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Relationship Between Alcohol Dependence and Neuropeptide Y *(NPY)* Gene Promoter Polymorphisms in A Turkish Sample

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SUMMARY

Objective: Neuropeptide Y (NPY) is a protein widely expressed in the central nervous system and involved in diverse physiological processes, such as emotional regulation, nutritional behavior, and stress. In some populations, studies on alcohol dependence (AD) and the *NPY* gene have found that *NPY* variations increase alcohol consumption and thus may potentially be associated with AD. In this study, we investigated the relationship between *NPY* gene promoter polymorphisms and phenotypes related to alcohol use.

Method: A total of 417 male participants comprising 252 individuals with AD and 165 healthy individuals were included in this study and phenotypic data were collected. Polymerase chain reaction-restriction fragment length polymorphism (PCR/RFLP) and DNA sequencing methods were used for genotyping the rs16147 and rs17149106 polymorphisms in the promoter region of the *NPY* gene. The data of 384 participants were analysed to evaluate the possible relationship between genotypes and the diagnosis of AD, family history of AD, the severity of AD using the Michigan Alcoholism Screening Test (MAST), the age of onset of problematic alcohol use, the average amount of alcohol consumed per day for the last six months and the lifetime maximum alcohol consumption in one day.

Results: A significant difference was found between the AD and control groups concerning rs16147 polymorphism genotype distribution (p=0.025). No association with polymorphisms and alcohol-related phenotypes were demonstrated in the AD group.

Conclusion: To our knowledge, this study shows for the first time in the literature that alcohol dependence is associated with *NPY* rs16147 polymorphism in the Turkish population.

Keywords: Alcoholism, genetics, *NPY*, polymorphism

INTRODUCTION

Alcohol dependence (AD) is associated with impaired social and professional functionality as the adverse consequences of compulsive alcohol seeking, loss of control over drinking and high rates of relapse (Cui et al. 2013, Akbar et al. 2018). Hence, AD is a mental health problem with important social and economic impact (Mathies et al. 2017). Studies using the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC) data have shown that the prevalence of lifetime alcohol dependence based on the DSM-IV criteria is 12.5% in the USA (Hasin et al. 2007). It is estimated that almost 15% of the total population of Europe have varying degrees of alcohol use disorder (Rehm et al. 2015). According to the Global Status Report on Alcohol, the 12-month prevalence of alcohol dependence in 2016 was 1.6% in Turkey (WHO 2018) which was lower than the prevalence in Europe. But, recent epidemiological data indicate an increasing trend in prevalence in the general Turkish population (Demirbaş 2015).

The incidence of comorbidities, the commonest being anxiety disorders and depression, are high in individuals with AD (Grant et al. 2004, Pacek et al. 2013, Hasin and Grant 2015). Although the definitive etiology is not clear, it

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has been suggested that heavy alcohol use leads to changes in different neurotransmitter and hormonal systems in the brain, thus contributing to the development of psychiatric disorders (Gabriels et al. 2019). Common genetic factors contributing to the coexistence of AD and major depression have been shown in family, twin and general community studies (Kendler et al. 1993, Swendsen and Merikangas 2000, Prescott et al. 2000, Andersen et al. 2017, Walters et al. 2018, Gandal et al. 2018), pointing to the presence of a common genetic and biological basis of AD and the investigated disorders (Zhou et al. 2017). Also, in individuals with mental disorders, alcohol use to relieve the symptoms of disease or for other reasons may lead to the development of AD, which may underlie the high incidence of comorbidity (Turner et al. 2018).

AD is a heterogeneous mental disorder affected by multiple genetic and environmental factors (Heath et al. 1997, Prescott ve Kendler 1999, Xian et al. 2008). Although studies on the genetics of AD were frequently on genes *ADH* and *ADLH* that encode enzymes involved in alcohol metabolism (Ehlers et al. 2012, Ayhan et al. 2015), genes involved in different physiological functions, such as *DRD2* (Bhaskar et al. 2010), *PECR* (Treutlein et al. 2009), AUTS2 (Schumann et al. 2011) and S*LC22A18* (Edenberg et al. 2010), *OPRM1*, *HTR1B*, and *SLC6A4,* have also been associated with AD (Franke et al. 2001, Feinn et al. 2005, Gurel et al. 2016, Wu et al. 2016).

