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Effects of *PDYN* 68-bpVNTR, *BDNF* rs6265, *OPRD1* rs569356 and *OPRM1* rs2075572 polymorphisms on opioid-methamphetamine co-use

Merve Ak [a,b,1], Gizem Özer-Uzaldı [a,b,1], Mukaddes Asena Yıldırım [b], Selin Özkan-Kotiloğlu [b,c], Mustafa Danışman [d], Dilek Kaya-Akyüzlü [b,*]

[a] *Ankara University, Graduate School of Health Sciences, Ankara, Turkiye*
[b] *Ankara University, Institute of Forensic Sciences, Ankara, Turkiye*
[c] *Kırşehir Ahi Evran University, Faculty of Science and Art, Department of Molecular Biology and Genetics, Kırşehir, Turkiye*
[d] *Ankara Training and Research Hospital AMATEM Clinic, Ankara, Turkiye*

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ABSTRACT

To investigate the impact of *PDYN* 68-bpVNTR, *BDNF* rs6265, *OPRD1* rs569356 and *OPRM1* rs2075572 variations on the susceptibility to opioid-methamphetamine co-use, a total of 532 individuals, including opioid (n = 104), and methamphetamine (n = 166) users as well as opioid and methamphetamine co- users (n = 158) and healthy individuals (n = 104), were included. *BDNF* rs6265, *OPRD1* rs569356 and *OPRM1* rs2075572 were genotyped by PCR-RFLP method, while *PDYN* 68-bp VNTR was genotyped by PCR. The assessment of impulsiveness, craving, withdrawal, anxiety and depressive symptoms was conducted using scales. A significant difference was identified between the substance-using groups and the control group in view of the alleles of *PDYN* 68-bp VNTR (p = 0.001). The allele frequencies of the *PDYN* 68-bp VNTR exhibited a difference between opioid and methamphetamine users (p = 0.018). The 5-repeat allele was first observed in the Turkish population. The analysed polymorphisms could contribute to the development of addiction, exerting an influence on impulsiveness, craving and withdrawal.

1. Introduction

Recent studies have indicated a rising trend in methamphetamine use among individuals with opioid use disorder, with a notable increase from 18.8 % to 34.2 % between 2011 and 2017 (Ellis et al., 2018; Glick et al., 2018). The concurrent use of heroin and methamphetamine, colloquially termed "goofball," carries with it a number of significant health risks, including elevated rates of HIV, overdose deaths, and severe health complications. This is particularly the case among individuals experiencing homelessness (Glick et al., 2021). This co-use, otherwise referred to as the "twin epidemics," has been demonstrated to result in a decline in treatment adherence for opioid disorder therapies, such as buprenorphine or methadone (Tsui et al., 2020). Research on the motivations behind the use of heroin and methamphetamine in conjunction reveals that many individuals use these substances together to experience a synergistic effect or to counterbalance the effects of one substance, thereby enabling them to function more "normally" in daily life (Boileau-Falardeau et al., 2022; Ondocsin et al., 2023). A considerable proportion of subjects reported consuming both drugs on the same day, often for almost half of each month. Furthermore, it was posited by a number of users that the consumption of methamphetamine had the effect of mitigating the symptoms associated with opioid withdrawal (Ondocsin et al., 2023). This phenomenon may be attributable to the drug's dual impact on the brain's dopamine reward systems (Ellis et al., 2018; Lopez et al., 2021). It has also been attributed to methamphetamine's capacity to alleviate opioid withdrawal symptoms through its impact on dopamine reward pathways (Ellis et al., 2018; Lopez et al., 2021).

Substance use disorders are known to be complex brain disorders influenced by both genetic and environmental factors (Edenberg and Kranzler, 2005). It is believed that identifying the genetic factors of substance use disorder and knowing the predisposition of individuals to use substances in advance will be useful in taking necessary precautions and developing different treatment methods (Deak and Johnson, 2022). The heritability of substance use disorder is not attributable to a single gene, but rather, it is the result of the interplay of multiple genes and

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* Correspondence to: Institute of Forensic Sciences, Ankara University, Dikimevi, Ankara 06590, Turkey.
E-mail addresses: kaya@ankara.edu.tr, kayadilek79@gmail.com (D. Kaya-Akyüzlü).

[1] Equal author contribution

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genetic polymorphisms. These genes, while contributing to the overall risk, play a relatively minor role in isolation (Ducci and Goldman, 2012).

Opioid receptors have been categorized into three distinct types: μ, κ, and δ. The μ receptor has been shown to facilitate pleasure and analgesia through dopamine stimulation, while the κ receptor has been linked to the induction of negative emotional effects (Fields and Margolis, 2015; Martin et al., 1976). The μ-opioid receptor (MOR), encoded by the *OPRM1* gene, is extensively distributed across the brain and is highly concentrated in areas such as the thalamus, caudate putamen, and globus pallidus (Reeves et al., 2022). MOR demonstrates a strong binding affinity for β-endorphin and enkephalin, while it's affinity for dynorphin—preferentially binding to the κ-opioid receptor—is relatively weaker (Gonzalez-Nunez et al., 2013). Moreover, MOR binds effectively to external opioid substances such as morphine, heroin, and methadone. MORs are believed to play a key role in mediating the effects of opioids, including euphoria, pain relief, and the withdrawal symptoms associated with opioid use (Ding et al., 2013). The δ-opioid receptor plays a key role in the rewarding effects of numerous addictive substances (Zhang et al., 2008). Dynorphin, a tridecapeptide that was first identified in 1979, is encoded by the *PDYN* gene and has been shown to bind to κ-opioid receptors (KORs) (Goldstein et al., 1979). This neuropeptide plays a crucial role in memory, emotional regulation, pain, and stress response across various brain regions, including the amygdala and the hippocampus (Goldstein et al., 1979; Schwarzer, 2009). The DYN/KOR system is responsible for the regulation of dopamine, a neurotransmitter that is associated with reward and motivation (Estave et al., 2022). Dysregulation of the DYN/KOR system, which is often due to stress or substance use, can result in the disruption of dopamine balance. This, in turn, can contribute to the development of addiction, depression, and schizophrenia (Karkhanis et al., 2017; Tejeda et al., 2021). While several studies have implicated the dysregulation of the DYN/KOR system in the pathophysiology of psychiatric disorders, including depression and anxiety, its specific role in the development of schizophrenia remains an area of active investigation (Abi-Dargham, 2021). Emerging evidence suggests that alterations in the DYN/KOR system may contribute to core symptoms of schizophrenia, particularly negative symptoms and cognitive dysfunction (Clark, 2020). Dynorphins have been shown to inhibit dopamine release in the reward pathways between the ventral tegmental area (VTA) and the nucleus accumbens (NAcc). This property renders the system a viable target for addiction therapies (Faisal et al., 2014; Shippenberg et al., 2007). Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, plays a pivotal role in the survival, differentiation, growth, and synaptic transmission regulation of midbrain dopaminergic neurons (Krieglstein, 2004). *BDNF* is highly expressed in the cortex and hippocampus, where it supports neurogenesis, neuron survival, and activity. A body of research has established a correlation between *BDNF* and mental and drug use disorders hereby suggesting a potential role for *BDNF* in treatment (Ornell et al., 2018).

This article explores the genetic underpinnings of co-use of heroin and methamphetamine, with a particular focus on genes such as *PDYN* and *BDNF*, as well as *OPRD1* and *OPRM1*. These genes are thought to play a role in the regulation of emotions, the response to stress and the plasticity of the nervous system, contributing to susceptibility to addiction and the outcomes of treatment (Anderson et al., 2013; Ornell et al., 2018; Al-Eitan et al., 2021). A survey of the extant literature reveals an absence of any prior research that has examined frequencies of the *PDYN* 68-bp VNTR, *BDNF* rs6265, *OPRD1* rs569356 and *OPRM1* rs2075572 polymorphisms in individuals who use opioid and methamphetamine concurrently. This study will be the first of its kind to be conducted in literature.

