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Detection of altered methylation of MB-COMT promotor and DRD2 gene in cannabinoid or synthetic cannabinoid use disorder regarding gene variants and clinical parameters

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ABSTRACT

This study aims to investigate the association between cannabinoid use disorder (CUD) or synthetic cannabinoid use disorder (SCUD) and methylation status of MB-COMT (membrane-bound catechol-O-methyltransferase) promotor or DRD2 gene considering gene variants and clinical parameters. Based on the DSM-5 criteria, 218 CUD/SCUD patients' diagnoses were confirmed with a positive urine test, and a control group consisting of 102 participants without substance use disorders was included. Methylation-specific PCR was used to identify the methylation of the MB-COMT promotor and DRD2 gene. DRD2-141C Ins/Del and COMT Val158Met gene variants were evaluated by using PCR-RFLP. When the DRD2 and MB-COMT promoter methylation of CUD/SCUD patients were compared with the control group, there was a significant difference between the MB-COMT promoter methylation status of the two groups. When comparing DRD2 gene methylation due to clinical parameters and DRD2 genotype distribution in patients, the methylation status was significantly different between the groups due to the family history. Again, comparing the MB-COMT promotor methylation due to the COMT Val158Met genotype distribution and clinical parameters in patients, the MB-COMT promoter methylation status was significantly different between the groups due to the presence of alcohol usage. In summary, whereas the MB-COMT promoter methylation may be associated with the CUD/SCUD, the methylation of the DRD2 gene was not related to CUD/SCUD.

KEYWORDS

Substance use disorder; cannabinoid use disorder; synthetic cannabinoid use disorder; DRD2; COMT; methylation; single-nucleotide polymorphism

Introduction

Substance use disorders (SUD) are chronic brain disorders characterized by repeated use of substance, despite adverse outcomes like poor health and detrimental social and economic consequences.[1] The heritability of SUD vulnerability is approximately 50% in twin and family linkage reports; therefore, the interaction of genetic and environmental factors determined SUD's risk.[2] An earlier meta-analysis of twin studies has also revealed significant heritability estimates of lifetime cannabis use of 40% for females and 48% for males.[3] Epigenetic mechanisms are included in the interaction between the environment and genome; they respond to changes in environmental factors (such as aging, diets, drugs, and exercise) and proceed to alter chromatin structure and therefore regulate gene expression.[4] Epigenetic modifications, such as DNA methylation contribute to complex features and diseases. The cytosine residues methylations in CpG dinucleotides are a crucial mechanism of variation and regulation in the genome.[5] Therefore, modulation of methylation at CpG sites within the human genome can result in an epigenetic pattern specific to individual environmental exposures, which may contribute to psychiatric disorders.[6] An increasing number of researchers have started to explain the role of DNA methylation in SUD. The DNA methylation detection and quantitation processes generally used in neuro-epigenetic research have recently

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been applied to addiction studies; therefore, besides few studies about genome-wide changes in DNA methylation, several candidate gene studies of DNA methylation changes following SUD have investigated.[7] Altered levels of DNA methylation are shown in response to different classes of substances such as alcohol,[8] cannabis,[9] cocaine,[10] methamphetamine,[11] opioids,[12] and tobacco.[13]

Catechol-O-methyltransferase (COMT) is the critical enzyme responsible for the metabolism of dopamine in the brain's cortical regions.[14] The COMT gene is placed on chromosome 22q11.21, has eight exons, and produces 271 amino acids, which metabolize catecholamines.[15] COMT gene polymorphisms are associated with the enzyme activity: higher activity is related to the COMT Val (valine) allele, and lower activity is associated with the COMT Met allele.[16,17] In codon 158 (in the rs4680 polymorphism) of the COMT gene, the Met allele's low enzymatic activity, which provides metabolic inactivation of dopamine, may be related to SUD. While COMT gene variants affect COMT activity, epigenetic modifications, especially DNA methylation of the COMT gene, may influence gene expression. Indeed, increased COMT gene methylation was related to decreased gene expression, and tobacco use disorder (TUD)[18] and alcohol use disorder (AUD)[2] were found to be related to higher membrane-bound catechol-O-methyltransferase (MB-COMT) promoter methylation, suggesting lower COMT gene activity.

