met+Met72Met genotipli AKB hastalarında belirgin bir azalma görülmüştür. Türk erkeklerinde 72Met alelinin varlığı ile AKB riski arasında bir ilişki belirlenmiştir.

Sonuç: Bu çalışmada, alkol kullanım bozukluğu olan bireylerde *GHRL* Leu72Met varyantı ile yüksek açil ghrelin düzeyi arasındaki ilişki ilk kez gösterilmiştir. Türk erkeklerinde, AKB açısından GHRL Leu72 alelinin, Met72 aleli ile kıyaslandığında koruyucu etkiye sahip olduğu söylenebilir. Çalışmaya dahil edilen örneklem büyüklüğü küçük olmasına rağmen, bu çalışmadan elde edilen bulgular GHRL Leu72Met polimorfizminin plazma açil ghrelin ve alkol bağımlılığı üzerinde etkisi olduğunu göstermiştir.

Anahtar kelimeler: Alkol Kullanım bozukluğu, GHRL Leu72Met polimorfizmi, açil ghrelin.

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Introduction

Alcohol use disorder (AUD), characterized by allostatic changes in the brain reward systems, is a chronic relapsing disease leading cause of morbidity and mortality worldwide. According to the data of the WHO in 2018, harmful use of alcohol caused the death of 3 million people and was responsible for 5.1% of the global disease burden. Chronic excessive consumption of alcohol changes the activity and function of many vital organs such as the liver and systems such as gastrointestinal and cardiovascular systems. The risk for various types of cerebrovascular disorders and different types of cancer increases due to chronic alcohol consumption. Regular alcohol intake leads to permanent neuro-adaptive changes causing serious and long-term consequences for brain functions (1). Additionally, the rate of alcohol-related crimes such as theft, rape and murder may rise, as the individual's decision-making ability is impaired and the risk of aggressive behaviour increases. Today, effective prevention strategies and treatment options for AUD, a chronic problem lasting for a lifetime, are still inadequate. Therefore, elucidating important genetic mechanisms in the pathophysiology of addiction in order to develop preventive and therapeutic approaches has now become one of the main goals of the research.

Multiple environmental and genetic factors contribute to the development and progression of AUD. Thus, it has a complex aetiology and pathophysiology. The components of the brain-gut axis such as neuroendocrine pathways are now thought to be involved in the pathophysiology of AUD. The hormone ghrelin, one of the important elements of the brain-gut axis, links the gastrointestinal and central nervous systems and its relationship with addictive behaviours has become crucial to investigate (2).

Ghrelin is a 28-amino acid peptide hormone synthesized mainly by the stomach enteroendocrine P/D1 oxyntic cells, but also, to a certain degree, in most human tissues such as kidney glomeruli and pancreas (2,3). The ghrelin peptide, produced as a 117 amino acid preproghrelin, is transformed into the active hormone with post-translational cleavage (2). There are 2 major forms of ghrelin in plasma: acyl ghrelin and des-acyl ghrelin. Acyl-ghrelin, also called n-octanoyl ghrelin or active ghrelin, has undergone noctanoylation at Ser3. Acyl-ghrelin activates the growth hormone secretagogue receptor 1a (GHS-R1a) which is widely expressed in both peripheral and central tissues (2,3). There is accumulating evidence suggesting that the ghrelin system is implicated in a variety of cellular and physiological processes (2). Moreover, during recent years, ghrelin has been shown to interact specifically with the mesolimbic dopaminergic system and play an important modulatory role in reward processing (2,4).