2. Materials and Methods

2.1. Case Selection

A total of 532 volunteers participated in this study. Inclusion criteria were: (1) age between 18 and 65 years, (2) meeting DSM-5 diagnostic criteria for opioid and/or methamphetamine use disorder, and (3) providing informed written consent. Exclusion criteria included: (1) diagnosis of a current psychotic disorder, (2) presence of severe neurological disease, and (3) inability to complete study assessments due to cognitive deficits or language barriers.

The participants were divided into groups according to the substances they used (opioid, methamphetamine, and opioid-methamphetamine co-use) and a healthy control group was also included in the study.

i. This group included a total of 104 individuals who met the DSM-5 diagnostic criteria for opioid use disorder exclusively and who had presented to Ankara Training and Research Hospital, Alcohol and Substance Addiction Research, Treatment and Training Centre (AMATEM) for treatment purposes. All participants in the OUD group underwent urine drug screening (UDS) upon admission to confirm recent opioid use. Individuals who have used substances other than opioids were excluded from the study. In addition, all participants in this group were active cigarette smokers.

ii. The group of methamphetamine users (n = 166) includes individuals who submitted applications to the AMATEM clinic for treatment and did not use any substance other than methamphetamine. Urine drug screening was also utilized to confirm recent methamphetamine exposure and to exclude use of other substances. Notably, tobacco use was prevalent across this group, with all individuals reporting regular cigarette smoking.

iii. The opioid-methamphetamine co-users group (n = 158) consisted of individuals diagnosed with opioid use disorder who also used methamphetamine and had applied to the AMATEM clinic for treatment. The substances utilised by the subjects were ascertained through the urine substance detection test conducted at the AMATEM clinic. Daily tobacco use was also reported consistently among members of this group.

iv. The healthy control group (n = 104) comprised individuals who had not used any illegal addictive substances in the past or currently. Since the individuals in the other three groups were smokers, the control group was also composed of smokers.

All individuals in the study groups were over 18 years of age, did not have any psychiatric disorder (schizophrenia, depression, bipolar disorder, psychotic disorders, and dementia), and did not use psychiatric medication, including both antidepressants and pharmacological treatments used in the management of substance use disorders, such as SSRIs, naltrexone, and buprenorphine.

Following the approval of the institutional ethics committee (Approval No.: I03–109–22 in 10 March 2022), blood samples were collected from all volunteers in the four groups. Blood samples were collected from the first three groups at the time of their application to AMATEM and from the control group when they underwent routine hospital checks. The sampling procedure was conducted in accordance with the principles outlined in the Declaration of Helsinki. Prior to participation, written informed consent was obtained from all subjects, and they were also administered a brief questionnaire containing questions regarding their socio-demographic information.

2.2. DNA Isolation and Genotyping

The isolation of genomic DNA was conducted in accordance with the stipulated instructions provided by the manufacturer of the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA). The storage of isolated DNA was conducted at a temperature of −20°C, prior to undergoing a polymerase chain reaction (PCR) analysis. With the exception of *PDYN* 68-bp VNTR, genotyping was conducted utilising the Polymerase Chain Reaction-Restriction Fragment Lenght Polymorphism (PCR-RFLP) method. The *PDYN* 68-bp VNTR polymorphism was analysed by PCR in accordance with the methodology previously described by Saify and Saadat (2015). Alleles containing 1, 2, 3, 4, or 5 repeats produced 379, 447, 515, 583, and 651 base pair (bp) PCR amplicons, respectively. Subsequent to this, the alleles of the *PDYN* 68-bp repeat polymorphism were grouped as follows: short/short 'SS' (1,1; 1,2; 2,2 copies), short/long 'SL' (1,3; 1,4; 2,3; 2,4 copies) and long/long 'LL' (3, 3; 3,4; 4,4 copies) repeat alleles.

The PCR-RFLPs of the *BDNF* rs6265, *OPRD1* rs569356, and *OPRM1* rs2075572 polymorphisms were performed as previously described by Skibinska et al. (2018), Kaya-Akyüzlü et al. (2023), and Ding et al. (2013), respectively. Supplementary Table 1 provides a comprehensive overview of the primer sequences, the PCR conditions, and the digestion conditions. In order to genotype the polymorphisms, it was necessary to separate the undigested and digested PCR products by means of gel electrophoresis on a 1–3 % agarose gel. The products were then visualised through ethidium bromide staining under a UV illuminator, and the resultant gel images were scanned and photographed using the Syngene Monitoring System (Figs. 1–4).

2.3. Measurement of the craving, opioid withdrawal, impulsiveness, and the intensity of depressive and anxiety symptoms

The impact of depression, anxiety, impulsiveness, and substance cravings on individuals was determined by means of an assessment using the following instruments: the Beck Depression Inventory II (BDI-II), the Beck Anxiety Inventory (BAI), the Barratt Impulsiveness Scale-11 (BIS-11), and the Substance Craving Scale (SCS), respectively. The Clinical Opiate Withdrawal Scale (COWS) was utilised to ascertain the severity of opioid withdrawal symptoms in individuals with OUD and opioid-methamphetamine co-users. The validity and reliability of the Turkish versions of these scales have been demonstrated in previous studies (Canan et al., 2015; Evren et al., 2011; Güleç et al., 2008; Hisli, 1989; Ulusoy et al., 1998).

2.4. Statistical Analysis

The data obtained from the study were subjected to statistical analysis using the SPSS program version 25.0. Initially, the distribution of the numerical continuous data was examined to ascertain its normality. This was accomplished by conducting a Kolmogorov-Smirnov test. The median and interquartile range (IQR) values are provided for data since all numerical continuous data were non-normally distributed. For sociodemographic data, such as marital status and employment status, frequencies and percentages are given. The hypothesis that the genotype frequencies of the *PDYN* 68 bp VNTR, *BDNF* rs6265, *OPRD1* rs569356, and *OPRM1* rs2075572 polymorphisms were in Hardy-Weinberg equilibrium was determined by chi-square test. The Hardy-Weinberg equilibrium test was applied to ensure genotype distributions were representative of the population, supporting the validity of our genetic comparisons. The association between these four polymorphisms and OUD, methamphetamine use, and opioid-methamphetamine co-use was evaluated by means of a logistic regression analysis. Logistic regression analysis was chosen to evaluate associations between genotype variants and substance use disorders due to its suitability for modeling binary and categorical outcomes. For each polymorphism, three genotypes were designated as follows: homozygous wild-type, heterozygous, and homozygous variant. Genotypes with low frequencies were combined to

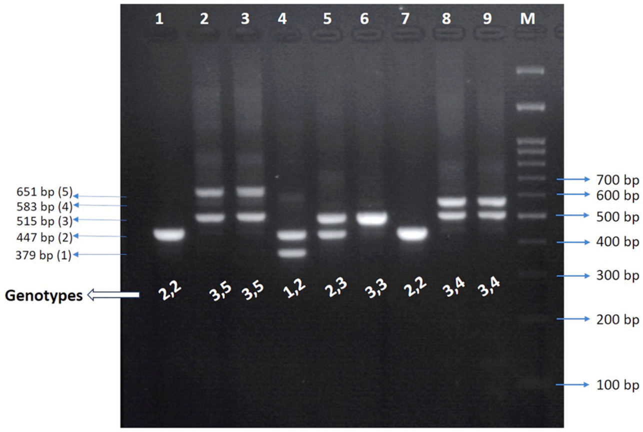

Fig. 1. Agarose gel electrophoresis showing the sizes of PCR products of the *PDYN* 68-bp VNTR (M: 100 bp ladder; Lanes 1 and 7: alleles containing 2 and 2 repeats (447 bp); Lanes 2 and 3: alleles containing 3 and 5 repeats (515 and 651 bp); Lane 4: alleles containing 1 and 2 repeats (379 and 447 bp); Lane 5: alleles containing 2 and 3 repeats (447 and 515 bp); Lane 6: alleles containing 3 and 3 repeats (515 bp); Lanes 8 and 9: alleles containing 3 and 4 repeats (515 and 583 bp).