The dopamine D2 receptor is involved in affect regulation, learning, motivation, reward processing, and decision-making,[19] all of which are crucial processes involved in SUD and other neuropsychiatric disorders such as mood disorders,[20] cognitive sequelae of schizophrenia,[21] and attention deficit hyperactivity disorder.[22] The dopamine receptor 2 (DRD2) gene localized on chromosome 11q23.2 can have Val96Ala, Leu141Leu, Val154Ile, Pro310Ser, Ser311Cys, TaqI A, A-241G, and -141C Ins/Del polymorphisms.[23] The -141C Ins/Del polymorphism is localized in the 5′ promoter region of the DRD2 gene and is the state of insertion (Ins) or deletion (Del) of cytosine at position -141.[24] Generally, analyzes were made in psychiatric studies has shown that this functional variant alters the expression of DRD2, and the expression of DRD2 is much lower in individuals carrying the Del allele in this region.[24] Another study reported that the DRD2-141C Ins/Del polymorphism could affect gene methylation states.[25] No earlier researches have clearly shown the interaction of methylation of MB-COMT promotor or DRD2 gene and COMT or DRD2 gene polymorphism on cannabinoid use disorder (CUD) and synthetic cannabinoid use disorder (SCUD) in the Turkish population. We hypothesized that the methylation of MB-COMT promotor or DRD2 gene and COMT or DRD2 gene polymorphism might be associated with the CUD/SCUD. We aimed to examine the relationship between CUD/SCUD and methylation of MB-COMT promotor or DRD2 gene and COMT or DRD2 gene polymorphism by comparing healthy controls.

Methods

Patient selection and diagnostic tools

This case-control study included 218 CUD/SCUD (44 patients with CUD and 174 patients with SCUD) and 102 age- and gender matched participants without SUD. CUD/SCUD patients were consecutively gathered from the 12th psychiatry clinic of Bakirkoy Mazhar Osman Mental Health and Neurology Training and Research Hospital between May 2019 and January 2020. The control group consisted of individuals with no medical or psychiatric diagnoses was recruited from the same location, the European side of Istanbul. Ethics committee approval was obtained from the Clinical Research Ethics Committee of the Istanbul Faculty of Medicine for the study (2019/87). We informed the patients in detail about the study's purpose, method, and procedures and obtained all the participants' written consent.

The interview was started by collecting sociodemographic and clinical information. Afterward, based on the DSM-5 criteria, the patients' diagnosis was confirmed with a positive urine test. In the toxicology laboratory of hospital for the determination of metabolites of various synthetic cannabinoids in urine; in-vitro pre-analytical tests; Immunalysis K2 Direct EIA Kit (JWH-018, JWH-073, AM-2201, UR-144 and their metabolites) and

Immunalysis Synthetic Cannabinoids-3 Urine Enzyme Immunoassay Kit (AB-PINACA and its metabolites), and Immunalysis for the detection of cannabinoid metabolites THC Urine Enzyme Immunoassay Kit is used.

We applied the family history method to examine patients about psychiatric disorders and SUDs in their relatives, despite its methodological limitations.[26,27] The patients were asked about the presence of SUDs (which included AUD and SUD but not TUD) in all first-degree relatives. SUDs were defined according to the DSM-5 criteria. All data about relatives came from the participants. No direct examinations of the first-degree relatives were conducted.