It is stated in the literature that *NPY* (Neuropeptide Y) plays a role in the regulation of alcohol consumption (Thiele et al. 1998, Pandey 2003, Thorsell 2006). *NPY* animal model studies have shown the effects of *NPY* on anxiety-related phenotypes, stress response and depression. The results obtained from these studies have been associated with the hypothesized that increased levels of *NPY* in the brain lead to a reduction in anxiety-related behavior and may lead to a reduction in alcohol intake (Minth et al. 1986, Heilig et al. 1989, Heilig et al. 2002, Rossetti et al. 2019). The effects of *NPY* on alcohol consumption have been demonstrated in human studies, as well as in animal models (Bice et al. 1998, Carr et al. 1998, Woldbye et al. 2002). Data from human studies show that individuals with alcohol dependence have lower levels of *NPY* in brain tissues compared to controls (Mayfield et al. 2002). Also, according to the results of a genome-wide association study (GWAS), the *NPY* gene region is associated with alcohol consumption and alcohol dependence in humans (Reich et al. 1998). Furthermore, it has been stated that excessive alcohol consumption in adolescence causes disorder in the *NPY* DNA methylation mechanisms in the amygdala and may cause anxiety and alcohol use disorders in adulthood (Sakharkar 2019). Considering the possible genetic link between *NPY* and alcohol abuse disorders together in humans, *NPY* has been proposed to be a promising target for treating problematic alcohol use and protecting individuals from relapse during abstinence (Robinson and Thiele 2017, Rodriguez and Covenas 2017).

Rs16147 (T/C) polymorphism in the promoter region of *NPY*, which is associated with emotion regulation and stress-related behavior, is known to affect *NPY* expression and *NPY* peptide levels (Itokawa et al. 2003, Buckland et al. 2005, Zhou et al. 2008, Shah et al. et al. 2009, Sommer et al. 2010). The other genomic variant rs17149106 (G/T) located in the promoter region of the *NPY* gene has been associated with medical disorders, such as alcohol dependence, asthma, and obesity, in the literature (Mottagui-Tabar et al. 2005, Yeung et al. 2011, Lu et al. 2016). Although this variant is assumed to be a functional variable (Lu et al. 2016), its specific effects on gene expression have not yet been fully explained (Sommer et al. 2010). That *NPY* plays a role in both anxiety/depression and alcohol use suggests that *NPY* has a possible mediatory effect in AD patients with comorbidity. However, so far, genetic association studies have focused on the diagnosis of alcohol dependence rather than the spectrum of alcohol-related phenotypes. Besides, the geographic representation of these studies is limited, and it is not clear whether comparable results will be obtained in different ethnic populations. Therefore, in this case-control study, the association between *NPY* gene promoter polymorphisms and phenotypes related to alcohol use were investigated for the first time in a Turkish population.

METHOD

Characteristics of the Participants and Data Collection

A total of 417 individuals including 252 patients diagnosed with the AD and 165 healthy male participants, were recruited for this study. The methods of selecting individuals included in this study have previously been detailed elsewhere (Gurel et al. 2016). The methods and tools used in our study are briefly described below. The AD group consisted of male individuals who applied to the Hacettepe University Faculty of Medicine (HUTF) Department of Psychiatry and Ankara Alcohol and Substance Addiction Treatment and Training Centre (AMATEM) for the treatment of AD and who did not have a psychiatric disorder. The control group was assembled from healthcare staff working in the HUTF by way of verbal announcement. The AD diagnoses of the participants were confirmed using the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). The SCID-I was developed and structured for the diagnosis of axis-I psychiatric disorders on the criteria of the DSM-IV (First et al. 1996) and was adapted to the Turkish language by Özkürkçügil et al. (1999). The B, C, D modules of SCID-I were used to exclude patients with psychotic and emotional disorders, and the E module to exclude participants with a diagnosis of substance dependence or substance abuse (other than nicotine). Participants who

were diagnosed with bipolar mood disorder, schizophrenia spectrum disorders, and substance abuse and dependence on any substance other than alcohol were excluded from this study. Participants who were not diagnosed with substance abuse and substance dependence other than alcohol, but had a history of substance use, were included in the study. Interviews were conducted with the patient group after the first week of hospitalization for alcohol detoxification following the alleviation of withdrawal symptoms.