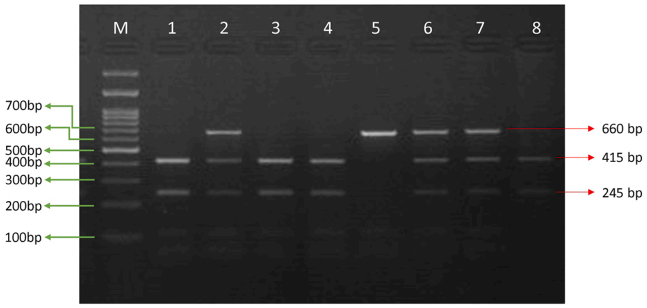

Fig. 2. Representative agarose gel (3 %) images of digested PCR products for the *BDNF* rs6265. 100 bp ladder; Lanes 1,3,4, and 8: homozygous wild-type genotype (GG) (415 bp and 245 bp); Lanes 2,6, and 7: heterozygote genotype (GC) (660 bp, 415 bp and 245 bp); Lane 5: homozygous variant genotype (CC) (660 bp).

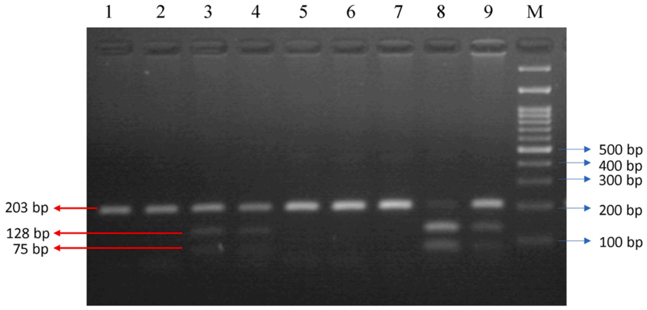

Fig. 3. Representative agarose gel (3 %) images of digested PCR products for the *OPRD1* rs569356. 100 bp ladder; Lanes 1,2,5,6, and 7: homozygous wild-type genotype (AA) (203 bp); Lanes 3,4, and 9: heterozygote genotype (AG) (203 bp, 128 bp, and 75 bp); Lane 8: homozygous variant genotype (GG) (128 bp and 75 bp).

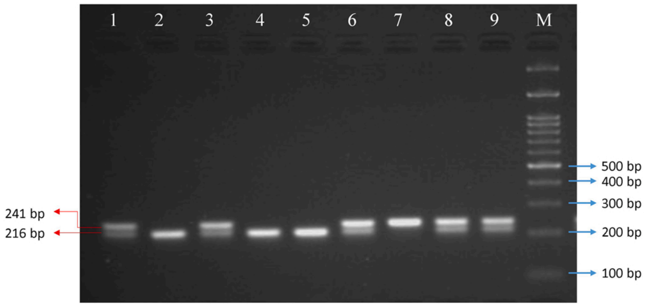

Fig. 4. Representative agarose gel (3 %) images of digested PCR products for the *OPRM1* rs2075572. 100 bp ladder; Lane 7: homozygous wild-type genotype (GG) (241 bp); Lanes 1,3,6,8, and 9: heterozygote genotype (GC) (241 bp, 216 bp, and 25 bp); Lanes 2,4, and 6: homozygous variant genotype (CC) (216 bp and 25 bp).

preserve statistical power, a common approach in genetic association studies. In this study, given that all numerical continuous data were non-normally distributed-as confirmed by the Kolmogorov-Smirnov test-, the non-parametric Kruskal-Wallis test was employed for pair-wise group comparisons, and the Mann-Whitney *U* test was used for three-group comparisons. The statistical significance of the findings was determined by the p-value, with a p-value less than 0.05 being considered statistically significant.

3. Results

3.1. Characteristics of the subjects studied

In total 104 individuals with OUD diagnosis (median ages 30 years, 17 females and 87 males), 166 methamphetamine users (median ages 30.5 years, 24 females and 142 males), 158 opioid-methamphetamine users (median ages 30 years, 22 females and 136 males**)** and 104 controls (median ages 34.5 years, 6 females and 98 males) were included in the study. The investigation revealed no statistically significant differences between the groups with regard to gender ($p = 0.099$). A significant difference was identified between the onset age of first substance use among opioid, methamphetamine, and opioid-methamphetamine users ($p = 0.04$). Post-hoc pairwise comparisons using Mann-Whitney U tests revealed a statistically significant difference between the methamphetamine use group and the opioid-methamphetamine co-use group ($p = 0.019$), with the co-use group initiating substance use at an earlier age. However, after applying Bonferroni correction for multiple comparisons (adjusted $p = 0.057$), this difference was no longer statistically significant. As illustrated in Table 1, the socio-demographic data set encompasses the mean age of the participants, in addition to information pertaining to their marital status, employment status, and educational attainment.

3.2. Distribution of the PDYN 68-bp VNTR promoter repeats

The number of VNTR promoter repeats were given in all groups in Table 2. A significant discrepancy was observed among the four groups ($p = 0.001$) as well as the addict groups and the control group ($p = 0.001$). Post hoc pairwise comparisons revealed a significant difference between the opioid and methamphetamine use groups ($p = 0.018$) in terms of the number of repetitions. It is noteworthy that 1,2 alleles were detected only in methamphetamine users (1.8 %), whereas 2,4 alleles were not detected only in opioid- methamphetamine users. It may also be noted that 5-repeat allele was detected in both individuals with OUD and methamphetamine users and the most frequent repeats were “2” and “3” repeats in the control group (49.9 %).

3.3. Genotype distribution of the BDNF rs6265, PDYN 68-bp VNTR, OPRD1 rs569356 and OPRM1 rs2075572 polymorphisms

Table 3 shows the genotype frequencies and Hardy–Weinberg equilibrium (HWE) of the four polymorphisms analysed in this study for the four groups included in the study. For each group, it was established that the genotype distributions of polymorphisms were in HWE.

The present study sought to evaluate the impact of *BDNF* rs6265, *PDYN* 68 bp VNTR, *OPRD1* rs569356, and *OPRM1* rs2075572 polymorphisms on opioid, methamphetamine, and opioid-methamphetamine co-use. Logistic regression analyses showed that there was no association between *BDNF* rs6265, *OPRD1* rs569356, and *OPRM1* rs2075572 polymorphisms and substance use disorder (SUD) ($p > 0.05$). Conversely, a trend association between *PDYN* 68-bp VNTR and substance use disorder was identified ($p = 0.06$). The *PDYN* 68-bp VNTR variant allele (S) frequency (38 %) was found to be higher in OUD patients in comparison to the other three groups. The lowest *PDYN* 68-bp VNTR variant allele frequency was identified in the control group (30 %) (Table 3).

3.4. Comparison of the groups in view of impulsiveness, craving and the intensity of anxiety and depressive symptoms

In the present study, the groups under investigation were compared with respect to impulsiveness, craving, and the intensity of symptoms of anxiety and depression (Supplementary Table 2). The severities of depressive (measured by the Beck Depression Inventory-II) and anxiety (measured by the Beck Anxiety Inventory) symptoms were significantly lower in the control group than in individuals with OUD,

Table 1

Demographics of the groups included in the study.