DNA analyses

Blood samples and DNA extraction

For the patients with CUD/SCUD and the control groups, 4 ml peripheral venous blood samples were collected in ethylenediaminetetraacetic acid tubes. Leukocyte isolation of all blood samples was performed first. Genomic DNA was extracted from leukocytes by using the Plus Blood Genomic DNA Purification test kit (GeneMark).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method

Genotyping of DRD2-141C Ins/Del and COMT Val158Met were determined using PCR and RFLP for patient and control groups. F: 5′-ACTGGCGAGCAGACGGTGAGGACCC-3′ and R: 5′-TGCGCGCGTGAGGCTGCCGGTTCGG-3′ was used as a primer for determining DRD2-141C Ins/Del variant.[24] F: 5′-ACTGTGGCTA CTCAGCTGTG-3′ and R: 5′-CCTTTTTCCA GGTCTGACAA-3′ was used as a primer for the determination of COMT Val158Met variant.[28] The BstNI enzyme was used for the DRD2-141 region, and the NlaIII enzyme was used for the COMT 158 region. Enzyme-digested PCR products were separated on 3% agarose gel.

Bisulfite treatment and methylation-specific polymerase chain reaction (MSP PCR)

Bisulfite modification is accepted as the gold standard to determine the methylation status of DNA.[29] To analyze, after isolation of DNA samples, we used the EZ-96 DNA Methylation-Gold kit according to manufacturer recommendations (Zymo Research). For methylation analysis of MB-COMT promotor[30] and DRD2[31] gene, two pairs of primers, one pair of methylated and one pair of unmethylated, were used for each region. Bisulfite modified DNA samples were amplified by PCR with Zymo Taq DNA polymerase (Zymo Research) with the primers at the following conditions: 10 min at 95 °C, 40 cycles (30 s at 95 °C, 40 s at annealing temperature for each primer and 45 s at 72 °C), and 72 °C for 7 min.

Statistical analyses

Statistical analysis was performed using IBM SPSS version 21.0 (IBM Corp. released 2012;

Table 1. Sociodemographic characteristics and clinical parameters of patients.

		SUD (*N*: 218) Mean ± SD	Control (*N*: 100) Mean ± SD
Age		28.63 ± 6.70	30.94 ± 10.56
		N	%
Sex	Male	218	100.0
	Female	0	0.0
Cigarette usage	No	133	61.0
	Yes	85	39.0
Alcohol usage	No	179	82.1
	Yes	39	17.9
Substance type	Synthetic cannabinoid	174	79.8
	Cannabinoid	44	20.2
Former polysubstance abuse	No	121	55.5
	Yes	97	44.5
Attempted suicide	No	152	69.7
	Yes	66	30.3
Psychotic symptom	No	95	43.5
	Yes	123	56.5
Family history	No	119	54.5
	Yes	99	45.5

Abbreviations: SD, standard deviation; SUD, substance use disorder.

Table 2. Comparison of frequencies of the DRD2 gene and MB-COMT promoter methylation between CUD/SCUD patients with controls.

Metilasyon	Genotype	CUD/SCUD	Healthy control	OR Exp. (*B*)	95% CI	*p*[a]
		n = 218 (%)	*n* = 102 (%)			
DRD2	Unmethylation	86 (39.5)	43 (42.2)			
	Partial methylation	132 (60.5)	59 (57.8)	1.202	0.645–2.237	0.536
COMT	Unmethylation	70 (32.1)	14 (13.7)			
	Partial methylation	148 (67.9)	88 (86.3)	5.912	2.054–17.020	0.001

[a]OR (95%CI) was adjusted by age and sex.

Table 3. Comparison of frequencies of DRD2-141C Ins/Del gene variants between CUD/SCUD patients with healthy controls.