The Michigan Alcoholism Screening Test (MAST) was used not only in the AD group but also in the control group to rule out present and past alcohol-related problems, such as alcohol misuse. The MAST is a self-report scale with 25 questions, developed to determine the severity of alcohol use disorder. The questions are marked as yes-no (Selzer 1971). The score determined for each question is specified in the instructions. In the validation study made in Turkey, a cut-off score in the 5-9 range corresponded with the highest level of AD discriminating capacity of the test. In this research, the Turkish language version of the MAST was used (Coşkunol et al. 1995). Although the MAST is designed as a screening test, high scores have been associated with severe addiction (Ogel et al. 2012). For better classification of the control group, the individuals with MAST scores above 5 were excluded from this study. In order to differentiate the ethnicity of the participants included in this study, the native language of each participant and of their parents were recorded. Only native Turkish speakers were intended to be included in this study. All participants were found to be native Turkish speakers. In both groups, the criteria of illiteracy and the presence of an additional disorder that might affect cognitive functions were also used for exclusion.

In the AD group of participants, the average amount of alcohol consumed per day for the last six months, the lifetime maximum alcohol consumption in one day and the family history obtained using the Family History-Research Diagnostic Criteria were evaluated (Andreasen et al. 1977). The presence and duration of at least two of the social complications comprising work-family-social problems related to alcohol, repeated absences from work or school-related to alcohol and violent behavior/arrest under the influence of alcohol were considered as criteria for determining the onset of alcohol use problems. As previous studies classified alcohol dependence according to the age of onset and used 20 and 25 years of age to discriminate early-onset (EO) and late-onset (LO) (Babor et al. 1992), the same cut-off points were used in this study to investigate the default effects of the age of onset. This research was carried out with the approval of the Hacettepe University Ethics Committee (GO 14 / 20-15), and informed consent was obtained from all the participants to participate in this research.

Genotyping

DNA was isolated from peripheral blood from all patients and controls using the QIamp DNA isolation kit (Qiagen GmbH, Hilden, Germany) and stored at -20ºC. Genotyping for *NPY* gene polymorphisms was carried out using PCR-RFLP and sequencing method. For rs16147, the sense and antisense primers were 5′-AGCTGCCTCCGACTTGTTCTA-3′ and 5′-TGCCAGAGATAGGAGCAGCC-3′, respectively. For rs17149106, the sense and antisense primers were 5′- CAGGTTTAACGCGATGAGCAAA-3′ and 5′-TGCCAGAGATAGGAGCAGCC-3′, respectively. Amplifications were carried out in a 25 µl reaction mixture containing 100 ng of genomic DNA, 2.5 µl of 10X PCR buffer (without Mg^{+2}), 1.5 µl of 25 mM $MgCI_2$, 1 µl of 2.5 mM dNTP mixture, 1 µl of 10 µM for each primer, 1U Taq DNA Polymerase Sigma ® (5U/µl) and 13.5 µl sterile distilled water. PCR products were examined by agarose gel electrophoresis. For rs16147 SNP promoter polymorphism, the amplicon was digested with 10 Units *CviK-I* (*Neb England Biolabs*). Restriction results were confirmed by DNA sequencing in randomly chosen 50 AD group and 50 control group participants. The rs17149106 polymorphism was screened by DNA sequencing in 251 AD group and 125 control group patients. The DNA sequencing reaction was carried out using the Big Dye Terminator cycle sequencing kit (3.2 version) on the ABI Prism 310 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA).

