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ORIGINAL ARTICLE

A case control study investigating the methylation levels of *GHRL* and *GHSR* genes in alcohol use disorder

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Abstract

Background Alcohol use disorder (AUD) is a relapsing disease described as excessive use of alcohol. Evidence of the role of DNA methylation in addiction is accumulating. Ghrelin is an important peptide known as appetite hormone and its role in addictive behavior has been identified. Here we aimed to determine the methylation levels of two crucial genes (*GHRL* and *GHSR*) in ghrelin signaling and further investigate the association between methylation ratios and plasma ghrelin levels.
Methods Individuals diagnosed with ($n=71$) and without ($n=82$) AUD were recruited in this study. DNA methylation levels were measured through methylation-sensitive high-resolution melting (MS-HRM). Acylated ghrelin levels were detected by ELISA. The *GHRL* rs696217 polymorphism was analyzed by the standard PCR-RFLP method.
Results *GHRL* was significantly hypermethylated ($P<0.0022$) in AUD between 25 and 50% methylation than in control subjects but no significant changes of *GHSR* methylation were observed. Moreover, *GHRL* showed significant positive correlation of methylation ratio between 25 and 50% with age. A significant positive correlation between *GHSR* methylation and ghrelin levels in the AUD group was determined ($P=0.037$). The level of *GHRL* methylation and the ghrelin levels showed a significant association in the control subjects ($P=0.042$).
Conclusion *GHSR* and *GHRL* methylation levels did not change significantly between control and AUD groups. However, *GHRL* and *GHSR* methylations seemed to have associations with plasma ghrelin levels in two groups. This is the first study investigating the DNA methylation of *GHRL* and *GHSR* genes in AUD.

Keywords Ghrelin · Growth hormone secretagogue receptor type A1 · DNA methylation · Alcohol use disorder · Addiction

Introduction

Alcohol use disorder (AUD), characterized by persistent consumption of excessive alcohol, is one of the leading causes of mortality globally and also has heavy economic impacts [1].

AUD is a prevalent relapsing disorder and the critical role of heritable factors in vulnerability to AUD has been shown in family and twin research [2]. It is known that the development of major psychiatric disorders including AUD is associated with both genetic and environmental factors which refer to epigenetics. Epigenetics is the term explaining the factors beyond the genetic code and the interaction between environment and genetics [3]. It is defined as genetic changes affecting gene expression without changing the DNA sequence. Several epigenetic mechanisms such as chromatin changes, histone modification, histone acetylation, DNA methylation and non-coding RNAs have been

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identified. DNA methylation, characterized by the addition of a methyl group onto the 5th carbon (5mC) of cytosine in cytosine-phosphate-guanine dinucleotide pairs (CpG), is a well-studied type of epigenetic modification [4]. This epigenetic process is generally related to transcriptional silencing via altering transcription factor binding and recognised as a key epigenetic regulator of cell growth, genomic stability and diseases such as cancer and psychiatric disorders [3, 5].

AUD is a multifactorial disease with complex pathophysiology and nowadays neuroendocrine pathways of the brain-gut axis are considered to likely be associated with its pathophysiology. Ghrelin is an orexigenic polypeptide composed of 28 amino acids, mainly secreted from gastric mucosa and encoded by *GHRL* gene. Ghrelin polypeptide is cleaved post-transcriptionally and forms acyl ghrelin which subsequently binds and activates a G-protein-coupled receptor namely the growth hormone secretagogue receptor type A1 (GHS-R1A) encoded by *GHSR* gene [6, 7]. This acyl ghrelin bond GHS-R1A modulates various central and peripheral physiological functions of systemic metabolism such as food intake, thermogenesis, cardiac functions, sleep-wake cycle, memory, stress, anxiety, depression and reward seeking behavior [8]. It was shown that intravenous administration of ghrelin led to increased reaction to food images in brain regions related to reward and appetite in healthy individuals [9]. Moreover, it has been shown that ghrelin regulates dopaminergic neuron signaling and may be involved in development and maintenance of addictive disease and also reward related craving [10, 11]. Therefore, it is wise to consider that ghrelin hormone connecting central nervous and gastrointestinal systems, is an important member of the brain-gut axis and revealing its role in addictive behavior is critical. In plentiful studies the effect of alcohol consumption on blood ghrelin levels has been reported [6, 12, 13]. In a randomized human laboratory study on heavy alcohol drinkers showed that ghrelin signaling modulates alcohol seeking behavior [14]. First population-based study investigating the association between ghrelin and alcohol consumption indicated that alcohol consumption increased serum ghrelin levels in non-dependent participants [15]. Besides, a recent large-scale study also stated that the levels of serum total ghrelin were higher in alcohol consumers than nonconsumers [16]. Moreover, Kaya-Akyüzlü and colleagues also reported that levels of acylated ghrelin were significantly higher in individuals with AUD when compared to control subjects [17]. Further, quite recent research indicated that intravenous infusion of alcohol significantly reduces serum ghrelin levels in heavy drinkers suggesting a dysregulation of the ghrelin system [18].