Pharmacogenetics is the study of understanding how genetic variations influence a person's disease susceptibility or drug response (5). In literature, several papers are investigating the association between AUD and genetic variability (6,7). In recent years, the effect of genes encoding ghrelin (*GHRL*) on alcohol addiction has drawn attention due to the relationship between ghrelin hormone and addictive behaviours. There have been only three studies linking certain genetic polymorphisms of the *GHRL* gene with AUD (8-10). However, these genetic association studies are contradictory, not conclusive and seem to be replicated with different populations. There have also been studies comparing the total or acylated ghrelin levels between healthy subjects and alcohol-dependent patients (11,12), none of them determined the effect of the *GHRL* Leu72Met polymorphism on plasma acylated ghrelin levels in AUD patients. Thus, the main purpose of this pilot case-control study is to determine whether the *GHRL* Leu72Met polymorphism has an effect on the plasma acylated ghrelin levels in alcohol addicts for the first time. Furthermore, the frequencies of the *GHRL* Leu72Met genotypes were compared between patients with AUD and healthy controls in Turkish men.

Methods

Study Population

Fifty male patients affected by current alcoholism according to ICD-10 (International Classification of Diseases-10) diagnostic criteria and also fulfilled the DSM-V criteria were included in the current investigation. The sample was composed of both inpatients and outpatients admitted to the hospital for the detoxification programme which lasted approximately twenty days. The exclusion criteria for study subjects were: (i) clinically significant comorbid psychiatric illness such as any psychotic disorders, severe depression, schizophrenia, mental retardation, and bipolar disorder, (ii) substance use disorders other than nicotine and alcohol dependence, (iii) BMI (body mass index) $\geq$ 30 Kg/m^2, (iv) any disorders or diseases including known diabetes, cirrhosis, abnormal fundic or duodenal histology and abnormal fasting glucose levels that can affect the plasma ghrelin concentration. Due to the fact that the prevalence of alcohol use was lower in females compared to males in Turkey, women with AUD were not included in the present pilot study. Controls comprised healthy individuals that satisfied the same exclusion criteria and had no past or current alcohol and/or substance use disorder (n=50). Controls were matched to AUD cases for gender and smoking habits. Only subjects met all of the criteria included in the study and, thus, none of the participants excluded from the study.

Only Turkish subjects were included in this pilot case-control study. A small questionnaire was employed to gather socio-demographic information on social factors such as marital, education and employment status, past and present substance use, family history of alcoholism, age onset of AUD was given to subjects. Written informed consent was obtained from each

subject who were eligible for the study. Ankara University Faculty of Medicine Clinical Research Ethics Committee approved the study design (approval no: 19-1300-18 in 2018).

Procedure

Sample Collection

The venous blood sample was taken from each subject into tubes with EDTA for DNA isolation and was kept at -20 ˚C while they were inactive use. To measure the plasma acylated ghrelin levels, blood samples from all subjects were collected into aprotinin (a protease inhibitor) tubes after an 8-h overnight fast. In the biochemistry laboratory, they were centrifuged at 2500 g for 10 minutes and stored at -80 ˚C until analysis.

Determination of the GHRL Leu72Met polymorphism by PCR-RFLP method

Genomic DNA was extracted from 200 µl whole blood samples using a Qiagen QIAamp DNA Blood Kit, according to the manufacturer's instructions. Genotyping was carried out using PCR-RFLP method. The sequences of the forward (F) and reverse (R) primers were 5'- GCTGGGCTCCTACCTGAGC-3' and 5'- GGACCCTGTTCACTGCCAC-3', respectively. PCR amplification was conducted in a final volume of 25 µL containing 200 ng of genomic DNA, 10XPCR buffer (Qiagen), 200 mM of dNTPs, 1.25 U of Hot Star Taq DNA polymerase (Qiagen) and 10 pmol each of the F and R primers, on a Techne Tc512 PCR System. The PCR cycling conditions consisted of an initial denaturation step of 15 min at 95 ˚C for; 36 cycles of 1 min of denaturation at 94 ˚C, 1.5 min of annealing at 65 ˚C, and 1 min of extension at 72 ˚C; and a final extension of 10 min at 72 ˚C. The PCR products of 618-bp were then digested with *BsrI* (New England Biolabs, Hertfordshire, UK) at 65 ˚C for 1.5 hours. A single 618 bp band stands for the presence of the 72Met allele; bands of 517 and 101 bp correspond to the Leu72 allele. The undigested and digested DNA fragments were separated by agarose gel electrophoresis on a 2% agarose gel, visualised by ethidium bromide (Figure 1).

