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OPRD1 rs569356 polymorphism has an effect on plasma norbuprenorphine levels and dose/kg-normalized norbuprenorphine values in individuals with opioid use disorder

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ABSTRACT

This study aimed to determine the effects of nine *OPRM1*, *OPRD1* and *OPRK1* polymorphisms on plasma BUP and norbuprenorphine (norBUP) concentrations and various treatment responses in a sample of 122 patients receiving BUP/naloxone. Plasma concentrations of BUP and norBUP were detected by LC–MS/MS. PCR-RFLP method was used to genotype polymorphisms. *OPRD1* rs569356 GG had significantly lower plasma norBUP concentration ($p = 0.018$), dose- ($p = 0.049$) and dose/kg-normalized norBUP values ($p = 0.036$) compared with AA. Craving and withdrawal symptoms were significantly higher in *OPRD1* rs569356 AG+GG relative to AA. There was a statistically significant difference between the *OPRD1* rs678849 genotypes in the intensity of anxiety (13.5 for CT+TT and 7.5 for TT). *OPRM1* rs648893 TT (18.8 ± 10.8) was significantly different to CC+CT (14.82 ± 11.3; $p = 0.049$) in view of the intensity of depression. This current study provides the first data on a prominent effect of the *OPRD1* rs569356 variation on BUP pharmacology due to its metabolite norBUP.

1. Introduction

Buprenorphine (BUP) is co-formulated with naloxone to discourage parenteral administration and is administered sublingually when used as a maintenance medication for OUD (Brown et al., 2011; Fihlman et al., 2018; Kreek et al., 2019; Seguí et al., 2020). BUP is metabolized via cytochrome P450 (CYP) 3A4/5-mediated N-dealkylation and its major and active metabolite norbuprenorphine (norBUP) is produced. Both BUP and norBUP are excreted in urine after conjugating to their 3-glucuronides by the UDP-glucuronosyl transferases (UGT), mainly UGT2B7, UGT1A1 and UGT1A3 (Fihlman et al., 2018). The pharmacological properties of BUP are complex and unique due to being a partial mu-opioid receptor agonist (Martin et al., 1976), delta- and kappa-opioid receptor antagonist and nociceptin receptor agonist (Leander et al., 1987; Negus et al., 2002). It is believed that not only BUP but also its metabolite norBUP may influence the pharmacological profile of BUP due to their high affinity for mu-, delta- and kappa-opioid receptors. Huang et al. (2001) examined the similarities and differences between BUP and norBUP in terms of their efficacy at opioid receptors using Chinese hamster ovary cells and found that norBUP had moderate efficacy at mu- and kappa-opioid receptors, while BUP had low efficacy. At delta-opioid receptors, norBUP was a full agonist, but not BUP (Huang et al., 2001). Additionally, Negus et al. (2002) showed that BUP had low efficacy at delta-opioid receptors despite its high affinity for this receptor in rhesus monkey brain membranes.

BUP is an FDA-approved drug in the treatment of opioid use disorder (OUD) (FDA, 2002). Although the long-term treatment of OUD with opioid agonists and/or antagonists is effective (Panlilio et al., 2019; Taqi et al., 2019), the rate of treatment failure is high among opioid users (Crist et al., 2018; Randesi et al., 2020; Wang et al., 2019) due to a number of parameters such as environmental and physiological factors including age, gender, weight, severity of disease, concomitant disease, and pharmacogenetics (Clarke et al., 2014; Yiannakopoulou, 2015). Individual genetic differences in genes encoding metabolism (Ettienne et al., 2017; Ettienne et al., 2019; Kaya-Akyüzlü et al., 2022a) or cellular drug targets (Imai et al., 2020; Kranzler et al., 2021; Randesi et al.,

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2020) could significantly explain the variability in BUP response among individuals. Therefore, understanding the effect of genetic background on BUP pharmacology is essential to boost the effectiveness of treatment and reduce the high rates of drop-out (Crist et al., 2018; Randesi et al., 2020).