As ghrelin is a promising target for the future potential treatments of AUD, it is further critical to understand the impacts of all factors including epigenetic changes involved in ghrelin signaling. Therefore, it is aimed to compare DNA methylation levels between control subjects and AUD patients and also investigate the association between DNA methylation ratios of *GHRL* and *GHSR* genes and serum acylated ghrelin levels.

Materials and methods

Study population and sample collection

This study recruited 82 healthy men volunteers and 71 men with alcohol use disorder (AUD) admitted to Ankara University Psychiatry Clinic. All of the individuals with AUD included in this study had the following inclusion criteria: (i) were between 18 and 65 years old, (ii) were diagnosed with AUD according to the ICD-10 (International Classification of Diseases-10) diagnostic criteria, iii) fulfilled the DSM-V criteria, (iv) were inpatients or outpatients. Exclusion criteria of the study were: (i) having clinically significant comorbid psychiatric and/or neurological illness, (ii) having substance use disorders other than nicotine and alcohol dependence (iii) having BMI (body mass index) ≥ 30 Kg/m^2, (iv) possessing any disorders or diseases such as diabetes that can affect the plasma ghrelin concentration. Controls composed of healthy volunteers that had no past or current alcohol and/or substance use disorder and also fulfilled the same exclusion criteria ($n = 82$). The study protocol was approved by the Ethics Committee of Ankara University (Approval number: 19-1300-18, 26.11.2018). All participants were informed about the research and they completed a questionnaire to gather sociodemographic information. Venous blood samples were collected according to the Helsinki Declaration. Whole blood samples were taken from each subject into EDTA tubes and kept at -20 °C until DNA isolation. Plasma acylated ghrelin levels were measured using blood samples collected into tubes containing aprotinin after an 8 h overnight fast following a centrifuge step at 2500 g for 10 min.

DNA isolation, bisulfite conversion and MS-HRM

DNA isolation from blood samples was performed with QIAamp DNA Blood kit (Qiagen, Germany) following the manufacturer's protocol. Isolated DNAs were aliquoted into two microtubes, one for genotyping and the other for methylation. The concentrations of isolated DNAs were measured using a Nanodrop spectrophotometer (Thermo Scientific). Following quantification, 200 ng of DNA from each sample was taken and bisulfite conversion was performed using EpiTect Bisulfite Kit (Qiagen, Germany) according to the

manufacturer's instructions. Bisulfite conversion for all samples was performed simultaneously in order to eliminate any potential batch effect. A sample of genomic DNA completely unmethylated (Qiagen, Germany) was used as control in order to check the efficiency of bisulfite conversion. Methylation levels of *GHRL* and *GHSR* genes were measured using methylation sensitive high resolution melting (MS-HRM) analysis in a Rotor GeneQ 5plex HRM system (Qiagen, Germany) where methylation independent primers and MS-HRM conditions were used as described in detail by Coppede and colleagues [19]. PCR reaction consisting of 5 µl of master mix (Qiagen, Germany), 10 pmol of each primer and 10 ng of bisulfite modified DNA template was performed in duplicate. For each separate run 10% of the samples independently to check the variability between assays. In order to estimate the methylation levels of each AUD and control subjects, standard DNA samples with known methylation ratios generated using fully methylated and unmethylated DNA (EpiTectH methylated and unmethylated human control DNA, bisulfite converted, Qiagen, Germany) were included in each run as previously detailed [20, 21].