Statistical Analyses

All statistical analyses were performed using the SPSS 22.0 package program (IBM Corp., Armonk, NY). The statistical difference in genotype and allele frequencies distribution of the AD and control group participants and analysis of its deviation from the Hardy–Weinberg equilibrium was assessed by using the chi-square test. Normality of data distribution was evaluated using the Shapiro-Wilk test. Depending on the data distribution, the relationships between genotype distribution and the amount of alcohol consumed, age of onset, family history, and MAST score were determined using the Student t-test for normally distributed data and the Mann-Whitney U test for the data with non-normal distribution. The Kruskal-Wallis H test was used for the comparisons of 3 groups of non-normally distributed data. The Pearson's Chi-square test and Fisher's Chi-square test were used to compare categorical variables. The effects of the age variable were checked by covariance analysis. Cohen's d statistics were used to determine the statistical power of this study and to show the significance of the difference between the results for both polymorphisms in the participating groups. The d values of less than 0.2, equal to 0.5 and higher than 0.8 were accepted as small, medium and large, respectively. Also, as a measure of the relationship between the variables, the "r" value of 0.10,

0.30 and 0.50 were accepted for small, medium and large effect size, respectively. A 'p' value of <0.05 obtained from statistical tests was accepted to indicate statistical significance.

RESULTS

A total of 252 AD group participants and 132 control group participants were genotyped after excluding the participants with high MAST scores of n=33 in the control group. The genotypic frequency distributions among the AD and the control group participants did not deviate significantly from the Hardy-Weinberg equilibrium (HWE).

The average age of the AD group (43.96±10.14) was significantly higher than the average age of the control group (34.93±7.73, t=9.943, df=345, p=0.025), whereas the average years of education in the patient group (8.94±3.80) was significantly lower than that in the control group (10.69±3.66, t=4357, df=390, p<0.0001).

Relationship Between Alcohol Dependence and the rs16147 and rs17149106 Polymorphisms

Genotype and allele distributions of *NPY* rs16147 and rs17149106 polymorphisms are shown in Table 1. In the co-dominant (TT, TC, CC) model, the genotype distribution of rs16147 was found to be significantly different between the AD and the control group (χ^2=7.341, df=2, p=0.025). In the T dominant genotype (TT + TC and CC) model, the CC genotype distribution was found to be significantly higher in the AD group (χ^2=4.156, df=1, p=0.041). However, the effect size estimated by Cohen's d analysis was small for rs16147 (t=0.677, df=382, p=0.499, Cohen's d=0.069, effect size r =0.0346).

Comparing the AD and the control groups according to the co-dominant model (GG, GT, TT), the rs17149106 genotype distribution did not differ between the groups (χ^2=2.360, df=2, p=0.307). Also, significant relationships were not determined between the AD and the control groups when compared on the rs17149106 polymorphism according to the G allele dominant genotype model (GG + GT and TT) (χ^2= -, df=1, p=0.35) and the T allele dominant genotype model (GT + TT and GG) (χ^2=0.0001, df=1, p=0.991).

Relationship Between Phenotypes Associated with Alcohol Dependence and rs16147 and rs17149106 Polymorphisms

The relationship between rs16147 and rs17149106 polymorphisms and mean and maximum alcohol consumption, MAST score, age of problematic alcohol use, and family history for the AD were evaluated. It was found that there was no significant relationship between the genotype distributions of both polymorphisms and maximum and mean daily alcohol consumption, MAST score, age of onset of problematic alcohol use, and family history. The findings are summarized in Table 2.

Table 1. Genotype and Allele Distribution of rs16147 and rs17149106 Polymorphisms in Alcohol Dependence (AD) and Control Groups

	AD (%)	Control (%)	χ^2	*df*	p
rs16147					
TT	63 (25.0)	27 (20.5)	7.341	2	0.025*
TC	113 (44.8)	78 (59.1)			
CC	76 (30.2)	27 (20.5)			
TT - TC	176 (69.8)	105 (79.5)	4.156	1	0.041*
CC	76 (30.2)	27 (20.5)			
rs17149106					
GG	229 (91.2)	114 (91.2)	2.360	2	0.307*
GT	12 (4.8)	9 (7.2)			
TT	10 (4.0)	2 (1.6)			
GG -GT	241 (96.0)	123 (98.4)	-	1	0.351†
TT	10 (4.0)	2 (1.6)			
GG	229 (91.2)	114 (91.2)	0.0001	1	0.991*
GT-TT	22 (8.8)	11 (8.8)			

* Pearson Chi-Square test
† Fisher-Exact Chi-Square test

Table 2. Association Between Genotype Distribution of rs16147 and rs17149106 Polymorphism and Alcohol-Related Phenotypes in the Alcohol Dependence (AD) Group.