To date, little is known about pharmacodynamics variants that would affect the efficacy of the sublingual BUP/naloxone combination in individuals with OUD. A limited number of papers examined some polymorphisms on opioid receptor genes including *OPRM1 (*Crist et al., 2018; Imai et al., 2020; Randesi et al., 2020), *OPRD1* (Clarke et al., 2014; Crist et al., 2013; Crist et al., 2019; Kranzler et al., 2021), and *OPRK1* (Gerra et al., 2014; Randesi et al., 2020). Imai et al. (2020) suggested that *OPRM1* A118G polymorphism could affect the pharmacological action of mu-opioid receptor in a study with 10 healthy men received a single intravenous dose of buprenorphine hydrochloride. Crist et al. (2018) demonstrated that *OPRM1* rs10485058 AA genotype, a non-coding transcript variant, was associated with less opioid-positive urine drug screens relative to AG+GG genotypes in the methadone treatment group, but not in the buprenorphine group. Randesi et al. (2020) reported that no significant main effects of gene variants (*OPRM1* A118G, *PDYN* VNTR and rs910080, and *OPRK1* rs6473797) on receiving first dose or last dose in a study comparing daily sublingual BUP/naloxone and monthly extended-release naltrexone injection in non-Hispanic Caucasians. *OPRD1* rs678849 was found to be associated with buprenorphine efficacy in African-Americans (Crist et al., 2013 and 2019) and in European-Americans (Kranzler et al., 2021) treated for OUD. To date, only two studies examined the effect of *OPRK1* gene variants (rs1051660 or rs6473797) on the buprenorphine treatment outcome such as early drop out, continuous use of heroin, severe behavioral or psychiatric problems, misbehavior or receiving first dose or last dose and no significant effects of these *OPRK1* polymorphisms on buprenorphine pharmacological action were reported (Gerra et al., 2014; Randesi et al., 2020). Due to these inconsistent results, the effect of these 3 pharmacodynamic genes (*OPRM1*, *OPRD1* and *OPRK1*) on BUP treatment responses remains unclear. Therefore, except *OPRD1* rs678849 and *OPRK1* rs6473797, the contribution of 8 new variants that were associated with drug response previously in *OPRM1*, *OPRD1* and *OPRK1* genes on BUP pharmacology by measuring plasma BUP and norBUP levels were examined for the first time in the current study.

Additionally, researchers have been investigating whether chronic depression and anxiety as well as negative craving and opioid withdrawal are correlated with the treatment failures of individuals receiving maintenance treatment such as buprenorphine and methadone (Kaya-Akyüzlü et al., 2022b; Panlilio et al., 2019). Thus, in the present study, we also explored the effect of *OPRM1*, *OPRD1* and *OPRK1* polymorphisms on the intensity of depression, anxiety, craving and withdrawal symptoms, whereas the prior published studies did not.

2. Patients and methods

2.1. Study population and sample collection

Subjects (n = 122) in this study were individuals who fulfilled the criteria for opioid use disorder according to The Diagnostic and Statistical Manual of Mental Disorders (DSM–5) diagnostic criteria and who had been receiving suboxone (sublingual BUP/naloxone combination) treatment at Ankara Training and Research Hospital in Ankara. The sample was composed of both inpatients and outpatients admitted to the AMATEM clinic for the OUD treatment. Inclusion criteria were as follows: (i) at least 18 years of age, (ii) fulfilled the criteria for opioid use disorder according to The Diagnostic and Statistical Manual of Mental Disorders (DSM–5) diagnostic criteria, (iii) at least 10 days of suboxone treatment for the steady-state plasma BUP concentrations, (iv) no acute health problems impairing renal or hepatic function. Subjects (i) with active drug addiction during BUP management therapy, (ii) with substance use disorders other than heroin and nicotine dependence such as other opioids, alcohol and/or benzodiazepines, (iii) with clinically significant comorbid psychiatric illness (any psychotic disorders, schizophrenia, mental retardation, bipolar disorder and severe depression) were not eligible for inclusion in the cohort. Furthermore, subjects who have been receiving any drugs (for example rifampicin, phenytoin, saquinavir, cimetidine, fluoxetine, ketoconazole, atazanavir, ritonavir) that might interact with the metabolism of BUP were excluded from the study. These drugs are inhibitors or inducers of CYP3A4 which metabolizes BUP. When one of these drugs is given with BUP, the metabolism of BUP may be inhibited or increased.