Genotyping of *GHRL* rs626917 polymorphism

Genotypes of individuals with and without AUD for *GHRL* Leu72Met polymorphism (rs626917) were determined by PCR-RFLP as described previously [17]. *GHRL* gene portions including rs626917 polymorphism were amplified and the 618 bp length amplicon was digested using *BsrI* enzyme for each individual (New England Biolabs, Hertfordshire, UK). Digestion products were then electrophoresed and visualized to determine the genotypes.

Determining plasma acylated ghrelin levels

In order to measure plasma acylated ghrelin levels, the method utilized by Kaya-Akyüzlü [17] was followed. A commercial ELISA kit (Spi-Bio, Bertin Pharma, France) was employed and plasma acylated ghrelin levels were determined by spectrophotometry (between 405 and 414 nm) and were given as pg/ml.

Statistical analysis

Both *GHRL* and *GHSR* methylation data were tested for normality using the Shapiro-Wilk test. Since methylation data demonstrated a normal distribution in our sample, to evaluate the association between the methylation status of *GHRL* and *GHSR* genes and ghrelin levels, we performed a paired test analysis between the methylation percentage as calculated by MS-HRM and ghrelin levels determined by ELISA. The normalization and melting curves were produced with 100, 75, 50, 25, 12.5 and 0% of methylation ratios. Correlation analysis between methylation and ghrelin levels was performed using the Pearson correlation and linear regression analysis were performed to search for correlation between age and methylation data by age at sampling as a covariate ANOVA utilizing R studio version 4.2.1. (cite as : RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/).

Results

General patient and control samples

Seventy one individuals with AUD and 82 healthy controls were administered in this study. The ages (mean ± standard deviation) of the control and AUD groups were 39.80 ± 9.40 and 45.78 ± 9.92, respectively. There was no difference between the groups for age ($P > 0.05$). There were six samples missing the ages providing that five with AUD patients, one was healthy volunteers. This study removed missing information along the samples.

Comparison of *GHSR* and *GHRL* methylation between individuals with and without AUD

GHRL showed an average methylation of 24.76 ± 1.20% in the healthy control (Fig. 1C) and of 23.53 ± 1.48% in AUD (Fig. 1A), not revealing statistically significant methylation of *GHRL* in AUD than in the healthy control ($P = 0.5195$) in Fig. 1E. *GHSR* methylation levels did not change significantly between the healthy control (Fig. 1D) and AUD patients (Fig. 1B) (1.86 ± 0.11% vs. 2.06 ± 0.16%; $P = 0.3302$) as shown in Fig. 1F. *GHRL* was significantly hypermethylated ($P < 0.0022$) in AUD between 25 and 50% methylation when compared to control subjects.

Correlation of *GHRL* and *GHSR* methylation with patient's age

No correlation between *GHSR* methylation in AUD patients and their age at sampling was observed (r = -0.054; $P = 0.79$). We also observed no correlation between increasing age at sampling and *GHSR* methylation in healthy controls (r = -0.09; $P = 0.59$), as clearly seen in Fig. 2A. *GHRL* methylation showed a less inverse correlation with advancing age in AUD (r = -0.097; $P = 0.041$) in Fig. 2B. However, we observed a modest positive correlation between increasing age at sampling and *GHRL* gene between 25% and