Measurement of Plasma Acylated Ghrelin Levels

A commercial ELISA kit (Spi-Bio, Bertin Pharma, France) was used to measure the plasma acylated ghrelin levels with spectrophotometry (between 405 and 414 nm). Plasma acylated ghrelin levels were given as pg/ml.

Statistical Analysis

The statistical analyses were performed using SPSS (version 21.0 for Windows). The Kolmogorow-Smirnow test was used to examine the normality of numerical variables. Data were shown as median or mean±standard deviation (S.D.) according to the normality of numerical data. For categorical data, numbers and percentages were given. The frequencies of the *GHRL* Leu72Met alleles and genotypes were calculated by the direct genotype counting method, and the departure from the Hardy-Weinberg equilibrium was tested by the Pearson chi-square test. The relationship between the *GHRL* Leu72Met polymorphism and AUD was modelled through binary logistic regression analysis. Odds ratio (OR) value and 95% confidence intervals were calculated for comparing the risk of dependence. Non-parametric Mann-Whitney U test was used for comparison of acylated ghrelin levels, pack years of smoking and age onset of alcohol. Two independent groups were compared by parametric Student's t-test in terms of metric variables such as age and BMI. A Chi-square test was used to compare categorical variables. The patients' plasma acylated ghrelin levels measured before (T_0) and after (T_1) detoxification programme were compared through Wilcoxon signed rank test. ANOVA was performed to test whether ghrelin genotype influence BMI and smoking years between the two groups (patients vs. control and Leu72Met genotype vs. Leu72Met and Met72Met genotypes) as independent variables. The Spearman rank correlation test was used to evaluate the linear relationship between baseline acylated ghrelin levels and standard drink per week. The relationship between the duration of smoking and years of alcohol addiction was analyzed using the Pearson correlation test. A p-value <0.05 was considered statistically significant.

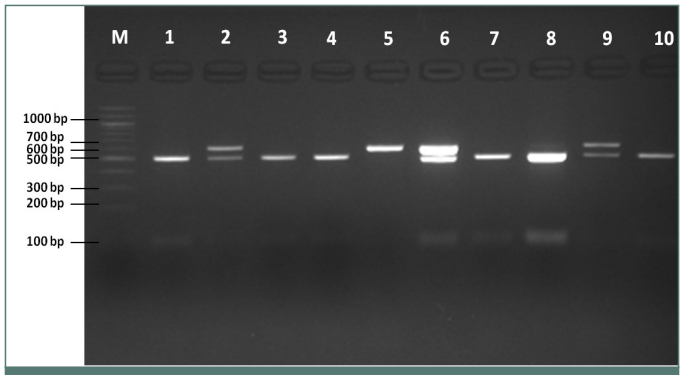

Figure 1. A representative agarose gel image of digested PCR products (618 bp) with BsrI for the *GHRL* Leu72Met polymorphism. M: 100 bp ladder; Lanes 1, 3, 4, 7, 8 and 10: homozygote typical (Leu72Leu) (517 bp and 101 bp); Lanes 2, 6 and 9: heterozygote genotype (Leu72Met) (618 bp, 517 bp and 101 bp); Lane 5: homozygote atypical (Met72Met) (618 bp)

Results

Fifty alcohol-dependent subjects (age: 46.46±10.28 years; BMI of 24.59±3.81 kg/m^2) and 50 healthy controls (age: 40.90±9.19 years; BMI of 26.74±3.34 kg/m^2) were enrolled in this study. Other demographic characteristics of Turkish males were given in Table 1. Pack years of smoking, a clinical quantification of cigarette smoking, was calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked. Patients with AUD had a significantly

higher duration of smoking (years) and pack years of smoking than healthy controls ($p<0.05$). In Table 2, the clinical features of patients with AUD were also shown. The Pearson correlation test revealed that there was a significant and positive correlation between the duration of smoking and the duration of alcohol addiction ($r=0.864$, $p<0.001$).