The institutional ethics committee approved the study design (Approval No: I6–385–21 in 2021). Written informed consent was obtained from each participant who was eligible for the study. Samplings were performed in accordance with the principles of The Declaration of Helsinki. Additionally, A small questionnaire used to gather demographic information on age, marital, education and employment status, age at onset of dependence, quantity of heroin consumed (g/day), family history of drug abuse and times and doses of sublingual BUP/naloxone was given to all subjects.

Four mL venous blood sample from each individual with OUD was collected into tubes with ethylenediaminetetraacetic acid (EDTA) after a minimum 10 days of continuous sublingual BUP/naloxone treatment and before taking daily BUP dose to measure the plasma buprenorphine level. Two mL of venous blood was also taken from each patient another tube with EDTA for DNA isolation. All samples were stored at − 20 °C until analysis.

2.2. Determination of Plasma Buprenorphine and Norbuprenorphine Levels

Plasma buprenorphine and its metabolite norbuprenorphine levels were measured by Liquid Chromatography Tandem Mass Spectrometry (LC–MS/MS). A triple quadrupole SCIEX 5500 Qtrap (AB Sciex, Darmstadt, Germany) was used. Substances were separated by a Shimadzu Prominence system with a RRHD Eclipse Plus C18 (95 Å, 1,8 μm, 2,1 ×50 mm, Zorbax, USA) column and detected by mass spectrometer (AB Sciex). For liquid–liquid extraction, “Abuse Drugs in Blood by LC/MS” (Eureka Lab Division) was used as previously described (Ferrari et al., 2018). The retention times for BUP and norBUP 6.52 min and 4.53 min with the LOD and LOQ for BUP and norBUP were 0.02 ng/mL and 0.125 ng/mL, respectively. Precision of the method was evaluated with the intra- and inter-day variations which were < 10% for both compounds.

2.3. Determination of the OPRM1, OPRD1 and OPRK1 polymorphisms

Genomic DNA was extracted from whole-blood samples collected in EDTA tubes using the QIAamp DNA blood-kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. Genotyping of *OPRM1* (rs648893, rs540825, rs510769), *OPRD1* (rs678849 and rs569356) and *OPRK1* (rs997917, rs12548098, rs6473797 and rs963549) polymorphisms was carried out using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). For the indel in the promoter region in the *OPRK1* (rs35566036), only PCR assay was applied to the isolated DNA samples. All PCR amplifications were conducted on a Techne Tc 512 PCR System in a 25-μL reaction mixture containing 0.16 mM of dNTPs, 10 pmol each of the forward (F) and reverse (R) primers, 1,25 U of Taq DNA polymerase (Ampliqon, Denmark), 10 ×PCR buffer (Ampliqon, Denmark) and 50 ng of genomic DNA. Then, the PCR products were digested in a reaction containing 5 U of restriction enzyme. Primer sequences, PCR conditions, restriction enzymes, and restriction fragment lengths are given in Supplementary Table S1. The undigested and digested PCR products were separated by gel electrophoresis on a 3% agarose gel, visualized by ethidium bromide staining under an UV illuminator, and then scanned and photographed using the Syngene Monitoring System.

2.4. Measurements

To measure craving, opioid withdrawal, the intensity of depression and anxiety, and the severity of addiction, individuals with OUD were assessed by Substance Craving Scale (SCS), Clinical Opiate Withdrawal Scale (COWS), Beck Depression Inventory-II (BDI-II), Beck Anxiety Inventory (BAI) and Addiction Profile Index (API), respectively. The validities and reliabilities of a Turkish version of these scales were demonstrated by Evren et al. (2011); Canan et al. (2015); Hisli et al., 1989; Ulusoy et al. (1998); Ögel et al. (2012), respectively.