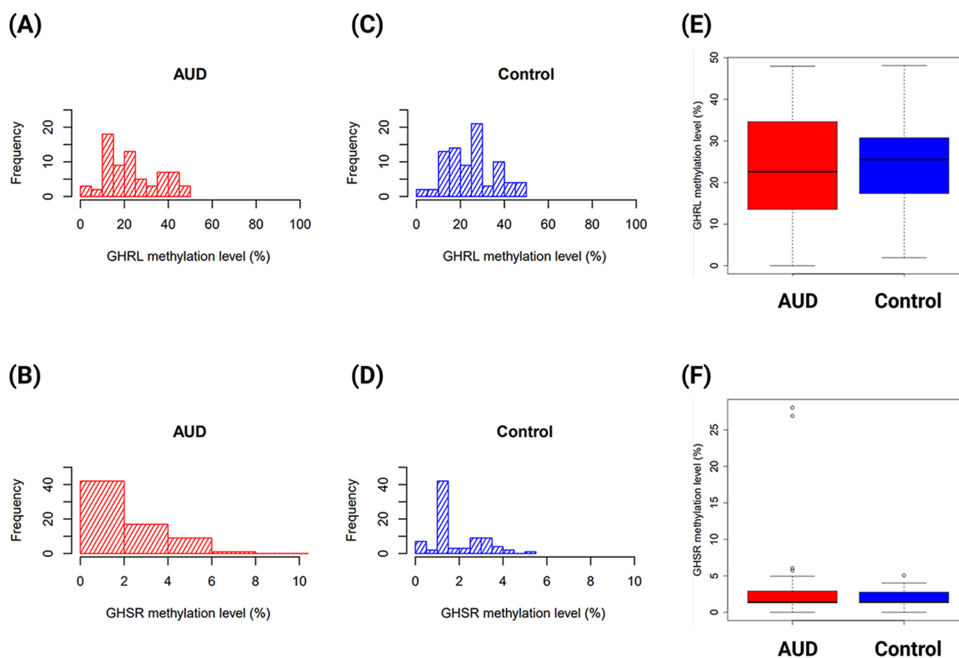

Fig. 1 The distributions of *GHRL* and *GSHR* methylation levels in AUD and healthy controls (**A**-**D**). Mean *GHRL* and *GHSR* methylation levels in AUD and healthy controls. (**E**) We observed an average *GHRL* methylation of 23.53 ± 1.48% AUD versus 24.76 ± 1.20% in the healthy controls. (P = 0.5195). (**F**) We observed an average *GHSR* methylation of 2.06 ± 0.16% AUD versus 1.86 ± 0.11% in the healthy controls. (P = 0.3302). Data are expressed as means ± standard errors of the means

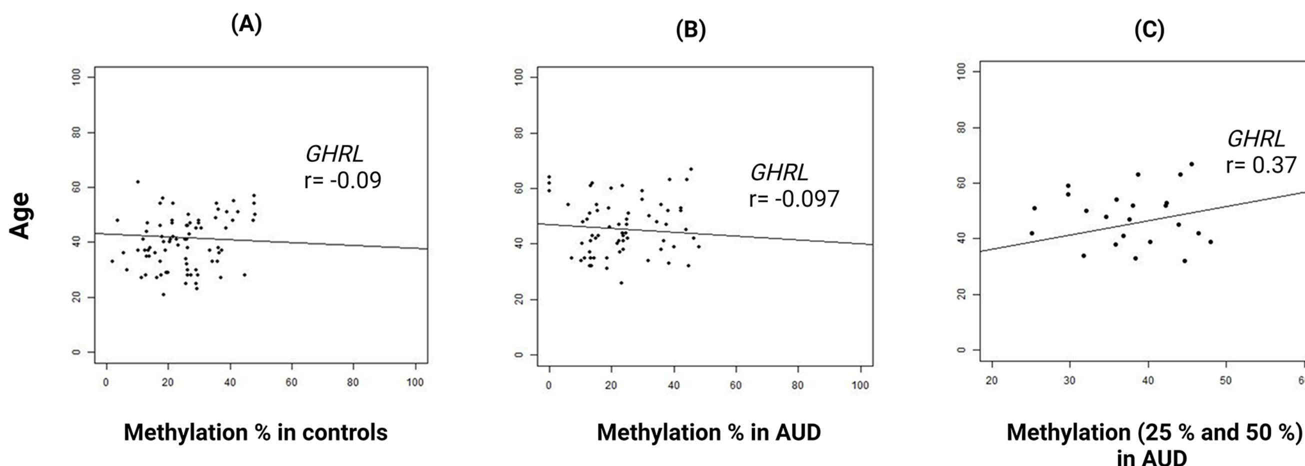

Fig. 2 Correlation between *GHRL* gene promoter methylation and patient's age. (**A**) No correlation between *GHRL* methylation in healthy controls and their age at sampling was observed (r = -0.09; P = 0.59). (**B**) An inverse correlation between *GHRL* methylation in AUD patients' and their age at sampling was observed (r = -0.097; P = 0.041). (**C**) A significant positive correlation between increasing age at sampling and *GHRL* gene between 50% and 25% methylation was observed in AUD patients (r = 0.37; P = 0.0026). r = Pearson's correlation coefficient