Parameters	OUD (n = 104)		Meth users (n = 166)		Opioid-Meth Co-users (n = 158)		Controls (n = 104)		p-value
Age (years) x̃ (IQR)	30,00 (26,00–35,00)		30,50 (26,00–35,00)		30,00 (27,00–34,00)		34,50 (26,75–42,00)		0.008
Weight (kg) x̃ (IQR)	70,00 (62,00–76,00)		67,00 (60,00–76,00)		66,00 (60,00–73,00)		84,00 (76,00–92,25)		0.001
Height (cm) x̃ (IQR)	174,50 (170,00–178,00)		172,50 (169,00–178,00)		173,00 (169,00–180,00)		178,00 (173,00–180,00)		0.001
Education	n	% frequency	n	% frequency	n	% frequency	n	% frequency	0.001
Primary	19	18,3	27	16,3	24	15,2	11	10,6	
Secondary	47	45,2	59	35,5	60	38,0	17	16,3	
High School	26	25,0	59	35,5	59	37,3	56	53,8	
Under-graduate	7	6,7	10	6,0	8	5,1	17	16,3	
Graduate	-	-	1	0,6	-	-	1	1,0	
Occupation	n	% frequency (95 % CI)	n	% frequency (95 % CI)	n	% frequency (95 % CI)	n	% frequency (95 % CI)	0.001
Working	69	66,3	72	43,4	74	46,8	92	88,5	
Not working	30	28,8	84	50,6	77	48,7	10	9,6	
Marital status	n	% frequency (95 % CI)	n	% frequency (95 % CI)	n	% frequency (95 % CI)	n	% frequency (95 % CI)	0.001
Single	66	63,5	78	47,0	101	63,9	39	37,5	
Married	25	24,0	63	38,0	38	24,1	62	59,6	
Widow/ Divorced	8	7,7	15	9,0	12	7,6	1	1,0	
The onset age of first substance use (years) x̃ (IQR)	18,00 (16,00–23,25)		19,00 (16,00–26,00)		17,00 (15,00–21,00)		-		**0.04**

n: sample size, x̃: median, IQR: Interquartile range, OUD: individuals with opioid use disorder diagnosis, Meth: Methamphetamine

Table 2
Genotypic distribution of *PDYN* 68-bp VNTR (rs35286281) polymorphism in controls and in OUD, Meth users and Opioid-Meth co-users.

***PDYN* 68 bp VNTR (rs35286281) Genotypes**	**OUD** (n = 104)		**Meth users** (n = 166)		**Opioid-Meth Co-users** (n = 158)		**Controls** (n = 104)	
	n	% frequency	n	% frequency	n	% frequency	n	% frequency
1,2	-	-	3	1.8	-	-	-	-
1,3	2	1.9	-	-	1	0.6	-	-
2,2	15	14.4	14	8.4	13	8.2	7	6.7
2,3	45	43.3	59	35.5	67	42.3	51	49.9
2,4	3	2.9	5	3.0	-	-	1	1.0
3,3	34	32.7	75	45.2	71	44.9	38	36.5
3,4	4	3.8	3	1.8	5	3.2	7	6.7
3,5	1	1.0	1	0.6	-	-	-	-

n: sample size, OUD: individuals with opioid use disorder diagnosis, Meth: Methamphetamine

Table 3
The genotype frequencies and Hardy–Weinberg equilibrium (HWE) according to genotypes of the *PDYN* 68 bp VNTR, *BDNF* rs6265, *OPRD1* rs569356 and *OPRM1* rs2075572 polymorphisms in all groups.

***PDYN* 68 bp VNTR (rs35286281) Genotypes**	**OUD** (n = 104)		**Meth users** (n = 166)		**Opioid-Meth Co-users** (n = 158)		**Controls** (n = 104)		**Logistic regression analysis**
	n	**% frequency**	**n**	**% frequency**	**n**	**% frequency**	**n**	**% frequency**	
LL	39	37.5	79	47.6	76	48.1	45	43.3	p = 0.06[§]
SL	50	48.1	64	38.6	68	43.0	52	50.0	
SS	15	14.4	17	10.2	13	8.2	7	6.7	
Variant allele freq.	38 %		31 %		30 %		32 %		
HWE	χ2 = 0.03; p = 0.87		χ2 = 0.55; p = 0.46		χ2 = 0.17; p = 0.68		χ2 = 2.47; p = 0.12		
BDNF rs6265 Genotypes	**OUD** (n = 102)		**Meth users** (n = 166)		**Opioid-Meth Co-users** (n = 138)		**Controls** (n = 98)		**Logistic regression analysis**
	n	**% frequency** (95 % CI)	**n**	**% frequency** (95 % CI)	**n**	**% frequency** (95 % CI)	**n**	**% frequency** (95 % CI)	
GG	75	72.1	118	71.1	94	59.5	69	66.3	p = 0.833[§]
GA	25	24.0	41	24.7	34	21.5	23	22.1	
AA	2	1.9	7	4.2	6	3.8	2	1.9	
Variant allele freq.	14 %		17 %		17 %		14 %		
HWE	χ2 = 0.002; p = 0.96		χ2 = 1.88; p = 0.17		χ2 = 1.55; p = 0.21		χ2 = 0.002; p = 0.96		
***OPRD1* rs569356 Genotypes**	**OUD** (n = 100)		**Meth users** (n = 122)		**Opioid-Meth Co-users** (n = 121)		**Controls** (n = 99)		**Logistic regression analysis**
	n	**% frequency** (95 % CI)	**n**	**% frequency** (95 % CI)	**n**	**% frequency** (95 % CI)	**n**	**% frequency** (95 % CI)	
AA	91	91.0	96	78.7	100	82.6	84	84.8	p = 0.139[§]
AG	8	8.0	23	18.8	19	15.7	14	14.1	
GG	1	1.0	3	2.5	2	1.7	1	1.01	
Variant allele freq.	0.05 %		12 %		10 %		8 %		
HWE	χ2 = 2.49; p = 0.11		χ2 = 1.22; p = 0.27		χ2 = 0.92; p = 0.34		χ2 = 0.23; p = 0.63		
***OPRM1* rs2075572 Genotypes**	**OUD** (n = 100)		**Meth users** (n = 125)		**Opioid-Meth Co-users** (n = 123)		**Controls** (n = 103)		**Logistic regression analysis**
	n	**% frequency** (95 % CI)	**n**	**% frequency** (95 % CI)	**n**	**% frequency** (95 % CI)	**n**	**% frequency** (95 % CI)	Odds Ratio (95 % CI)
CC	35	35.0	51	40.8	37	30.08	32	31.06	p = 0.851[§]
CG	50	50.0	54	43.2	59	47.96	53	51.45	
GG	15	15.0	20	16.0	27	21.95	18	17.48	
Variant allele freq.	40 %		38 %		46 %		43 %		
HWE	χ2 = 0.17; p = 0.68		χ2 = 0.79; p = 0.37		χ2 = 0.14; p = 0.70		χ2 = 0.24; p = 0.62		

n: sample size, OUD: individuals with opioid use disorder diagnosis, Meth: Methamphetamine. [§]The control group was used as the reference category in the logistic regression analysis.

methamphetamine users, and opioid-methamphetamine co-users ($p < 0.05$). It is worth noting that the intensity of depressive and anxiety symptoms was a bit lower among opioid-methamphetamine co-users (28.0 and 17.5) than among opioid (29.0 and 20.0) and methamphetamine users (29.0 and 20.0), although this is not statistically significant ($p > 0.05$).

3.5. Impulsiveness, craving and the intensity of anxiety and depressive symptoms, across PDYN 68-bp VNTR genotypes

In this study, the genotypes of *PDYN* 68-bp VNTR, *BDNF* rs6265, *OPRD1* rs569356 and *OPRM1* rs2075572 polymorphisms were statistically compared in terms of impulsiveness, craving, anxiety and depressive symptom scale scores.