2.5. Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 21.0 software for Windows was used for the statistical analyses. The normality of numerical variables was examined by the Kolmogorov-Smirnov test. Data were presented as the mean and standard deviation (SD) or median and the interquartile range (IQR) according to the normality of the data. For categorical data, numbers, percentages and 95% confidence interval (CI) were given. The frequencies of the *OPRM1* (rs648893, rs540825, rs510769), *OPRD1* (rs678849 and rs569356) and *OPRK1* (rs35566036, rs997917, rs12548098, rs6473797 and rs963549) alleles and genotypes were obtained by direct count, and the departure from the Hardy-Weinberg equilibrium ($p2 + 2pq+q2 = 1$) was evaluated by the chi-square test. Plasma BUP and norBUP levels were given as ng/mL. BUP values were normalized by adjusting with patients' weight and daily dose. Dose-normalized BUP concentrations (BUP/D) and dose/kg-normalized BUP concentrations (BUP/D.kg^{-1}) were calculated using the following equations: BUP concentration (ng/mL)/BUP daily dose (mg/day) and BUP concentration (ng/mL)/BUP daily dose per body mass kilogram (mg/kg/day). The metabolite-to-parent drug ratios (individual metabolic ratios-MR) were calculated using the following equation: metabolite concentration (ng/mL)/parent drug concentration (ng/mL). The patients' BUP concentrations and dose- and dose/kg-normalized plasma BUP concentrations among different *OPRM1, OPRD1* and *OPRK1* functional genotypes were compared using Mann Whitney test or Kruskal-Wallis test, as appropriate. The relationships between scores of measures and BUP or norBUP concentrations and dose- and dose/kg-normalized BUP or norBUP values were analyzed by the Spearman correlation test. Bonferroni post-hoc test was also used to locate the origin for any significant difference in plasma norBUP levels as well as total scores of measurements between genotypes. Linkage disequilibrium (LD) and haplotype analysis were performed using SHEsis-Plus software to evaluate the combined effect of all polymorphisms (Li et al., 2009; Shi and He, 2005). Haplotypes with frequencies $< 3\%$ were omitted. Linkage disequilibrium was followed by the D′ statistic, and a D′ value of ≥ 0.8 indicated that related SNPs formed one block. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Characteristics and demographics of subjects

There was a total of 122 individuals with OUD receiving sublingual BUP/Naloxone treatment in the cohort, and of them, 114 were males (93.4%) and 8 were females (6.6%). The median age of all subjects was 29.0 years (Interquartile range (*IQR*): 26.0–34.0 years). All subjects were smokers. The characteristics of the subjects regarding weight, height, marital, education and employment status ($n = 122$) were presented on Table 1.

Daily doses of BUP ranged from 1.0 mg/day to 8.0 mg/day (median 6.01 mg/day; IQR: 4.0–8.0 mg/day). The median steady-state serum concentration of BUP was 0.061 ng/mL (*IQR*:0.04–0.11 ng/mL). After normalized by dose and by dose/body weight, they were changed to 0.011 ng/mL per mg/day and to 0.74 ng/mL per mg/kg/day, respectively. The median plasma norBUP level was 1.54 ng/mL (*IQR*:0.93–2.82 ng/mL). Plasma norBUP level was also normalized by dose and dose/body weight. Dose-normalized norBUP and dose/body weight normalized norBUP values were 0.31 ng/mL per mg/day (*IQR*:0.14–0.57) and 20.29 ng/mL per mg/kg/day (*IQR*: 10.01–39.85), respectively. Additionally, the median norBUP/BUP value representing the metabolic activity of BUP to norBUP conversion was 23.65 (*IQR*: 13.4–42.5).

Table 1
The characteristics of the individuals with OUD.