Table 1 Genotype and allele frequencies of *GHRL* rs626917 in patients with and without AUD

GHRL rs626917 genotypes	AUD patients (n = 71)		Healthy controls (n = 82)	
	n	% (95% CI)	n	% (95% CI)
GG	54	76 (66.1–85.9)	69	84 (76,1-91.9)
GC	16	22.6 (12.9–32.3)	13	16 (8.1–23.9)
CC	1	1.4 (0.6–2.2)	0	NA
Variant allele frequency	13%		8%	
HWE	P = 0.88 χ^2 = 0.02		P = 0.44 χ^2 = 0.61	

*NA: non-available because calculation of CI requires $n \geq 5$; n: sample size, CI: Confidence Interval, HWE: Hardy-Weinberg Equilibrium

50% methylation was observed in AUD patients (r = 0.37; P = 0.0026) Fig. 2C.

GHRL genotype distribution with *GHRL* methylation

The allele and genotype frequencies in both groups were shown in Table 1 for *GHRL* rs626917. The frequencies of wild type G and polymorphic C alleles were 92% and 8% in alcohol dependent individuals, and 87% and 13% in control subjects, respectively. The genotype frequencies of *GHRL* rs626917 polymorphism were consistent with

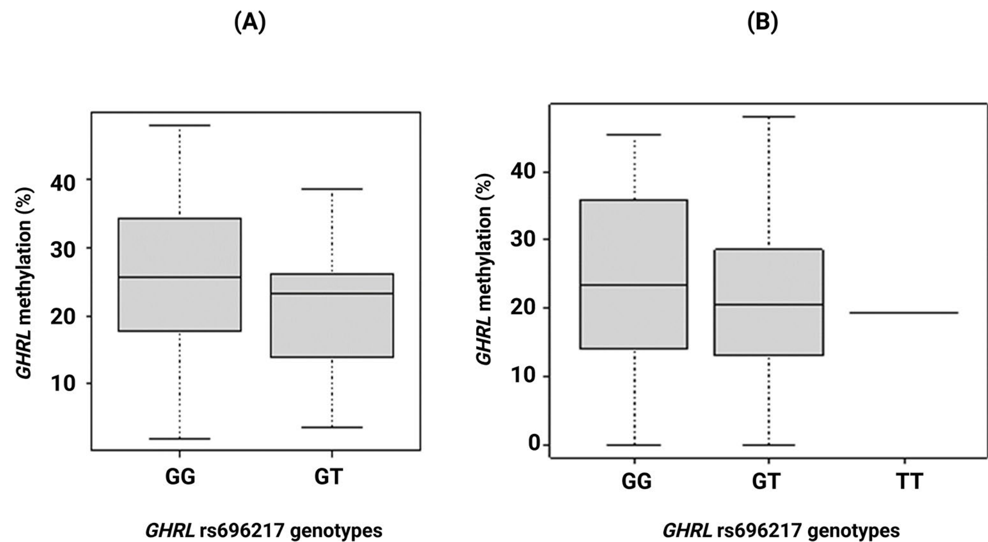

Fig. 3 *GHRL* genotype distribution with *GHRL* methylation (**A**) controls, (**B**) AUD patients. No association between *GHRL* genotypes and *GHRL* methylation levels in control and AUD subjects was determined

Table 2 AUD characteristics of all subjects in the genotypes of *GHRL* rs626917 (G > T) polymorphism. [Data are expressed as mean ± SD. *P* values < 0.05 are significant.]

GHRL-AUD	GG (*n* = 54)	GT (*n* = 16)	TT (*n* = 1)	*P* Value
Age (years)	45.12 ± 9.95	47.81 ± 10.11	46	0.36
Methylation	22.76 ± 12.10	25.80 ± 13.00	19.40	0.41
Ghrelin level	21.84 ± 14.41	28.96 ± 14.84	23.72	0.10

Table 3 Control characteristics of all subjects in the genotypes of *GHRL* rs626917 (G > T) polymorphism. [Data are expressed as mean ± SD. *P* values < 0.05 are significant.]