	Genotype	Alcohol consumption		Age of onset (20)		Age of onset (25)		MAST Score
		Maximum (Unit)	Mean (Unit)	EO (%)	LO (%)	EO (%)	LO (%)	
rs16147	TT	87.08	97.94	7 (17.9)	35 (26.1)	19 (20.4)	23 (29.1)	76.36
	TC	87.04	86.69	22 (56.5)	61 (45.5)	44 (47.3)	38 (48.1)	81.49
	CC	95.43	90.36	10 (25.6)	38 (28.4)	30 (32.3)	18 (22.8)	84.06
p		0.609**	0.501**	0.437*		0.259*		0.741**
rs17149106	GG	88.60	88.88	37 (94.9)	118 (90.8)	85 (93.4)	70 (89.7)	79.82
	GT	78.55	95.20	2 (5.1)	8 (6.3)	4 (4.4)	6 (7.7)	88.75
	TT	105.10	98.40	0 (0.0)	4 (3.1)	2 (2.2)	2 (2.6)	69.13
p		0.629**	0.859**	0.520†		0.652†		0.773**

*Pearson Chi-Square Test, ** Kruskal-Wallis, † Fisher's Final Chi-Square Test
EO: Early-onset, LO: Late-onset, MAST: Michigan Alcohol Screening Test
Unit: The amount of alcohol consumed wascalculated in terms of standard units. Accordingly, raki, whiskey,gin, brandy, and vodka were considered to contain approximately equal amounts of alcohol, and 70 cl of high spirits were calculated as 30 units. 0.33 L of beer and 0.15 L of wine were recorded as 1 unit.

DISCUSSION

To date, many polymorphisms have been identified in the *NPY* gene that have been associated with AD in different populations (Mottagui-Tabar et al. 2005, Bhaskar et al. 2013). To our knowledge, ours is the first study examining the relation of *NPY* rs16147 and rs17149106 polymorphisms and alcohol dependence in the Turkish population. The primary finding of our study is that the genotype frequency of rs16147 is significantly different between patients and healthy individuals.

NPY, which is widely expressed in the central nervous system, has been reported in many studies to suppress anxiety-related behaviors and reduce alcohol consumption with its direct anxiolytic effect (Pleil et al. 2015, Thorsell and Mathé 2017). In a study by Mayfield et al. (2002), it was found that the *NPY* protein levels in the brain tissues of AB patients were lower than the control group. Also, studies on animals have shown an inverse relationship between *NPY* levels in the central nervous system and alcohol preference (Badia-Elder et al. 2001). Rossetti et al. (2019) concluded that the genetic susceptibility of Sardinian alcohol-preferring (sP) rats consume high quantities of alcohol could be associated with the low brain levels of *NPY* as compared to the Sardinian alcohol non-preferring (sNP) rats. The rs16147 polymorphism in the *NPY* gene promoter region has been shown to significantly improve the transcriptional activity of the *NPY* gene (Itokawa et al. 2003, Buckland et al. 2005). Therefore, the excess of TT + TC genotype in AD participants may indicate the presence of decreased expression of *NPY* levels, which may lead to alcohol preference and the risk of developing AD. Although it was reported that the number of days of heavy alcohol consumption and the alcoholism severity scores of AD patients carrying two copies of the T allele were significantly low (Vergne et al. 2010), a relationship of the amount and severity of alcohol consumption with the genotypes was not demonstrated in our study.

Some other polymorphisms other than the promoter region in the *NPY* gene have been analyzed in relation to the AD, but the results are controversial (Okubo and Harada 2001, Koehnke et al. 2002, Zhu et al. 2003). Zill et al. (2008) investigated three single nucleotide polymorphisms (SNP) in the *NPY* gene promoter region, including rs16147 and rs17149106, and could not detect a significant relationship. In another study, 4 SNPs in the *NPY* gene were investigated in 577 individuals with AB in the Nordic population, and it was found that the rs16147 polymorphism of the *NPY* gene was significantly associated with alcohol dependence (Mottagui-Tabar et al. 2005). Our results are in line with the studies of Mottagui-Tabar et al., where it was reported that there was a significant relationship between rs16147 and AD.