	Genotype	CUD/SCUD	Healthy control	OR Exp. (*B*)	95% CI	*p*
		n = 218 (%)	*n* = 100 (%)			
DRD2-141C	Ins/Ins	174 (79.8)	84 (84)	3.892[a]	0.532–28.475[a]	0.181[a]
	Ins/Del	42 (19.3)	14 (14)	6.031[a]	0.725–50.191[a]	0.096[a]
	Del/Del	2 (0.9)	2 (2)	0.454[b]	0.063–3.268[b]	0.593[b]
Allele	Ins	390 (80.1)	164 (82.0)			
	Del	46 (19.5)	32 (18.0)	0.619[b]	0.381–1.007[b]	0.068[b]

[a]OR (95%CI) was adjusted by age and sex, [b]Fisher's exact test.

Armonk, NY, USA). Quantitative data (clinical parameters, COMT and DRD2 genotype distributions, and MB-COMT promotor and DRD2 genes methylation) represented as descriptive statistics included the minimum, maximum, mean, standard deviation, frequency, and percentage. The Pearson chi-square or Fisher's exact test analyzed comparisons of COMT and DRD2 genotype distribution and MB-COMT promotor and DRD2 gene methylation of patients. Statistical significance was accepted as $p < 0.05$ for the results of all analyses. We performed the power analysis with the "G*power" software (version 3.0.5, http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3/), post hoc goodness of fit $\chi 2$ test, with an "-error" probability of 0.05. The possible presence of population stratification bias has been gauged according to Lee and Wang,[32] considering COMT or DRD2 frequencies reported for Turkish populations[33,34] and incidence rates of SUD in Turkey, as reported by Ilhan et al.[35]

Results

The CUD/SCUD patients were evaluated according to sociodemographic characteristics and clinical parameters, as shown in Table 1. 60.5% ($n = 132$) of the patients had partial methylated, 39.5% ($n = 86$) had unmethylated DRD2 gene, again 57.8% ($n = 59$) of the healthy participants had partial methylated, 42.2% ($n = 43$) had unmethylated DRD2 gene. 67.9% ($n = 148$) of the patients had partially methylated, 32.1% ($n = 70$) had unmethylated MB-COMT promoter, again 86.3% ($n = 88$) of the healthy participants had partially methylated, 13.7% ($n = 14$) had unmethylated MB-COMT promoter. When the DRD2 gene and MB-COMT promoter methylation status of SUD patients were compared with the control group, there was a significant difference between groups (OR: 5.912; 95% CI: 2.054–17.020; $p = 0.001$). The percentages of unmethylated MB-COMT promotor were significantly higher than in CUD/SCUD patients (Table 2).

Within the DRD2-141C Ins/Del genotype distribution, 79.8% ($n = 174$) of the patients had Ins/Ins, 19.3% ($n = 42$) had Ins/Del, and 0.9% ($n = 2$) had Del/Del genotypes. In the DRD2-141C Ins/Del genotype distribution, 84.0% ($n = 84$) of the healthy participants had Ins/Ins, 14% ($n = 14$) had Ins/Del, and 2% ($n = 2$) had Del/Del genotypes. When the DRD2-141C Ins/Del genotype and the allele frequency distributions of the CUD/SCUD patients were compared to the healthy controls, there was no significant difference between the two groups ($p > 0.05$) (Table 3). Again in the COMT Val158Met genotype distribution, 24% ($n = 52$) of the patients had Met/Met, 45.6% ($n = 99$) had Met/Val, and 30.4% ($n = 66$) had Val/Val genotypes. In the

Table 4. Comparison of frequencies of COMT Val158Met gene variants between CUD/SCUD patients with healthy controls.

Metilasyon	Genotype	CUD/SCUD	Healthy control	OR Exp. (*B*)	95% CI	*p*
		n = 217 (%)	*n* = 79 (%)			
COMT Val158Met	Met/Met	52 (24.0)	24 (30.4)	0.961[a]	0.408–2.267[a]	0.928[a]
	Met/Val	99 (45.6)	29 (36.7)	1.639[a]	0.722–3.722[a]	0.238[a]
	Val/Val	66 (30.4)	26 (32.9)	0.861[b]	0.513–1.546[b]	0.673[b]
Allele	Met	203 (46.8)	77 (48.7)			
	Val	231 (53.2)	81 (51.3)	0.924[b]	0.642–1.331[b]	0.710[b]

[a]OR (95%CI) was adjusted by age and sex, [b]Fisher's exact test.