Methamphetamine users with the SS genotype had significantly higher BIS-11 scores compared to those with the SL+LL genotype (37.5 versus 32.0) ($p = 0.04$) (Table 4). When opioid, methamphetamine and opioid-methamphetamine co-users with SS allele of the *PDYN* 68-bp VNTR were compared for impulsiveness, a statistically significant difference was found between the groups ($p = 0.04$), with the lowest impulsiveness in the opioid- methamphetamine (29.0 vs. 32.0 and 37.5) group. There was no significant difference in the intensity of impulsiveness, craving, anxiety and depressive symptoms between *PDYN* 68 bp VNTR genotypes in individuals with OUD and opioid-methamphetamine co-users ($p > 0.05$). Although no statistically significant difference was found ($p = 0.113$), it was found that impulsiveness was lower in people with the SS genotype (29.0; 25.5–31.5) among opioid-methamphetamine co-users than in people carrying at least one L

Table 4

Comparison of genotypes the *PDYN* 68-bp VNTR of the groups included in the study in terms of impulsivity, craving, withdrawal, anxiety and depressive symptoms.

Scales	**OUD (n = 104)**					**Meth users (n = 160)**					**Opioid-Meth Co-users (n = 157)**				
	Co-dominant model					Co-dominant model					Co-dominant model				
	SS (n = 15)	SL (n = 50)	LL (n = 39)	SL+LL (n = 89)	SL+SS (n = 65)	SS (n = 17)	SL (n = 64)	LL (n = 79)	SL+LL (n = 143)	SL+SS (n = 81)	SS (n = 13)	SL (n = 68)	LL (n = 76)	SL+LL (n = 144)	SL+SS (n = 81)
Impulsiveness Median (IQR)	32.0 (30.0–37.0)	30.0 (25.0–35.5)	31.0 (26.0–38.0)	31.0 (25.0–36.75)	31.0 (25.25–36.00)	37.5 (32.0–41.75)	33.0 (26.00–38.0)	32.0 (27.5–38.5)	32.0 (27.0–38.0)	33.0 (28.0–39.0)	29.0 (25.5–31.5)	32.0 (27.0–37.0)	31.0 (25.0–39.0)	38.0 (26.0–38.0)	31.0 (27.0–36.5)
p value	χ2 = 1.378 p = 0.502			U= 523.5 p = 0.597 Z = −0.528	U= 18789.5 p = 0.868 Z = −0.166	χ2 = 4.429 p = 0.109			U= 724.5 **p = 0.040** Z = −2.049	U= 2671.5 p = 0.800 Z = −0.253	χ2 = 2.842 p = 0.242			U= 643.5 p = 0.113 Z = −1.587	U= 2715.0 p = 0.943 Z = −0.071
SCS Median (IQR)	18.0 (13.0–22.0)	18.5 (12.75–29.25)	18.0 (14.0–25.0)	18.0 (14.0–29.0)	18.00 (13.00–29.00)	20.00 (8.00–27.00)	16.50 (8.50–24.00)	18.00 (11.00–25.00)	18.00 (10.00–25.00)	18.00 (9.00–25.50)	21.00 (14.00–26.00)	16.00 (12.00–26.00)	21.00 (13.00–29.50)	18.00 (13.00–28.00)	17.00 (13.00–26.00)
p value	χ2 = 0.279 p = 0.870			U= 548.5 p = 0.597 Z = −0.528	U= 15400.5 p = 0.339 Z = −0.957	χ2 = 0.290 p = 0.865			U= 548.5 p = 0.597 Z = −0.528	U= 2160.5 p = 0.953 Z = −0.059	χ2 = 3.391 p = 0.184			U= 685.5 p = 0.652 Z = −0.451	U= 1956.5 p = 0.121 Z = −1.552
COWS Median (IQR)	3.00 (1.00–11.00)	1.00 (0.00–4.00)	3.00 (1.00–7.00)	2.00 (0.00–4.00)	2.00 (0.00–4.00)	3.50 (1.25–6.00)	4.00 (2.00–9.00)	3.00 (1.00–7.00)	3.00 (1.00–7.00)	4.00 (2.00–7.00)	2.00 (1.00–3.00)	3.00 (1.00–7.00)	4.00 (2.00–6.00)	3.00 (1.00–7.00)	3.00 (1.00–7.00)
p value	χ2 = 5.121 p = 0.077			U= 480.0 p = 0.138 Z = −1.483	U= 18825.5 p = 0.622 Z = −0.493	χ2 = 1.480 p = 0.477			U= 1028,5 p = 0.942 Z = −0.073	U= 2382.5 p = 0.266 Z = −1.112	χ2 = 2.738 p = 0.254			U= 645.0 p = 0.106 Z = −1.617	U= 2557.0 p = 0.418 Z = −0.811
BDI-II Median (IQR)	29.00 (20.00–36.00)	29.00 (18.00–36.25)	31.00 (22.5–43.00)	29.00 (19.00–38.00)	29.00 (18.5–36.00)	24.50 (10.00–41.00)	31.00 (18.00–43.00)	29.50 (19.0–38.75)	30.00 (19.00–42.00)	29.00 (17.5–42.50)	29.00 (14.00–40.50)	28.00 (22.25–37.00)	27.00 (17.25–35.75)	28.00 (19.25–36.75)	28.00 (20.5–37.50)
p value	χ2 = 1.061 p = 0.588			U= 612.5 p = 0.921 Z = −0.099	U= 29003.5 p = 0.644 Z = −0.462	χ2 = 1.200 p = 0.549			U= 783.5 p = 0.371 Z = −0.894	U= 2550.0 p = 0.758 Z = −0.309	χ2 = 1.073 p = 0.585			U= 825.0 p = 0.819 Z = −0.228	U= 2391.5 p = 0.369 Z = −0.898
BAI Median (IQR)	21.00 (13.00–31.00)	14.00 (6.00–30.00)	21.00 (12.00–32.50)	19.00 (10.00–30.75)	16.00 (8.00–30.00)	18.00 (5.75–42.25)	19.50 (10.00–38.00)	22.00 (7.50–33.00)	21.00 (10.00–33.50)	19.50 (10.00–38.00)	14.00 (9.50–19.00)	20.00 (12.25–29.25)	16.00 (7.25–32.50)	18.00 (9.00–30.00)	18.00 (10.00–29.00)
p value	χ2 = 3.691 p = 0.158			U= 551.5 p = 0.443 Z = −0.767	U= 29827.0 p = 0.740 Z = −0.332	χ2 = 0.177 p = 0.915			U= 1020,5 p = 0.790 Z = −0.267	U= 2717.5 p = 0.830 Z = −0.215	χ2 = 2.260 p = 0.323			U= 700.5 p = 0.275 Z = −1.091	U= 2459.5 p = 0.512 Z = −0.655

n: sample size, IQR: Interquartile range, OUD: individuals with opioid use disorder diagnosis, Meth: Methamphetamine, SCS: Substance Craving Scale, COWS: Clinical Opioid Withdrawal Scale, BDI-II: Beck Depression Inventory-II, BAI: Beck Anxiety Inventory.

allele (SL+LL) (38.0; 26.0–38.0).

3.6. Impulsiveness, craving and the intensity of anxiety and depressive symptoms, across BDNF rs6265 genotypes

As can be seen in Supplementary Table 3, there was no significant difference in the scale scores between the *BDNF* rs6265 genotypes in any of the groups ($p > 0.05$). When opioid, methamphetamine and opioid-methamphetamine users with the *BDNF* rs6265 GA+AA genotype were compared, it was found that craving was significantly lower in methamphetamine users with the GA+AA genotype (15.0; 9.5–25.5) compared with opioid- methamphetamine co-users with the GA+AA genotype (23.0; 13.5–30.0) ($p = 0.034$).