The allele and genotype frequencies in both study and control subjects were shown in Table 3 for Leu72Met polymorphism in the *GHRL* gene. The frequencies of the Leu72 and 72Met alleles were 95.0% and 5.0% in healthy controls, and 86% and 14.0% in alcohol dependent individuals, respectively. The genotype frequencies of *GHRL* Leu72Met polymorphism were consistent with Hardy–Weinberg equilibrium both in patients ($\chi2=0.0006$; $p=0.98$) and controls ($\chi2=0.13$; $p=0.71$).

The *GHRL* Met72Met genotype was detected in only one patient with AUD and no control subject had this genotype. Thus, subjects having the Met72Met genotype was merged with those having Leu72Met genotype and compared statistically to those having Leu72Leu. The relationship between the *GHRL* Leu72Met genotypes and AUD was examined by logistic regression analysis. Leu72Met and Met72Met genotypes were found to lead to a 3.16 times greater alcohol dependence risk (OR=3.162; 95% CI=1.032-9.685) (Table 3).

Plasma acylated ghrelin levels of controls (n=50) and patients (n=36; T_0) were also measured using an ELISA kit (Table 4). Since 14 of 50 patients were not hungry when they first applied, no blood sample could be taken for the measurement of acylated ghrelin. The median plasma acylated ghrelin levels were significantly higher in patients ($\tilde{x}$=23.72 pg/ml; range=161.01) than in controls ($\tilde{x}$=20.11 pg/ml; range=21.66) ($p=0.012$). In AUD patients, the median plasma acylated ghrelin levels were significantly lower in patients with Leu72Leu genotype ($\tilde{x}$=21.43 pg/ml; range=161.01) than those with Leu72Met and Met72Met genotypes ($\tilde{x}$=34.79 pg/ml; range=46.21) ($p=0.033$). In healthy subjects, although median plasma acylated ghrelin levels were higher in subjects with Leu72Met + Met72Met genotypes ($\tilde{x}$=23.24 pg/ml; range=7.5) than those with Leu72Leu genotype ($\tilde{x}$=20.11 pg/ml; range=21.66), there was not a significant difference between the *GHRL* Leu72Met polymorphism genotypes in terms of the plasma acylated ghrelin levels ($p=0.106$).

During the 20 days of the detoxification, 10 patients (27.8%) dropped out defined as those patients who consumed any

Table 1. Socio-demographics characteristics

Parameter	AUD (n=50)		Control (n=50)		p-value
Education					
Primary/Secondary	14	28 (%)	8	16 (%)	$\chi2=6.76$ $p=0.009$
High School	17	34 (%)	10	20 (%)	
Undergraduate	13	26 (%)	19	38 (%)	
Graduate	6	12 (%)	13	26 (%)	
Occupation					
Working	28	56 (%)	47	94 (%)	$\chi2=19.25$ $p=0.000$
Not working	10	20 (%)	1	2 (%)	
Retired	12	24 (%)	2	4 (%)	
Marital status					
Single	10	20 (%)	14	28 (%)	$\chi2=4.17$ $p=0.041$
Married	25	50 (%)	35	70 (%)	
Widow/Divorced	15	30 (%)	1	2 (%)	
Current smoker					
Yes	48	96 (%)	48	96 (%)	
No	2	4 (%)	2	4 (%)	
Smoking (years) Mean±S.D. ($\tilde{x}$, min.-max.)	25.69±10.57 25 (5-46)		16.87±10.84 15 (0-40)		z=-3.627 p=0.000
Pack years of smoking Mean±S.D. ($\tilde{x}$, min.-max.)	34.46±22.99 30 (0-111)		15.65±12.76 10 (0-45)		z=-4,515 p=0.000

AUD: Alcohol use disorder, n: sample size, S.D.: standard deviation, $\tilde{X}$: median, min: minimum, max: maximum

amount of alcohol. The remaining 26 patients (72.2%) completed all visits and plasma acylated ghrelin levels were measured again (T_1). Median plasma acylated ghrelin levels of patients having both Leu72Leu (21.43 vs. 19.15) and Leu72Met+Met72Met (34.79 vs. 19.87) decreased at T_1 (Table 4). There was a dramatic decrease in acylated ghrelin levels between T_0 and T_1 in patients with Leu72Met+Met72Met genotypes (p=0.059).