Table 5. Comparison of DRD2 gene methylation status of patients due to the genotype distribution.

Genotype	Methylation status		OR Exp. (*B*)	95% CI	p^b
DRD2-141C Ins/Del	Unmethylated, *n* = 69 (%)	Partial methylated, *n* = 132 (%)			
Ins/Ins	71 (82.6)	103 (78.0)	1.448	0.715–2.932	0.384
Ins/Del	15 (17.4)	27 (20.5)	1.322	0.649–2.695	0.482
Del/Del	0 (0)	2 (1.5)	1.015	0.994–1.037	0.520

[b]Fisher's exact test.

Table 6. Comparison of DRD2 gene methylation status of patients due to the clinical parameters.

		Methylation status		OR Exp. (*B*)	95% CI	p^a
		Unmethylated, *n* = 86(%)	Partial methylated, *n* = 132 (%)			
Substance type	Synthetic cannabinoid	66 (76.7)	108 (81.8)			
	Cannabinoid	20 (23.3)	24 (18.2)	0.724	0.368–1.424	0.319
Polysubstance abuse	Yes	50 (58.1)	71 (53.8)			
	No	36 (41.9)	61 (46.2)	1.194	0.690–2.067	0.526
Alcohol usage	Yes	17 (19.7)	22 (16.6)			
	No	69 (80.3)	110 (83.4)	0.812	0.403–1.637	0.560
Cigarette usage	Yes	84 (97.6)	1 (0.75)			
	No	2 (2.4)	131 (99.2)	3.113	0.277–34.973	0.358
Psychotic symptom	Yes	53 (60.2)	70 (53.0)			
	No	33 (39.8)	62 (47.0)	0.693	0.395–1.148	0.200
Suicide attempt	Yes	31 (36.0)	35 (26.5)			
	No	55 (64.0)	97 (73.5)	0.648	0.358–1.175	0.153
Family history	Yes	50 (58.1)	49 (37.1)			
	No	36 (41.9)	83 (62.9)	0.425[b]	0.244–0.741[b]	0.003[b]

[a]OR (95%CI) was adjusted by age and sex, [b]Fisher's exact test.

Table 7. Comparison of MB-COMT promotor methylation status of patients due to the genotype distribution.

Genotype	Methylation status		OR Exp. (*B*)	95% CI	*p*
COMT Val158Met	Unmethylated, *n* = 69 (%)	Partial methylated, *n* = 148 (%)			
Met/Met	15 (21.7)	37 (25.0)	0.945[a]	0.424–2.103[a]	0.889[a]
Met/Val	34 (49.3)	65 (43.9)	1.211[a]	0.620–2.368[a]	0.575[a]
Val/Val	20 (29.0)	46 (31.1)	1.105[b]	0.591–2.066[b]	0.874[b]

[a]OR (95%CI) was adjusted by age and sex, [b]Fisher's exact test.

COMT Val158Met genotype distribution, 30.4% (*n* = 24) of the healthy participants had Met/Met, 36.7% (*n* = 29) had Met/Val, and 32.9% (*n* = 26) had Val/Val genotypes. When the COMT Val158Met genotype and the allele frequency distributions of the CUD/SCUD patients were compared to the healthy controls, there was no significant difference between the two groups ($p > 0.05$) (Table 4).

When comparing DRD2 gene methylation (partial methylation, unmethylation) due to the DRD2-141C Ins/Del genotype distribution (Table 5) and clinical parameters (substance type, the presence of polysubstance misuse, alcohol or cigarette usage, attempted suicide, psychotic symptom, family history, and frequency of substance usage) in patients with CUD/SCUD, the DRD2 gene methylation was significantly different between the groups due to the family history (OR: 0.425; 95% CI: 0.244–0.741; $p = 0.003$) (Table 6). Comparing the MB-COMT promoter methylation (partial methylation, unmethylation)

Table 8. Comparison of MB-COMT promotor methylation of patients due to the clinical parameters.