Variable	Median	Mean	IQR	S.D.
Age (years)	29.00	-	26.00–34.00	-
Weight (kg)	70.5	-	63.00–75.00	-
Height (cm)	-	174.30	-	7.29
Variable	**n (% frequency) (95% CI)**			
Education	Primary	Secondary	High School	Under-graduate
	24 (19.6%) (12.6–26.6)	55 (45.1%) (36.3–53.9)	35 (28.7%) (0.21–0.37)	8 (6.6%) (2.2–11,0)
Occupation	Not working		Working	
	57 (46.7%) (37.8–55.6)		65 (53.3%) (44.4–62.2)	
Marital status	Married		Single Widow/divorced	
	37 (60.3%) (22.1–38.5)		79 (64.8%) 6 (4.9%) (56.3–73.2) (1.1–8.7)	
Gender	Female		Male	
	8 (6.6%) (57.6–74.4)		114 (93.4%) (0.89–97.8)	
Prison	Yes		No	
	74 (60.7%) (52.0–69.4)		48 (39.3%) (30.6–50.0)	
Disciplinary action at school	Yes		No	
	23 (18.9%) (11.9–25.8)		99 (81.1%) (75.9–86.3)	

IQR: Inter Quartile Range, S.D.: standard deviation, n: sample size, CI: Confidence interval

The median SCS score was 8 (*IQR*: 4–12) with a minimum of zero and a maximum of 27. The median withdrawal instance was 4 (*IQR*: 2–6), with a minimum of zero and a maximum of 13 instances. The median BAI and API scores were 13 (*IQR*: 6.0–20.25) and 11.18 (*IQR*:9.05–13.12), respectively. The mean BDI-II score was 17.01 ± 11.17, with a minimum of zero and a maximum of 45. In addition, a significant and positive correlation was found between depression and anxiety (r^2=+0.468, $p = 0.001$), between depression and craving (r^2=+0.223, $p = 0.014$), between opioid withdrawal and depression (r^2=+0.186, $p = 0.04$), withdrawal and anxiety (r^2=+0.381, $p = 0.001$) and withdrawal and craving (r^2=+0.372, $p = 0.001$). Moreover, there were significant and positive correlations between the severity of addiction and addiction (r^2=+0.248, $p = 0.006$) and craving (r^2=+0.330, $p = 0.001$).

3.2. Genotype distribution of the OPRM1, OPRD1 and OPRK1 polymorphisms

OPRM1, *OPRD1* and *OPRK1* SNP's information, major and minor allele frequencies and Hardy-Weinberg equilibrium (HWE) of these polymorphisms among individuals with OUD were presented in Table 2. Except *OPRD1* rs569356 and *OPRK1* rs35566036, the genotype frequencies of the polymorphisms genotyped in this study in all samples were consistent with Hardy–Weinberg equilibrium ($p > 0.05$).

For each polymorphism, homozygote wild type, heterozygote and homozygote variant type were compared in view of plasma BUP and norBUP concentrations, BUP/D, BUP/D.kg^{-1}, norBUP/D, norBUP/D.kg^{-1} values, MR, total scores of measures (craving, depression, anxiety, withdrawal, and the severity of addiction) and characteristics of heroin use such as duration of heroin use (years), age at onset of dependence (years), quantity of heroin consumed (g/day).

Table 2
SNPs information, major allele, and the frequencies of minor alleles among individuals with opioid use disorder.

Gene	SNP	SNP position	OUD (*n*=122)			
			Major allele	Minor allele	Minor allele frequency	HWE *p*-value
OPRM1	rs648893	6:154117494	A	G	0.72	0.11
	rs540825	6:154093311	A	T	0.71	0.07
	rs510769	6:154040884	C	T	0.25	0.50
OPRD1	rs569356	1:28810174	A	G	0.13	0.001
	rs678849	1:28818676	C	T	0.43	0.27
OPRK1	rs35566036	INDEL	L	S	0.59	0.03
	rs997917	8:53239818	T	C	0.45	0.31
	rs6473797	8:53240422	T	C	0.40	0.30
	rs12548098	8:53242895	T	C	0.23	0.15
	rs963549	8:53229264	C	T	0.19	0.14

OUD: Opioid use disorder, HWE: Hardy-Weinberg Equilibrium

3.3. BUP and norBUP plasma concentrations and dose- and dose/weight-normalized BUP and norBUP values, across OPRD1 rs569356 genotype subgroups