GHRL-Control	GG (*n* = 69)	GT (*n* = 13)	TT (*n* = 0)	*P* Value
Age (years)	40.37 ± 9.23	36.85 ± 9.56	-	0.23
Methylation	25.41 ± 11.10	26.94 ± 10.23	-	0.57
Ghrelin level	20.91 ± 10.02	23.88 ± 8.03	-	0.25

Hardy–Weinberg equilibrium both in patients ($\chi^2 = 0.02$; $P = 0.88$) and controls ($\chi^2 = 0.61$; $P = 0.44$).

As seen in Fig. 3A and B, we observed no association between *GHRL* genotypes and *GHRL* methylation levels in control and AUD subjects, respectively. Tables 2 and 3 serve information about AUD and control characteristics of all subjects in the genotypes of *GHRL* rs626917 (G > T) polymorphism, respectively. As seen clearly, no association has been detected between any subgroups ($P > 0.05$).

Correlation of *GHRL* and *GHSR* methylation with ghrelin levels

No significant correlation between the level of *GHRL* methylation and the ghrelin levels in AUD patients. Whereas the level of *GHRL* methylation and the ghrelin levels showed a significant correlation in control subjects ($r^2 = +0.33$, $P = 0.042$; Fig. 4A). There was a significant positive correlation between *GHSR* methylation and ghrelin levels in the AUD group ($r^2 = +0.79$, $P = 0.037$; Fig. 4B).

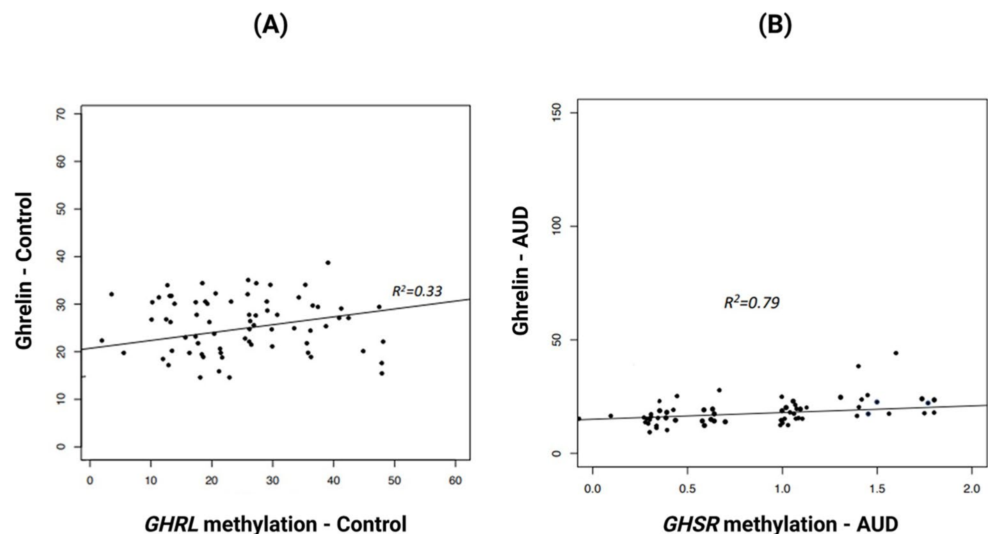

Fig. 4 Correlation of *GHRL* and *GHSR* methylation with ghrelin levels. (**A**) The level of *GHRL* methylation and the ghrelin levels showed a significant association in control subjects ($P = 0.042$). (**B**) There was a significant positive correlation between *GHSR* methylation and ghrelin levels in the AUD group ($P = 0.037$)

Discussion

We examined the methylation levels of *GHRL* and *GHSR* genes in AUD subjects and age-, gender- and body mass index- matched controls, as well as the levels of plasma acyl ghrelin, as biomarkers to search for the association between these biomarkers and AUD.