Genotype	Genotype	Methylation status		OR Exp. (*B*)	95% CI	p^a
		Unmethylated, $n = 70$(%)	Partial methylated, $n = 148$ (%)			
Substance type	Synthetic cannabinoid	59 (84.2)	115 (77.7)			
	Cannabinoid	11 (15.8)	33 (22.3)	1.584	0.740–3.392	0.236
Polysubstance abuse	Yes	28 (40.0)	69 (46.6)			
	No	42 (60.0)	79 (53.4)	1.308	0.734–2.329	0.363
Alcohol usage	Yes	4 (5.7)	35 (23.6)			
	No	66 (94.3)	113 (76.4)	5.107	1.737–15.010	0.003
Cigarette usage	Yes	69 (98.6)	2 (1.4)			
	No	1 (1.4)	146 (98.6)	1.078	0.096–12.136	0.952
Psychotic symptom	Yes	40 (57.1)	83 (56.1)			
	No	30 (42.9)	65 (43.9)	0.971	0.542–1.738	0.920
Suicide attempt	Yes	21 (30.0)	44 (29.7)			
	No	49 (70.0)	104 (70.3)	1.014	0.542–1.897	0.965
Family history	Yes	36 (51.4)	63 (42.5)			
	No	34 (48.6)	85 (57.5)	0.696	0.393–1.233	0.214

[a]OR (95%CI) was adjusted by age and sex.

due to the COMT Val158Met genotype distribution (Table 7) and clinical parameters (substance type, the presence of polysubstance abuse, alcohol or cigarette usage, attempted suicide, psychotic symptom, family history, and frequency of substance usage) in patients with CUD/SCUD, the MB-COMT promoter methylation was significantly different between the groups due to the presence of alcohol usage (OR: 5.107; 95% CI:1.737–15.010; $p = 0.003$) (Table 8).

Discussion

An increasing number of studies have started to explain the role of DNA methylation in SUD.[36] Firstly, substance usage can alter DNA methylation by repeated administration. The substances have been reported to modify methylation patterns in the brain's reward areas.[37] Secondly, DNA methylation can cause addiction's pathophysiology by affecting genes' expression related to synaptic plasticity and memory. This situation results in long-term neuroadaptations underlying the onset and endurance of addictive behaviors.[38] Thirdly, the literature includes studies on maternal substance use and fetal genetic methylation. They showed that epigenetic alteration had transferred to the first generation without direct exposure to any drug. Throughout the first half of pregnancy, alcohol usage can change epigenetic patterns in the embryo, leading to reduced fetal growth and congenital abnormalities following SUD risk.[39]

Numerous researches have tried to identify specific genetic risk factors connected to CUD/SCUD. Although no individual single-nucleotide polymorphisms reached genome-wide significance, gene-based tests identified four genes significantly associated with lifetime cannabis use: CADM2, KCNT2, NCAM1, and SCOC.[40] On the other hand, in research examining the COMT (rs4680), CNR2 (rs2501432), CNR2 (rs2229579), UCP2 (rs659366), and IL-17F (rs763780) gene variants in SCUD patients have been reported that the CNR2 (rs2229579) and UCP2 (rs659366) variants were associated with SCUD.[33] Although previous studies on methylation have been performed mostly in the context of AUD, our recently published study reported that the global DNA methylation might be associated with the SCUD.[41] Earlier researches have also shown that cannabinoids significantly alter histone methylation and acetylation. Prini et al. demonstrated that Δ^9-THC alters histone modifications in the prefrontal cortex, essentially methylation of H3K9, and the expression of a subset of plasticity genes crucial for the improvement of adult cognitive deficits.[42]