Some clinical and demographic characteristics of patients with AUD according to the *GHRL* Leu72Met genotypes were shown in Table 5. Patients with Leu72Met+Met72Met genotypes had higher BMI (25.96±3.76 kg/m^2), longer duration of smoking (27.92±9.42 years) and longer duration of alcohol addiction (32.00±10.35 years) than those with the Leu72Leu genotype (24.11±3.77 kg/m^2, 24.97±10.94 years, 27.54±10.04 years, respectively). Patients with Leu72Met+Met72Met genotypes had lower age of onset (17.61±4.54) than those with Leu72Leu genotype (18.05±3.76 years). However, when the main effect of ghrelin genotype on BMI was tested using ANOVA, no main effects of genotype (F=1.517, p=0.221), diagnosis (patients and control) (F=3.754, p=0.056) and diagnosis*genotype interactions (F=0.340, p=0.561) were detected. On the other hand, statistical comparisons of smoking duration (years) values revealed the main effects of genotype (F =3.988, p=0.049) with no main effect of diagnosis (F=3.697, p=0.058) and no significant diagnosis*genotype interactions (F=1.068, p=0.304). Analysis of the patients with AUD according to the *GHRL* Leu72Met genotypes revealed no differences between patients having the Leu72Met genotype and those having Leu72Met and Met72Met genotypes pack years of smoking (p=0.618), age of onset (p=0.327), years of alcohol addiction (p=0.178) and standard drink per week (p=0.325) (Table 5). The Spearman rank correlation test determined no correlation between baseline acylated ghrelin levels and standard drink per week (r=-0.034, p=0.845).

Discussion

To the best of our knowledge, the present pilot case-control study was the first to assess the effect of pro-ghrelin Leu72Met polymorphism on plasma acylated ghrelin levels in alcohol-dependent individuals. Plasma acylated ghrelin levels were significantly lower in AUD patients with Leu72Leu genotype than those with Leu72Met and Met72Met genotypes. Furthermore, a dramatic decrease was seen in AUD patients having Leu72Met+Met72Met (34.79 vs. 19.87) as compared to those having Leu72Leu (21.43 vs. 19.15), suggesting the effect of the *GHRL* Leu72Met variant on acylated ghrelin levels. In humans, the *GHRL* gene (Gene ID: 51738) is located in 3p25-26 and comprises 7 exons. The five exons code the precursor preproghrelin with 117 amino acids and exons 1 and 2 encode the functional 28 amino-acid region (3,13-15). The *GHRL* gene is highly polymorphic and has non-coding or coding region SNPs (more than 300) (3). Among them, the Leu72Met (+408C>A) polymorphism of the *GHRL* gene, located on the second exon, replaces the leucine at the 72nd amino acid with a methionine by switching cytosine to adenine (15). The *GHRL* Leu72Met polymorphism does not change the sequence of mature ghrelin since it lies outside the region where the mature ghrelin protein is encoded. Although its functional significance is still unknown, it is hypothesized that the *GHRL* Leu72Met polymorphism affects ghrelin secretion and activity by changing to protein synthesis or mRNA stability (14,15).