There was a statistically significant difference between *OPRD1* rs569356 genotype subgroups with regard to median plasma norBUP concentration, dose normalized norBUP (norBUP/D) and dose/kg-normalized norBUP (norBUP/D.kg^{-1}) values as determined by Kruskal-Wallis test (Fig. 1). As it was seen in Table 3, Bonferroni's Post hoc test showed differences in plasma norBUP concentration, values of norBUP/D and norBUP/D.kg^{-1} comparing subjects with typical genotypes— AA:AG, AG:GG, and AA:GG. Statistically significant differences were found in plasma norBUP levels (GG:AA, p = 0.027; GG:AG, p = 0.017), in norBUP/D value (GG:AG, p = 0.05), and in norBUP/D.kg^{-1} value (GG:AA, p = 0.04; GG:AG, p = 0.043). On the other hand, the median plasma concentration of BUP, BUP/D and BUP/D.kg^{-1} values and norBUP/BUP ratio did not differ significantly among three *OPRD1* rs569356 genotype subgroups; although the median values of BUP, BUP/D and BUP/D.kg^{-1} was lower in GG genotype subgroup compared with the AA and AG subgroups (Table 3). In addition to that, the median norBUP/BUP value representing the metabolic activity of BUP to norBUP conversion was highest in AG genotype subgroup compared to AA and GG subgroups. *OPRM1* and *OPRK1* polymorphisms genotyped in the current study had no statistically significant effects on plasma BUP and norBUP levels and dose- and dose/kg-normalized BUP and norBUP values according to co-dominant (Supplementary Tables S2 and S3), wild-type recessive and wild type-dominant models (data not shown) (p > 0.05).

3.4. The total scores of SCS, COWS, BDI-II, BAI and API, across OPRM1 rs648893, OPRD1 rs569356 and rs678849 genotype subgroups

The intensities of craving and withdrawal, depression and anxiety as well as the severity of addiction were also compared according to the *OPRD1* rs569356 genotype subgroups and were presented in Table 4. Kruskal-Wallis test revealed that there was statistically significant difference between *OPRD1* rs569356 genotype subgroups in view of the intensity of craving according to the co-dominant model (p = 0.048) (Fig. 2). Bonferroni's Post hoc test showed a significant difference in the total score of SCS comparing subjects with AA:GG genotypes (p = 0.05). Additionally, according to the A-recessive model (AG+GG vs. AA), the median SCS and COWS were statistically higher in *OPRD1* rs569356 AG+GG (9, IQR: 3.5–20.5; 5, IQR:2.5–8.5, respectively) as compared to AA subgroup (7, IQR:4.0–10.5; 4, IQR:2.0–5.0, respectively) (Fig. 2).

Mann-Whitney U test showed that there was a statistically significant difference between the *OPRD1* rs678849 genotype subgroups in median BAI score, with a median BAI score of 13.5 (*IQR*:7.0–22.25) for CT+TT genotype and 7.5 (*IQR*:4.5–14.25) for TT genotype (Table 4). No significant associations were found between other scales such as SCS, COWS, BDI-II and API and *OPRD1* rs678849 genotype subgroups