In the present study, while the MB-COMT promoter methylation (partial methylation, unmethylation) of the CUD/SCUD patients was significantly different from the control, there was no statistically significant difference between the DRD2 gene methylation of the CUD/SCUD patients with the control group. The participants carrying the unmethylated MB-COMT promotor had a higher risk of a CUD/SCUD. Therefore, we speculate that our results suggest that epigenetic aberrations of the MB-COMT promotor may be associated with CUD/SCUD's pathophysiology in

the Turkish population. Because in our study, the participants had CUD or SCUD dominance, the present study is the first in this field to show the relationship between the COMT promoter methylation and adult CUD or SCUD patients. When the literature about COMT promoter methylation associated with CUD or SCUD is reviewed, only one study involving adolescents was seen. Van der Knaap et al. reported that hypermethylation of COMT promotor in adolescents is related to a reduced risk of CUD.[43] Our results were statistically compatible with Van der Knaap et al.'s study. Comparing the methylation of MB-COMT promotor in CUD/SCUD patients according to clinical parameters (polysubstance misuse, the presence of alcohol usage or smoking, attempted suicide, psychotic symptom, family history, duration, and frequency of substance usage) demonstrated that there was a significant difference between the two groups due to the alcohol usage. In literature, some studies have found relationships between methylation of genes in the dopamine system and AUD. Hillemacher et al. published higher rates of methylation of the dopamine transporter gene in AUD.[44] Again, Philibert et al. reported that methylation of monoamine oxidase-A is associated with AUD in women.[45] On the other hand, Van der Knaap et al. found that MB-COMT promoter methylation was not related to daily smoking and AUD.[43]

Recent investigations described the associations between DRD2 gene methylation and different psychiatric disorders (eating disorders,[46] Gilles de la Tourette syndrome,[47] etc.), SUD, and non-SUDs like pathologic gambling.[48] There was no statistically significant difference found between DRD2 gene methylation (partial methylation, unmethylation) of the CUD/SCUD patients with the control group in our study. When the literature was reviewed, only one study on the association between DRD2 gene methylation and CUD has been found. Gerra et al. found a significantly higher level of DNA methylation in CUD patients than controls at exon 8 of the DRD2 gene.[49] Therefore our study is the first in this field to examine the relationship between DRD2 gene methylation and CUD or SCUD patients together. Also, the research examining the connection between altered methylation of the dopamine transporter gene (DAT1) promoter CpG sites and CUD have been revealed no statistical differences in the general methylation status of the DAT1 gene promoter CpG island between the patients and controls similar to our study.[50]

Comparing the methylation of DRD2 genes in CUD/SCUD patients according to clinical parameters (polysubstance misuse, the presence of alcohol usage or smoking, attempted suicide, psychotic symptom, family history, duration, and frequency of substance usage) demonstrated that there was a statistically significant difference between the DRD2 gene methylation groups due to the family history. Although fewer studies have focused on addiction, evidence suggests that first-degree relatives of SUD patients have higher addiction rates than controls. Especially, adult first-degree relatives of patients with dependence on various substances, including cannabis, cocaine, ethanol, and opiates, demonstrated an eight-fold increased risk of SUD.[51] Additionally, SUD patients' generation was three times more likely to develop an addiction to ethanol or marijuana than children of unaffected parents.[52] It was thought that epigenetic modifications were erased and not transferred on to the following generations until recently. However, epigenetic alterations (DNA methylation, histone post-translational modifications, and non-coding RNAs) that were acquired in one generation can be inherited in the next generation.[53] DiNieri et al. reported that maternal cannabis usage alters the developmental regulation of mesolimbic D2 receptor in offspring by epigenetic mechanisms that regulate histone lysine methylation, and the following reduction of D2 receptor may contribute to addiction vulnerability later in life.[54] This result is parallel to our findings in which SUD's family history is associated with DRD2 gene methylation in CUD/SCUD patients. In contrast to the current study, Fransquet et al. published that there is no strong evidence that maternal cannabis use in pregnancy is related to offspring DRD4 methylation.[55]