3.7. Impulsiveness, craving and the intensity of anxiety and depressive symptoms, across OPRD1 rs569356 genotypes

The effect of the *OPRD1* rs569356 polymorphism on impulsiveness, craving, opioid withdrawal, anxiety and depressive symptoms was shown in Supplementary Table 4. Individuals with OUD with the AA genotype of the *OPRD1* rs569356 had significantly lower SCS score compared to those with the AG+GG genotype (18.0 versus 30.0) ($p = 0.037$). In this study, individuals with OUD, methamphetamine users and opioid-methamphetamine co-users with the AG+GG genotype of the *OPRD1* rs569356 were also compared in terms of impulsiveness, craving and the intensity of anxiety and depressive symptoms. There was a statistically significant difference between the three groups, with the highest score on the craving scale (30.0 vs. 20.0 and 23.0) being found in individuals with OUD ($p = 0.038$). When comparing the subgroups, a significant difference was found between individuals with OUD and methamphetamine users ($p = 0.014$) and between individuals with OUD and opioid-methamphetamine co-users ($p = 0.05$) in terms of craving. There was also a trend towards a significant relationship between anxiety and AG+GG genotype of the *OPRD1* rs569356 between the individuals with OUD and the opioid-methamphetamine co-users ($p = 0.067$).

3.8. Impulsiveness, craving and the intensity of anxiety and depressive symptoms, across OPRM1 rs2075572 genotypes

In Supplementary Table 5, the genotypes of the *OPRM1* rs2075572 are compared in terms of their emotional status. Individuals with OUD with the CC genotype of the *OPRM1* rs2075572 had significantly higher COWS score compared to those with the GG+GC genotype (3.0 versus 1.5) ($p = 0.018$). No significant differences were observed between the *OPRM1* s2075572 genotypes in the OUD group with regard to impulsivity, craving, anxiety and symptoms of depression ($p > 0.05$). There was no significant differences between the genotypes of the *OPRM1* rs2075572 in view of impulsiveness, craving, opioid withdrawal, anxiety and depressive symptoms in the methamphetamine group and in the opioid-methamphetamine co-users ($p > 0.05$).

3.9. Age onset of first opioid/methamphetamine use, duration of opioid/methamphetamine use and quantity of opioid/methamphetamine consumed, across PDYN 68-bp VNTR genotypes

The effects of *BDNF* rs6265, *PDYN* 68 bp VNTR, *OPRD1* rs569356 and *OPRM1* rs2075572 genotypes on the age onset of first substance use and the amount of substance used were also evaluated in this study. The effect of *PDYN* 68-bp VNTR genotypes is shown in Table 5. The quantity of methamphetamine consumed was higher among individuals with the SS genotype (3.25 g/day and 5.0 g/day, respectively) of the *PDYN* 68-bp VNTR compared with those with the SL+LL genotype (2.0 g/day in each group). This difference tends to be significant ($p = 0.07$) in methamphetamine group, but it is statistically significant in the opioid-methamphetamine co-users ($p = 0.037$). With regard to the age onset of first substance use, there was no significant difference between the *PDYN* 68-bp VNTR genotypes ($p > 0.05$).

3.10. Age onset of first opioid/methamphetamine use, duration of opioid/methamphetamine use and quantity of opioid/methamphetamine consumed, across BDNF rs6265 genotypes

Supplementary Table 6 shows the effect of *BDNF* re6265 genotypes on the age onset of first substance use and the amount of daily substance used. As seen in the table, the duration of opioid use in individuals with OUD with the *BDNF* rs6265 GG genotype (8.0 years; 6.0–10.0 years) was statistically significantly higher compared to GA+AA (6.0 years; 3.25–11.0 years) ($p = 0.046$). Furthermore, in the opioid-methamphetamine co-users, the duration of methamphetamine use (years) was higher in individuals with the GG genotype (2.0 years; 1.0–7.25 years) of the *BDNF* rs6265 compared with GA+AA (1.5 years; 0.5–3.0 years). This difference was statistically significant ($p = 0.02$). In terms of the age onset of first substance use and the amount of daily substance used, there was no significant difference between *BDNF* rs6265 genotypes ($p > 0.05$).

3.11. Age onset of first opioid/methamphetamine use, duration of opioid/methamphetamine use and quantity of opioid/methamphetamine consumed, across OPRD1 rs569356 and OPRM1 rs2075572 genotypes

There was no significant difference between the *OPRD1* rs569356 and *OPRM1* rs2075572 genotypes regarding the age onset of first substance use and the amount of daily substance used ($p > 0.05$) (Supplementary Tables 7 and 8).

4. Discussion

Genetic variations in opioid-related genes, such as *OPRM1*, *OPRD1,* and *PDYN*, have been demonstrated to be associated with an elevated risk of developing opioid and alcohol use disorders (Votinov and Goerlich, 2020). However, this study investigated for the first time the impact of the *PDYN* 68-bp VNTR, *BDNF* rs6265, *OPRD1* rs569356, and *OPRM1* rs2075572 polymorphisms in individuals who are engaging in the concurrent use of opioid and methamphetamine due to significant increase in the combined use of heroin and methamphetamine in recent years.

One of the most extensively studied polymorphisms in the *PDYN* gene, associated with substance use disorders, is the 68-base pair VNTR polymorphism (rs35286281) located at 1250 base pairs upstream of exon 1 (Butelman et al., 2012; Horikawa et al., 1983). It is evident that the variations of the *PDYN* gene are linked to emotional responses and, as such, play a significant role in the research conducted within the domains of mood disorders and epilepsy (Chen et al., 2002). Previous studies have widely examined the associations between single substance use (e.g., opioids or methamphetamine) and genetic polymorphisms such as the *PDYN* 68-bp VNTR and they have produced mixed results across substances like cocaine, methamphetamine, and opioid. The extant literature encompasses an analysis of a 68-base pair repeat polymorphism in the promoter region of the *PDYN* gene among methamphetamine-dependent Japanese men and women. They established that the 3- or 4-repeat (L) alleles were significantly more prevalent in individuals dependent on methamphetamine than in the control group (Nomura et al., 2006). In contrast, an earlier study on European-, African-, and Hispanic-American cocaine users reported that the 1- or 2-repeat (S) alleles were more prevalent in cases than in controls (Bousman et al., 2009). A larger study focusing on a uniform ethnic group revealed that the H allele (three or four copies) of the *PDYN* 68-bp VNTR was significantly more prevalent among individuals with heroin dependence compared to controls (Wei et al., 2011). However, to our knowledge, there are no studies specifically examining the association of *PDYN* 68-bp VNTR polymorphism with the co-use of opioids and

Table 5

Comparison of genotypes of the *PDYN* 68-bp VNTR in all groups in view of the age onset of first substance use, duration of heroin/meth use and quantity of heroin/meth consumed.