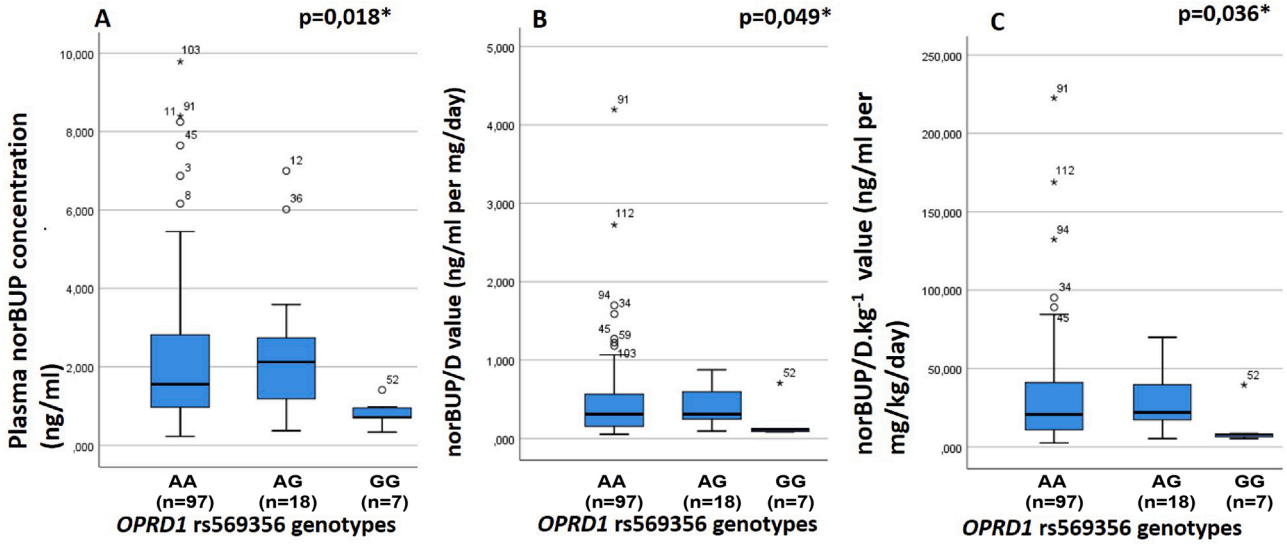

Fig. 1. *OPRD1* rs569356 genotypes and plasma norBUP concentrations and, dose-normalized and dose/kg-normalized norBUP values. (**A**) Distribution of median plasma norBUP concentration, **(B)** dose normalized norBUP and (**C**) dose/kg- normalized BUP values in patients with TT, TC and CC. X axis: *OPRD1* rs569356 genotypes with patient numbers; Y axis: median plasma norBUP concentrations (A), median values of dose-normalized (B) and dose/kg-normalized norBUP (C).

Table 3
Comparison of individuals with OUD according to the *OPRD1* rs569356 and rs678849 genotypes in view of plasma BUP- and norBUP-related concentrations.