When our study was compared CUD/SCUD patients' COMT or DRD2 genotype distribution with the control group and the MB-COMT promotor or DRD2 gene methylation due to the genotype distributions in CUD/SCUD patients, there were no significant differences between

groups. In one of our latest studies about this subject, again, we observed no significant association between COMT Val158Met polymorphism and SCUD, while the COMT Val158Met variant was associated with attempted suicide in SCUD patients.[33] Again, our results were compatible with the Nennicioglu et al.'s research that reported no statistically significant difference between SCUD patients and the control group in terms of COMT gene Val158Met polymorphism.[56] The rs6277 polymorphism of the DRD2 gene could also have an essential function in CUD susceptibility due to its crucial role in altering dopamine receptor 2 gene expression and density in the thalamus and cortex. An association between Taq1A and Taq1B DRD2 polymorphisms and CUD was evaluated by Nacak et al. in the 112 CUD and 130 healthy control subjects; they observed a significant association between Taq1A gene polymorphism and CUD compared to the control subjects.[34] In contrast, Creemers et al. showed that carrying the A1 allele of the DRD2 TaqIA polymorphism, or the 7 repeat DRD4, was not directly related to AUD or CUD, similar to our study.[57]

Our study's strength is the first study showing the interaction of methylation of MB-COMT promotor or DRD2 gene and COMT or DRD2 gene polymorphism on CUD/SCUD in the Turkish population; therefore, both genetic and epigenetic variations of these genes provide a complete picture of the role in CUD/SCUD. Secondly, since CUD/SCUD patients and healthy participants were collected from the same location, the European side of Istanbul, our study's findings more valuable. Besides the strengths of the present research, there are also several limitations. The first limitation was the small sample size, which can limit the statistical power. Secondly, the significance of epigenetic findings based on blood samples must be carefully considered when making inferences about specific mechanistic pathways for brain-related disorders, as in our research. Only DNA methylation was analyzed in our study, while other epigenetic mechanisms such as histone deacetylation and hyperacetylation or miRNA were not studied. The present cross-sectional study prevented us from evaluating whether methylation is an outcome of substance use or whether methylation predisposes drug-seeking behavior and CUD/SCUD. Finally, including only male CUD/SCUD patients in our study prevented gathering information related to a female-male gender comparison.

In conclusion, while the MB-COMT promoter methylation might be related to the CUD/SCUD, the DRD2 gene methylation and COMT or DRD2 polymorphisms were not found to be associated with CUD/SCUD. These findings prove that epigenetic alterations especially MB-COMT promoter methylations may be useful biomarkers to identify susceptibility for substance use. Besides, they may be useful in the prevention of CUD/SCUD. Confirming these findings with different ethnicities will better investigate the association between these epigenetic alterations and CUD/SCUD, therefore, they may contribute to improving effective behavioral or pharmacological interventions for CUD/SCUD.

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Disclosure statement

All authors declare not to have any conflicts of interest that might be interpreted as influencing the manuscript's content.

Ethical Approval

The authors declare that all methods contributing to this work comply with the relevant national and institutional committees' ethical standards on human experimentation and the Helsinki Declaration of 1975, as revised in 2013.[58]

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Contributions of authors

Y.O., H.M.A., and S.P. are responsible for the formulation of overarching research goals and aims. Y.O., S.P., and H.M.A. conceived and designed the study. Y.O., S.P., and P.C.A. are responsible for the provision of study materials and laboratory samples. O.P. acquired, analyzed, and interpreted all data. H.M.A. drafted the manuscript; all of the

authors critically revised the manuscript. S.P. and P.C.A. supervised the study.

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Data availability statement

The authors confirm that all relevant data are included in the article, which does not contain any supplementary material.

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