Table 2. Clinical features of people with alcohol dependence (n=50)

Clinical features	Min.	Max.	Mean±S.D.	Median
The onset age of alcohol	10	30	17.94±3.93	17.00
Standard drink*/week	4.80	98.56	25.51±23.82	18.48
Years of alcohol use	7	51	28.70±10.21	27.5

* A standard drink contains 17.05 mL pure ethanol. S.D.: standard deviation, min: minimum, max: maximum

Table 3. The *GHRL* Leu72Met polymorphism genotype frequencies of patients with AUD and healthy subjects

GHRL Leu72Met Genotypes	AUD		Control		p-value	Odds Ratio (95% CI)
	n	%	n	%		
Leu72Leu	37	74	45	90		Reference
Leu72Met + Met72Met	13	26	5	10		
Total	50	100	50	100	**p=0.044**	3.162 (1.032-9.685)
Variant allele freq.	14%		5%			
HWE p-value	χ2=0.0006; p=0.98		χ2=0.13; p=0.71			

AUD: Alcohol use disorder; n: sample size, CI: confidence interval, χ2: chi-square

To date, there have been many studies indicating the relationship between SNPs on the *GHRL* gene and various disorders (17-20). However, only three human studies searched the association of the SNPs on the *GHRL* gene with AUD, but their findings were contradictory (9,10,21). Suchankova et al. suggested that the 72Met allele may have a protective role against AUD (9). Unlike Suchankova's study, Leggio et al. observed 72Met allele frequency was higher in alcohol-dependent subjects (21.4%) than in controls (14.7%) (10). In another study, healthy women social drinkers (n=212) were compared with women having AUD (n=113) given different *GHRL* and *GHSR* polymorphisms and suggested that these genes did not seem to be major susceptibility genes for female alcohol dependence (21). Among these studies, the results of Suchankova's study was similar to the findings of studies concerning the effect of the *GHRL* Leu72Met polymorphism on insulin metabolism and obesity. Similar to Leggio et al. (2012), our pilot case-control study found that the frequency of the 72Met allele is statistically higher in AUD patients (0.14%) than in controls (0.05%). Furthermore, Leu72Met and Met72Met genotypes were found to lead to 3.16 times greater alcohol dependence risk in the present study (Table 3). These conflicting results suggest that the *GHRL* Leu72Met polymorphism seemed

Table 4. Association of the *GHRL* Leu72Met polymorphism with plasma acylated ghrelin levels in both AUD and healthy subjects

Plasma Acylated Ghrelin levels (pg/ml)	AUD				Control (n=50)	
	T0 (n=36)		T1 (n=26)			
	Leu72Leu (n=26)	Leu72Met+ Met72Met (n=10)	Leu72Leu (n=16)	Leu72Met+ Met72Met (n=10)	Leu72Leu (n=45)	Leu72Met+ Met72Met (n=5)
Min.	13.13	17.46	13.10	14.33	13.61	17.94
Max.	174.14	63.67	155.37	77.39	35.27	25.40
Mean± S.D.	31.22± 32.96	35.75± 14.67	38.94± 42.36	28.61± 19.70	20.38± 20.11	22.32± 2.77
Median	21.43	34.79	19.15	19.87	20.11	23.24
p value	z=-2.137; p=0.033		z=-0.132; p=0.895		z=-1.618; p=0.106	

AUD: Alcohol use disorder, n: sample size, S.D.: standard deviation, min: minimum, max: maximum

Table 5. Comparison of AUD patients according to the *GHRL* Leu72Met genotypes given some parameters

Parameters	*GHRL* Leu72Met Genotypes						p-value
	Leu72Leu (n=37)			Leu72Met + Met72Met (n=13)			
	Min.	Max.	Mean±S.D. / median	Min.	Max.	Mean±S.D. / median	
BMI	16.37	33.77	24.11±3.77	16.41	31.26	25.96±3.76	t=-1.526 p=0.134
Smoking (years)	5	46	24.97±10.94	15	45	27.92±9.42	t=-0.836 p=0.408
Pack years of smoking	0.7	111.0	35.44±25.18	0.00	60.00	31.69±15.64	t=0.501 p=0.618
Alcohol addiction (years)	7	47	27.54±10.04	16	51	32±10.35	t=-1.367 p=0.178
Age onset of alcohol (years)	10	30	18.05±3.76/ 17.00	13	27	17.61±4.54/ 16.00	z=-0.980 p=0.327
Standart drink*/ week	5.74	98.56	28.39±26.32/ 18.48	4.80	40.74	17.31±11.88/ 18.48	z=-0.985 p=0.325

* A standard drink contains 17.05 mL pure ethanol. n: sample size, S.D.: standard deviation, min: minimum, max: maximum

to affect AUD by as yet unknown mechanisms. Future studies with larger populations using different techniques are needed to understand the effect of this polymorphism and, also, the underlying mechanisms.