The association between rs16147 polymorphism and AD has been investigated in different populations and races, with both negative (Zill et al. 2008) and positive results (Ilveskoski et al. 2001, Mottagui-Tabar et al. 2005). According to the National Center for Biotechnology Information. (NCBI) database, the T allele in the rs16147 is more frequent in African populations and quite the opposite, with the C allele being more frequent, in Asian and American population samples. The allelic distribution of rs16147 in our study population was similar to those reported in most other European populations, which is about 0.5:0.5. (Mottagui-Tabar et al. 2005, Zill et al. 2008) (http://www.ensembl.org/). The frequency of this polymorphism result is similar to our previous studies related to previous findings showing the allelic distributions of ADH and MOR receptor genes in the Turkish population to be similar to other European populations (Ayhan et al. 2015, Gurel et al. 2016).

In this study, we also investigated the association between *NPY* promoter region rs17149106 polymorphism and AD. In a previous study, it was found that an *NPY* promoter region SNP, (rs17149106) was associated with AD (Mottagui-Tabar et al. 2005). However, there are a limited number of studies on this polymorphism and its role in modifying the *NPY* levels has not been comprehensively studied. According to the Ensemble database, allelic distribution of rs17149106 G/T is about 0.9:0.1, and the G allele has been observed to be more common in all populations (http://www.ensembl.org/). In our study, we also found that the G allele is more common in both the control and AD group participants. This finding is in line with the results of others (Mottagui-Tabar et al. 2005, Zill et al. 2008), but the relationship of this polymorphism with the AD unlike the results of Mottagui-Tabar (2005) was not detected in our study.

Relationships between rs16147 and rs17149106 polymorphisms and the maximum and average alcohol consumptions, the MAST score, onset age of problematic alcohol consumption and family history were also not demonstrated in our study. The relationship between rs16147 and rs17149106 and these clinical features has been investigated in a limited number of studies. Mottagui-Tabar et al. (2005) examined the relationship of *NPY* gene polymorphisms, including rs16147 and rs17149106, with early and late-onset AD and stated that rs17149106 polymorphism is an essential marker for late-onset alcohol addiction in Nordic populations, which our results on the Turkish population do not agree with.

Sampling characteristics may be one of the reasons for contradictory results in the literature related to *NPY* gene polymorphisms and alcohol dependence. In this study, the main limitation is the characteristics of the sample population. Although our study sample was selected only from native Turkish speakers, the ethnic origin of the participants may differ, and the *NPY* genotype distributions investigated may differ on the basis of the differences in ethnic backgrounds. Another limitation of this study is the inclusion of only male participants. In a study population consisting of male and

female individuals, rs17149106 polymorphism was found to have a strong relationship with AD, especially among women, although the number of female participants is one-third of men (Mottagui-Tabar et al. 2005). Based on this finding, it would be more appropriate to examine rs17149106 polymorphism in a sample, including women.

There are also differences in the sociodemographic characteristics of the AD and the control group of participants. The control group mean age was significantly lower than that of the AD group, which suggests that some of the control group participants may still have a risk of developing AD. Also, when the education levels of the AD and the control group are compared, the education level in the AD group is lower. While the relationship of *NPY* with learning and memory has been demonstrated, the relationship between polymorphisms examined in our study and verbal learning and memory has not been described in the literature (Gotzsche and Woldby 2016, Kornhuber and Zoicas 2017). The relationship has mostly been shown with emotional memory (Zhou et al. 2008, Mickey et al. 2011, Hormer et al. 2018). Thus, there is no evidence that a mental function that may directly affect the level of education would be affected by polymorphisms examined in our study and to the best of our knowledge, there is not any study that found to show the relationship between *NPY* polymorphisms and education level.

In this study, information for family history was collected by means of an interview-based method. Interview-based methods are often open to misinterpretation. Making use of national or regional records or direct discussions with the interviewees would have been better methods for acquiring more precise results. Another important limitation of this study is the size of the participant population, which is an important factor in incidence report studies. The relationship between genotype and alcohol dependence should be examined in a larger sample.

In conclusion, to the best of our knowledge, this is the first study to show the association between *NPY* gene rs16147 polymorphism and AD in a Turkish population. Well-designed case-control studies based on large sample size are required to investigate and evaluate the relationship between *NPY* gene polymorphisms and the risk of AD.

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