	OUD (n = 104)					**Meth users (n = 160)**					**Opioid-Meth Co-users (n = 157)**				
	Co-dominant model					Co-dominant model					Co-dominant model				
	SS (n = 15)	SL (n = 50)	LL (n = 39)	SL+LL (n = 89)	SL+SS (n = 65)	SS (n = 17)	SL (n = 64)	LL (n = 79)	SL+LL (n = 143)	SL+SS (n = 81)	SS (n = 13)	SL (n = 68)	LL (n = 76)	SL+LL (n = 144)	SL+SS (n = 81)
The onset age of first substance use (years) Median (IQR)	17.0 (16.0–21.0)	19.0 (16.5–24.0)	18.0 (16.0–23.5)	19.0 (16.0–24.0)	18.5 (16.0–23.5)	18.5 (16.0–28.25)	20.5 (16.0–28.0)	18.0 (15.0–24.0)	19.0 (15.0–26.0)	20.0 (16.0–28.0)	18.0 (15.5–23.0)	17.0 (15.0–22.0)	17.5 (16.0–20.0)	17.0 (15.0–21.0)	17.5 (15.0–22.0)
p value	χ2 = 1663 p = 0.435			U= 472.0 p = 0.212 Z = −1.249	U= 1006.5 p = 0.915 Z = −0.107	χ2 = 2.092 p = 0.234			U= 1062.0 p = 0.951 Z = −0.061	U= 2392.5 p = 0.114 Z = −1.580	χ2 = 0.448 p = 0.799			U= 798.0 p = 0.535 Z = −1.580	U= 2792.5 p = 0.953 Z = −0.058
Duration of heroin use (years) Median (IQR)	7.0 (4.75–10)	8.0 (6.0–10.0)	8.0 (7.0–12.0)	8.0 (7.0–12.0)	8.0 (5.0–10.0)	-	-	-	-	-	12.0 (9.5–13.0)	9.0 (3.0–15.0)	9.5 (5.0–12.0)	9.0 (4.5–13.0)	10.0 (4.13–13.75)
p value	χ2 = 3.102 p = 0.212			U= 462.0 p = 0.22	U= 886.5 p = 0.113	-			-	-	χ2 = 1.756 p = 0.416			U= 689.0 p = 0.200 Z = −1.282	U= 2552.0 p = 0.479 Z = −0.708
Duration of Meth use (years) Median (IQR)	-	-	-	-	-	3.25 (1.12–9.25)	2.0 (1.0–3.0)	2.0 (1.0–4.0)	2.0 (1.0–4.0)	2.0 (1.0–4.0)	5.0 (1.25–12.0)	2.0 (1.0–3.0)	2.0 (1.0–4.0)	2.0 (1.0–4.0)	2.0 (1.0–5.0)
p value	-			-	-	χ2 = 3.438 p = 0.179			U= 765.0 **p = 0.07** Z = −1.814	U= 2481.5 p = 0.321 Z = −0.993	χ2 = 4.729 p = 0.0094			U= 466.5 **p = 0.037** Z = −2.081	U= 2233.0 p = 0.959 Z = −0.051
Quantity of heroin consumed (g/day) Median (IQR)	3.0 (2.0–5.0)	2.0 (1.0–4.0)	2.0 (1.5–4.88)	2.0 (1.0–4.5)	2.0 (1.38–4.63)	-	-	-	-	-	2.0 (1.0–4.0)	2.5 (1.0–5.0)	2.0 (1.0–4.0)	2.5 (1.0–5.0)	2.0 (1.0–5.0)
p value	χ2 = 1.543 p = 0.462			U= 515.0 p = 0.285 Z = −1.068	U= 1006.5 p = 0.107 Z = −0.915	-			-	-	χ2 = 2.658 p = 0.265			U= 694.5 p = 0.199 Z = −1.285	U= 2421.5 p = 0.180 Z = −1.342
Quantity of Meth consumed (g/day) Median (IQR)	-	-	-	-	-	1.0 (1.0–2.0)	1.0 (0.5–2.0)	1.5 (1.0–2.5)	1.0 (1.0–2.0)	1.0 (0.9–2.0)	1.0 (0.5–1.0)	1.0 (0.5–1.0)	0.8 (0.5–1.0)	1.0 (0.5–1.0)	1.0 (0.5–1.0)
p value	-			-	-	χ2 = 3.919 p = 0.141			U= 967.0 p = 0.805 Z = −0.246	U= 2322.0 p = 0.077 Z = −1.767	χ2 = 0.952 p = 0.621			U= 778.5 p = 0.601 Z = −0.523	U= 2440.5 p = 0.535 Z = −0.620

n: sample size, IQR: Interquartile range, OUD: individuals with opioid use disorder diagnosis, Meth: Methamphetamine.

methamphetamine. This represents a significant gap in the literature, especially given the high prevalence and clinical severity of polysubstance use. In our study, no statistically significant association was found between *PDYN* 68-bp VNTR genotype distribution and opioid-methamphetamine co-use. Interestingly, the frequency of the S allele in the co-use group (30 %) was lower compared to opioid-only users (38 %), but almost similar to the methamphetamine-only group (31 %). Compared to healthy controls (32 %), however, the co-use group—showed a lower frequency of the S allele. These findings partially align with prior research linking the S allele to increased vulnerability to substance use, particularly opioid dependence (Yuferov et al., 2019). However, the absence of a distinct genetic pattern in the co-use group suggests that *PDYN* 68-bp VNTR may not specifically contribute to the risk of combining opioids with stimulants, but rather to a broader predisposition to substance use in general. In addition, the methamphetamine and co-use groups showed lower frequencies of the S allele (31 % and 30 %, respectively), closer to that of the control group, which may reflect different neurobiological pathways or risk mechanisms underlying stimulant versus opioid use.Our findings contribute to the limited literature on the genetic underpinnings of polysubstance use and highlight the need for further studies investigating gene–environment interactions and their role in complex patterns of drug use.

When the number of *PDYN* repetitions was compared between the 4 groups, a significant discrepancy was observed between the substance-using groups and the control group in terms of the number of repetitions in our study ($p = 0.001$). This finding aligns with prior research suggesting that variations in the *PDYN* gene may play a role in the susceptibility to SUDs by modulating the expression of prodynorphin, a precursor to dynorphins that interact with kappa-opioid receptors involved in stress regulation and reward processing. Additionally, a significant difference in allele frequency between opioid and methamphetamine users ($p = 0.018$) suggests that different substances may influence or be influenced by distinct genetic vulnerability profiles. Moreover, it should be noted that this is the first time that the 5-repeat allele has been observed in the Turkish population, both in users of opioids and in users of methamphetamine. In this study, the first data on the opioid and methamphetamine co-users have also been obtained. Although rare, the 5-repeat allele may represent a novel or underexplored variant that warrants further investigation in larger cohorts, particularly given its absence in the control group. These findings will contribute to the functional analyses of less common variants like the 5-repeat allele.

A significant difference was found between methamphetamine users and opioid-methamphetamine co-users in the age of onset of substance use ($p = 0.019$). It was observed that the age of onset of substance use was 18 years (IQR: 16.0–23.25) for individuals with OUD, 19 years (IQR: 16.0–26.0) for methamphetamine users and 17 years (IQR: 15.0–21.0) for opioid-methamphetamine co-users. These findings suggest that individuals who initiate opioid use at a younger age may be at increased risk of progressing to methamphetamine use, highlighting the potential for a sequential pattern in polysubstance involvement. This trend is consistent with previous research showing that early exposure to one substance may lower the threshold for experimentation with others, possibly due to neurodevelopmental vulnerability, altered reward sensitivity, or increased exposure to high-risk social environments (Jordan and Andersen, 2017). Although a statistically significant difference was observed between the groups ($p = 0.04$), the variability in onset age, as indicated by the broad range, suggests that this difference may not be clinically meaningful. But future studies with longitudinal designs may help clarify whether this difference holds meaningful implications for treatment or prognosis. Such studies could also explore whether targeted interventions for younger users may reduce the likelihood of transitioning from single to multiple substance use.