AUD is a complex disease related to both social and medical problems. It is a known fact that environmental and genetic factors contribute to its etiology and also increasing evidence suggests that epigenetic modification is another component of AUD via altering gene expression, DNA methylation and noncoding RNAs [2]. Transcription of the genes involved in the processes of the central nervous system have been shown to be altered by methylation [22]. There has been a growing body of studies showing that epigenetic should be the focused point for prevention and novel treatment strategies for AUD [2]. Among epigenetic modifications, DNA methylation has emerged as one of the most remarkable ones associated with alcohol dependence and an important area of study due to possessing potential to be used as a biomarker [4]. Therefore, a noticeable amount of studies investigating AUD and DNA methylation have been done in the past decade [2, 23]. Various genes including mu opioid receptor (*OPRM1*) and alpha synuclein (*SNAC*) have been found to be associated with altered methylation levels in the context of alcohol dependence and craving [3, 22, 24, 25]. Pro-opiomelanocortin gene (*POMC*) is one of the genes investigated in terms of methylation level in alcohol use disorder and has been found to be associated with craving in AUD patients [26]. Similarly, it has been indicated that there was a significant association between methylation of monoamine oxidase A (*MAOA*) gene and alcohol dependence in women [27]. Along with these positive examples which had small sample size, in general, the results of promoter methylation studies were reported to be either negative or inconsistent [2]. A noticeable amount of previous studies have detected no association between gene methylation and AUD for genes such as *Orexin A* and dopamine transporter (*DAT)* [2, 23, 28–30]. To date, there have been various studies stating the role of gene methylation in AUD, however, none of them investigated *GHRL* and *GHSR* genes. In our study, methylation levels of *GHRL* and *GHSR* genes were determined in AUD and control subjects and it was revealed that they did not differ between two groups. Conversely, hypermethylation (25% and 50% ratio) of *GHRL* gene was significantly higher in AUD than control subjects. In this range of methylation, ghrelin levels were higher in controls than AUD subjects, but this difference was not significant ($P = 0.5$). Hypermethylation of the *GHRL* gene may lead to lower ghrelin levels in AUD patients. This is the first study showing the effect of *GHRL* methylation on plasma ghrelin levels. Furthermore, a positive correlation between increasing age and *GHRL* gene in the subgroup of hypermethylation (25% and 50%) was detected. This suggested that methylation of the *GHRL* gene was associated with increased age, which could be a result of aging and alcohol consumption.

Ghrelin, a brain-gut hormone consisting of 28 amino acids, is located in brain regions and expressed in the dopaminergic system, and therefore is related to occurrence and development of addiction. It plays a crucial role in addiction by means of stimulating the cholinergic-dopaminergic reward system [1, 12]. Elevated ghrelin levels have been reported in alcoholism and alcohol withdrawal but not in alcohol craving [31]. However, a positive correlation between acetylated ghrelin levels and alcohol craving has been stated in alcohol dependent males, whereas no association was found between total ghrelin levels and alcohol craving [11]. It has been stated that ghrelin levels were found to be increased during withdrawal but decreased on regular alcohol consumption in AUD patients [31, 32]. Ralevski et al. [18] suggested a dysregulation of the ghrelin system in heavy drinkers as abnormalities in ghrelin levels were observed when intravenous alcohol intake was applied. Since ghrelin seems crucial to prevent craving and withdrawal in the treatment of alcohol dependence, current study focused on the genes related to ghrelin pathway.

Ghrelin is the ligand of growth hormone secretagogue receptor 1a (GHS-R1A), a nuclear receptor encoded by *GHSR* gene. Therefore, the capability of the cells to respond to ghrelin hormone is regulated by GHS-R1A. In case of methylation of *GHSR*, the expression of the gene is expected to decrease. *GHSR* hypermethylation is quite common in many types of cancers and therefore it is considered to be a potential biomarker [33]. Moreover, *GHSR* gene hypermethylation was observed in patients with anorexia nervosa which is a psychiatric disorder [34, 35]. Previously, it has been reported that alcohol intake was negatively correlated with *GHSR* expression in mice and rats [36, 37]. Suchankova et al. [37] has also investigated the association between alcohol consumption and *GHSR* gene methylation levels and observed no differences in methylation levels between rats consuming low and high levels of alcohol. Moreover, it has been reported that the deletion of ghrelin receptor decreases the consumption of alcohol in rats [38]. Our investigation revealed that the methylation ratio of *GHSR* gene was quite low in both control and AUD subjects. This indicated that the *GHSR* gene was hypomethylated in both groups without any significant difference. This outcome demonstrated that alcohol consumption had no effect on the methylation of *GHSR* gene unlike animal studies in the literature. This should be further confirmed by novel studies with larger sample sizes.