In this study, the BMI of patients with AUD were compared between genotypes of the *GHRL* Leu72Met polymorphism (Table 5). Patients with Leu72Leu genotype have lower BMI (24.11±3.77 kg/m^2) than those with Leu72Met+Met72Met genotypes (25.96±3.76 kg/m^2), but ANOVA showed no main effect of genotypes on BMI. Ukkola et al. (2002) suggested that the Met72 allele seems to be protective against fat accumulation (13). However, Kuzuya et al. (2006) observed a significant effect of 72Met allele on overweight/obesity only in middle-aged men, but not in older men or women (22). Previous studies reporting a significant association between the 72Met allele and earlier age of onset of obesity have included only children and adolescent subjects (23,24). There is a limited study examining the association between Leu72Met polymorphism and overweight in middle-aged subjects. In the present study, all the patients with AUD were men and middle aged (46.46±10.28 years), which seemed to support Kuzuya's study in terms of BMI. Both the duration of smoking and the years of alcohol addiction were higher in AUD patients with Leu72Met and Met72Met genotypes than those with Leu72Leu genotype (Table 5). We speculated that the 72Met allele could be associated with any kind of addiction such as nicotine and alcohol as in this study. In addition, the age onset of alcoholism was lower in patients with Leu72Met and Met72Met genotypes. These findings were consistent with our finding that showed the greater alcohol dependence risk in individuals with Leu72Met and Met72Met genotypes. Although there have been studies investigating the relationship between smoking and fasting total and/or acylated ghrelin levels, to our knowledge, no study has yet searched the association of the GHRL Leu72Met polymorphism with nicotine dependence.

The other interesting finding was that pack years of smoking and standard drinking per week were higher in AUD patients with Leu72Leu genotype compared to those with Leu72Met and Met72Met genotypes (Table 5). According to these remarkable differences, it may be speculated that AUD patients with Leu72Leu genotype may consume more alcohol to compensate for the energy expenditure due to lower plasma acylated ghrelin levels compared to those with Leu72Met and Met72Met genotypes. They may also smoke more cigarettes due to increased alcohol consumption. A positive and significant correlation between alcohol and nicotine dependence was also found in this study.

The major limitation of the present study is the small number of participants. The power of the sample after the study was not calculated because of the fact that the expected number of individuals could not participate due to Sars-Cov-2 pandemic. Therefore, this was presented as a pilot case-control study. Still, the small population did not negate the importance of the *GHRL* Leu72Met polymorphism for the level of acylated ghrelin and the predisposition of AUD. Since the prevalence of alcohol use was lower in females compared to males in Turkey due to social and economic reasons, women were not included in the present pilot study. With a larger sample, including women with AUD and social drinkers, a clear generalized statement about the role of the *GHRL* Leu72Met polymorphism can be done. Second, the findings of this study are descriptive and correlational, but do not establish causality. Though its limitations, this pilot case-control study contributes additional evidence suggesting the effects of chronic alcohol consumption on peripheral acylated ghrelin levels.

In conclusion, the *GHRL* Leu72Leu genotype was found to be associated with lower plasma acylated levels in AUD patients for the first time, but its role requires clarification in studies involving large numbers of AUD patients. Additionally, Turkish men with the *GHRL* Met72 allele seemed to be more susceptible to AUD as compared to those with the Leu72 allele. Taken together, despite the small number of subjects evaluated, the findings in this study further strengthen the possible role of the *GHRL* Leu72Met polymorphism in AUD and especially acylated ghrelin levels.

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