In the present study, the impulsivity scale score was significantly higher in methamphetamine users with the SS genotype (median: 37.5) of the *PDYN* 68-bp VNTR than in those with the SL+LL genotype (median:32.0; $p = 0.04$). It is known that dysfunction of dopamine neurotransmission due to any reason such as addiction has been implicated in the mediation of impulsivity (Pine et al., 2010). One of the main effects of methamphetamine is known to be an increase in the concentration of dopamine in synaptic clefts (Ujike, 2002). Increased dopamine release in the mesolimbic region causes stimulation of D1 dopamine receptors, leading to increased *PDYN* gene expression and release of dynorphin peptides (Cole et al., 1995; Gerfen et al., 1990; Steiner and Gerfen, 1996). With repeated methamphetamine use, dynorphin levels are significantly increased in the dorsal and ventral striatum (Hanson et al., 1988; Li et al., 1988; Steiner and Gerfen, 1993). Dynorphin peptides stimulate the kappa-opioid receptors responsible for dysphoria, and this stimulation reduces dopamine release (Bals-Kubik et al., 1993; Pfeiffer et al., 1986; Shippenberg and Rea, 1997). Chronic use of methamphetamine increases expression of the *PDYN* gene, which is responsible for dynorphin peptides; dynorphin peptides bind to kappa opioid receptors and this interaction causes dysphoria by inhibiting dopamine release. The *PDYN* 68-bp VNTR polymorphism may influence this feedback loop. The expression level of *PDYN* may vary depending on the number of repeats of the 68-bp VNTR polymorphism (Rouault et al., 2011; Zimprich et al., 2000). *PDYN* promoter activity was higher in alleles with 3 or 4 repeats of the 68-bp VNTR polymorphism than in alleles with 1 or 2 repeats (Zimprich et al., 2000). Therefore, the increase in dopamine caused by decreased *PDYN* expression in individuals with the SS genotype of the *PDYN* 68-bp VNTR may have led to an increase in the BIS-11 score determining impulsivity. In contrast, the opioid-methamphetamine co-user group exhibited an opposite pattern. This unexpected finding may be attributable to the modulatory effects of opioid use on dopamine pathways. Opioids have a strong positive reinforcement property because they activate the downregulation of dopamine receptors, a key element of the brain's reward circuitry (Sharma et al., 2016). This enhanced dopaminergic tone may counterbalance the lower PDYN expression seen in SS carriers, leading to a blunted impulsivity phenotype. A notable finding of the study was that the duration of methamphetamine use in opioid-methamphetamine co-users was statistically significantly higher in individuals with SS genotype (5.0 years) of the *PDYN* 68-bp VNTR compared to individuals with SL+LL genotype (2.0 years), suggesting that although impulsivity may be lower, other mechanisms (e.g., neuroadaptations or reinforcement sensitivity) may sustain long-term use in this group. Taken together, these findings suggest a complex interaction between *PDYN* genotype, dopaminergic signaling, and polysubstance use patterns. This is the first study, to our knowledge, to report genotype-related impulsivity differences in opioid-methamphetamine co-users, underscoring the need for further investigation of genetic modulation in polysubstance dependence.

The *OPRD1* gene, located on chromosome 1 (1p36.1-p34.3), is the first human opioid receptor gene to be cloned (Uhl et al., 1994). Among its variants, *OPRD1* rs569356, positioned 1968 bp upstream of the transcription start site, was the only variant identified in the promoter region of *OPRD1*. Zhang et al. (2008) found that the minor G allele of the *OPRD1* rs569356 has higher levels of luciferase expression compared to the major A allele. Individuals with OUD with at least one G allele (AG+GG) had significantly higher levels of craving (30.0) compared with those with an A allele (18.0) ($p = 0.037$), consistent with our previous findings (Kaya-Akyüzlü et al., 2023). Although there was no statistically significant difference in the methamphetamine users and opioid-methamphetamine co-user groups ($p > 0.05$), a similar trend was observed—individuals carrying the G allele tended to report higher craving levels than those with the A allele. These findings suggest that the G allele may enhance delta-opioid receptor (DOR) expression, potentially modulating reward sensitivity and increasing substance craving. It is plausible that elevated DOR availability may amplify dopaminergic tone or reduce the threshold for achieving euphoric effects, thereby driving increased substance use behavior to attain the desired reward state. Further functional studies are warranted to elucidate the precise mechanisms underlying this genotype-behavior relationship.

Over the past few years, many pharmacogenetic studies have examined the impact of genetic polymorphisms on opioid use, treatment response, and side effect profiles. Genetic variations in *OPRM1*, which encodes the μ-opioid receptor (a key pharmacodynamic target for heroin and prescription opioids), have also been demonstrated to influence addiction susceptibility and the efficacy of pharmacological treatments. For example, *OPRM1* rs2075572, located in intron 2, has been investigated in relation to postoperative opioid consumption, pain perception, and adverse drug reactions (ADRs) (Xie et al., 2020). However, in the present study, no statistically significant association was found between *OPRM1* rs2075572 and OUD, methamphetamine use, or predisposition to opioid-methamphetamine co-use ($p > 0.05$, logistic regression). On the other hand, the *OPRM1* rs2075572 CC subjects exhibited a significantly elevated opioid withdrawal (median: 3.0) in comparison to the GG+GC subjects (median: 1.5), according to the Mann-Whitney *U* test ($p = 0.018$). The *OPRM1* rs2075572 locus is situated within intron 2 and exhibits high linkage disequilibrium with the rs9479757 locus (Huang et al., 2008; Smith et al., 2005). Xu et al. (2014) posited that *OPRM1* rs9479757 is responsible for alterations in the expression of hMOR-1 proteins by modulating exon 2 splicing. It can be hypothesised that the reduced binding of heroin to MORs in OUD patients with minor C allele as compared to major G allele, resulting from decreased mu-opioid receptor expression, may lead to increased withdrawal. The present results are in line with previous research, including our earlier findings (Kaya-Akyüzlü et al., 2024) and those of Wang et al. (2012), reinforcing the potential role of *OPRM1* rs2075572 as a genetic marker associated with opioid withdrawal severity, even in the absence of direct effects on OUD diagnosis or co-use behavior.

The present study found that the duration of opioid use was significantly higher in individuals with the *BDNF* GG genotype (median: 8.0 years) compared to individuals with the GA+AA genotype (median: 6.0 years), in a sample of individuals with OUD ($p = 0.046$). A similar increase in the duration of methamphetamine use was found in opioid-methamphetamine co-users, with individuals having the GG genotype (median: 2.0 years) using methamphetamine for a longer duration than those having the GA+AA genotype (1.5 years) (0.02). The valine (196 G) allele has been demonstrated to be linked to a heightened susceptibility to the development of substance dependency, while the GG genotype has been shown to be associated with late-onset dependency (Cheng et al., 2005). The human *BDNF* gene comprises six $5'$ exons with distinct promoter regions, which are spliced into a single $3'$ terminal exon to form the mature mRNA. The *BDNF* Val66Met polymorphism (rs6265; G196A) is located on chromosome 11p13 and involves a substitution of valine (Val) with methionine (Met) in the prodomain. This affects intracellular trafficking and activity-dependent secretion of *BDNF* (Egan et al., 2003; Li et al., 2005). This distinctive configuration underscores the capacity for the regulation of *BDNF* expression through transcription (Russo-Neustadt, 2003). As demonstrated in animal models of dopamine neuron damage, *BDNF* in dopamine neurons enhances D3 receptor expression in the nucleus accumbens and contributes to behavioral sensitization (Kaymaz et al., 2013). Together, these findings suggest that the *BDNF* GG genotype may influence addiction trajectories by affecting neuroplasticity and dopaminergic regulation, potentially contributing to longer durations of opioid and methamphetamine use.

One limitation of this study is the relatively small sample size within certain genotype subgroups, which may have limited the statistical power to detect smaller effect sizes. As such, the results should be considered preliminary and warrant replication in larger samples.

In conclusion, this is the first genetic study of the concurrent use of opioid and methamphetamine in the current literature. Among the four polymorphisms examined, only the *PDYN* 68-bp VNTR polymorphism showed a significant deviation from the control group. A noteworthy difference in allele distribution was identified between the OUD and methamphetamine groups in view of the *PDYN* 68-bp VNTR repeats. Importantly, this study is the first to report the presence of 5-repeat alleles of the *PDYN* gene in the Turkish population.

Furthermore, although *BDNF* rs6265, *OPRD1* rs569356, and OPRM1 rs2075572 were not directly associated with substance use diagnosis, findings suggest that they may contribute to the risk of developing substance use disorders through their influence on impulsivity, craving, and withdrawal symptoms, respectively. These preliminary findings underscore the need for future research in larger, more diverse cohorts to validate and expand upon these observations.

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CRediT authorship contribution statement

Ak Merve: Writing – original draft, Resources, Methodology. **Kaya-Akyüzlü Dilek:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Danışman Mustafa:** Resources. **Özkan-Kotiloğlu Selin:** Methodology, Investigation, Data curation. **Yıldırım Mukaddes Asena:** Methodology. **Özer-Uzaldı Gizem:** Writing – original draft, Methodology.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dilek Kaya-Akyuzlu reports financial support was provided by Ankara University Scientific Research Projects Coordination Unit. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2025.104714.

Data Availability

Data will be made available on request.

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