In the current study, a positive correlation between *GHSR* methylation and ghrelin levels in AUD subjects was determined. Although methylation ratio is low, the correlation between *GHSR* methylation and ghrelin levels may indicate that *GHSR* gene was suppressed due to methylation and ghrelin could not bind to its receptor and as a result, the level of plasma ghrelin increased. In the literature, it has been reported that genetic suppression of *Ghsr1a* gene was found to be associated with decreased alcohol consumption in mice and rats [39–42]. In order to better explain this correlation, further studies are required to be done.

In order to address the potential role of the genetic background to *GHRL* promoter methylation, we explored whether *GHRL* rs626917 polymorphism affected the methylation levels of *GHRL* gene for the first time. To the best of our knowledge, no studies have investigated the influence of any *GHRL* SNPs on *GHRL* methylation. It has been previously reported that *GHRL* rs626917 polymorphism was associated with alcohol dependence and also with higher plasma acylated ghrelin levels [17]. Nonetheless, no impact of *GHRL* rs626917 polymorphism on *GHRL* methylation was observed in both groups in our study. The influence of genetic variations on gene methylation have been reported in alcohol dependence previously. Pathak et al. [43] showed an interaction between methylation of aldehyde dehydrogenase 2 (*ALDH2*), an important gene in ethanol metabolism, and rs886205 SNP. On the contrary, a study on individuals with alcohol-nicotine codependence reported no association between 13 SNPs and the methylation level of 5-hydroxytryptamine receptor 2B (*HTR2B*) gene encoding serotonin receptor [44].

The limitation of this study is that the expression levels of *GHRL* and *GHSR* genes have not been evaluated. This could not be possible due to economic and operational conditions however it could be informative to better understand the impact of methylation. Albeit, no correlation was observed between the expression and methylation level of *Ghsr* gene in low and high alcohol consuming rats. It was concluded that the regulation of *Ghsr* gene expression might be independent of the methylation mechanism in rats. As it has been shown that AUD is a disease related to ghrelin signaling and GHS-R1A possesses the features to be a promising candidate, *GHSR* antagonists are being tested as therapeutic agents against AUD [32, 37]. In our study, *GHSR* gene remained unmetyhlated in both groups. Even though it has been shown that *GHSR* methylation had no influence on *GHSR* expression in rats, more studies on humans should be done to reveal whether the role of *GHSR* in alcohol dependence depends on methylation or might be regulated by other epigenetic mechanisms.

Epigenetic modifications have been recognised to have a crucial role in initiation, maintenance and progression of the pathophysiology of AUD [4]. Therefore, it is critical to reveal potential epigenetic modifications to better understand AUD and to develop novel therapies for AUD by targeting different epigenetic pathways of various genes having role in AUD pathophysiology.

In conclusion, more studies are required to be done in larger study sizes in order to better understand the methylation status of the genes of the ghrelin system and possible association with alcohol use disorder. Furthermore, in future studies, other epigenetic mechanisms regulating gene expression such as microRNAs are needed to be investigated to reveal the potential effect of the ghrelin system in AUD development.

Author contributions Conceptualization: Selin Özkan-Kotiloğlu, Dilek Kaya-Akyüzlü; İnci Özgür-İlhan; Methodology: Selin Özkan-Kotiloğlu, Dilek Kaya-Akyüzlü, Özlem Doğan; Funding acquistion: Dilek Kaya-Akyüzlü; Sample collection: İnci Özgür-İlhan, Ece Ağtaş-Ertan; Formal analysis: Selin Özkan-Kotiloğlu; Data analysis: Emine Güven; Writing-original draft: Selin Özkan-Kotiloğlu; Writing - review & editing: Selin Özkan-Kotiloğlu, Dilek Kaya-Akyüzlü.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethical approval The study protocol was approved by the Ethics Committee of Ankara University (Approval number: 19-1300-18, 26.11.2018).

Conflict of interest The authors declare no conflict of interest.

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