SNP ID	Genetic Model	N	Median (IQR)	p-value
Plasma BUP levels (ng/mL)				
OPRD1	Co-dominant			
rs569356	AA	97	0.066 (0.036–0.11)	$\chi2 = 2.6$ $p = 0.28$
	AG	18	0.059 (0.041–0.18)	
	GG	7	0.036 (0.03–0.05)	
	G-dominant			
	AG+GG	25	0.052 (0.04–0.15)	U= 1161.0, Z = −0.327 $p = 0.74$
	AA	97	0.066 (0.036–0.11)	
OPRD1	Co-dominant			
rs678849	CC	36	0.062 (0.035–0.11)	$\chi2 = 0.900$ $p = 0.64$
	CT	66	0.066 (0.04–0.1)	
	TT	20	0.041 (0.03–0.16)	
Plasma norBUP levels (ng/mL)				
OPRD1	Co-dominant			
rs569356	AA	97	1.56 (0.96–2.84)	$\chi2 = 7.99$
	AG	18	2.12 (1.12–2.8)	**p= 0.018**
	GG	7	0.71 (0.69–0.99)	
	G-dominant			
	AG+GG	25	1.51 (0.78–2.6)	U= 1114.0, Z = −0.625 $p = 0.53$
	AA	97	1.56 (0.96–2.84)	
OPRD1	Co-dominant			
rs678849	CC	36	1.56 (1.05–2.97)	$\chi2 = 0.208$ $p = 0.90$
	CT	66	1.497 (0.94–2.61)	
	TT	20	2.13 (0.68–2.99)	
BUP/D (ng/mL per mg/day)				
OPRD1	Co-dominant			
rs569356	AA	97	0.011 (0.006–0.03)	$\chi2 = 1.81$ $p = 0.40$
	AG	18	0.012 (0.007–0.03)	
	GG	7	0.005 (0.005–0.024)	
	G-dominant			
	AG+GG	25	0.009 (0.006–0.03)	U= 1128.5, Z = −0.533 $p = 0.59$
	AA	97	0.011 (0.006–0.03)	
OPRD1	Co-dominant			
rs678849	CC	36	0.0105 (0.006–0.02)	$\chi2 = 0.287$ $p = 0.87$
	CT	66	0.011 (0.006–0.029)	
	TT	20	0.0105 (0.004–0.04)	
BUP/D.kg^{-1} (ng/mL per mg/kg/day)				
OPRD1	Co-dominant			
rs569356	AA	97	0.74 (0.44–2.35)	$\chi2 = 1.97$
	AG	18	1.034 (0.47–1.91)	$p = 0.37$
	GG	7	0.36 (0.28–1.48)	
	G-dominant			
	AG+GG	25	0.73 (0.37–1.62)	U= 1149.5, Z = −0.400 $p = 0.69$
	AA	97	0.74 (0.44–2.35)	
OPRD1	Co-dominant			
rs678849	CC	36	0.68 (0.43–1.54)	$\chi2 = 0.705$
	CT	66	0.76 (0.45–2.01)	$p = 0.70$
	TT	20	0.67 (0.29–2.18)	
norBUP/D (ng/mL per mg/day)				
OPRD1	Co-dominant			
rs569356	AA	97	0.31 (0.16–0.57)	$\chi2 = 6.03$
	AG	18	0.31 (0.24–0.62)	**p= 0.049**
	GG	7	0.12 (0.09–0.12)	
	G-dominant			
	AG+GG	25	0.26 (0.11–0.52)	U= 1088.0, Z = −0.79 $p = 0.43$
	AA	97	0.31 (0.16–0.57)	
OPRD1	Co-dominant			
rs678849	CC	36	0.28 (0.13–0.65)	$\chi2 = 0.015$
	CT	66	0.30 (0.15–0.57)	$p = 0.99$
	TT	20	0.36 (0.12–0.56)	
norBUP/D.kg^{-1} (ng/mL per mg/kg/day)				
OPRD1	Co-dominant			
rs569356	AA	97	20.75 (10.95–41.16)	$\chi2 = 6.66$ **p= 0.036**
	AG	18	22.03 (16.8–43.5)	
	GG	7	8.00 (6.37–8.46)	
	A-recessive			
	AG+GG	25	19.28 (7.46–36.6)	U= 1083.0, Z = −0.821 $p = 0.41$
	AA	97	20.75 (10.95–41.16)	
OPRD1	Co-dominant			
rs678849	CC	36	18.04 (9.76–52.55)	$\chi2 = 0.015$ $p = 0.99$
	CT	66	20.29 (10.9–39.7)	
	TT	20	22.6 (7.73–35.96)	
norBUP/BUP (metabolic ratio)				
OPRD1	Co-dominant			
rs569356	AA	97	23.14 (13.7–44.03)	$\chi2 = 1.07$ $p = 0.59$
	AG	18	29.56 (10.74–54.18)	
	GG	7	23.5 (9.44–24.8)	
	G-dominant			
	AG+GG	25	23.8 (10.45–41.4)	U= 1195.0, Z = −0.111 $p = 0.91$
	AA	97	23.14 (13.7–44.03)	
OPRD1	Co-dominant			
rs678849	CC	36	22.88 (12.07–44.65)	$\chi2 = 0.089$ $p = 0.95$
	CT	66	24.52 (12.05–42.2)	
	TT	20	22.87 (15.26–56.14)	

($p > 0.05$).

OPRM1 rs648893 variation showed a trend association with increased intensity of depression ($p = 0.08$), with a mean BDI-II score of 11.93 ± 7.6 for CC genotype, 15.07 ± 12.2 for CT genotype and 18.8 ± 10.8 for TT genotype. According to the C-dominant model (TT vs. CC+CT), independent t-test revealed that *OPRM1* rs648893 TT subgroup (18.8 ± 10.8) was significantly different to CC+CT subgroup (14,82 ± 11,3; $p = 0.049$) (Table 4). There were no significant associations between *OPRM1* (rs540825, rs510769) and *OPRK1* (rs35566036, rs997917, rs6473797, rs12548098, rs963549) variations and measurements such as SCS, COWS, BAI and API ($p > 0.05$) (Supplementary Tables S4 and S5).

3.5. Haplotype Analysis

Fig. 3 showed the patterns of LD in the *OPRM1, OPRD1* and *OPRK1* genes, with their $|D'|$ and *r2* values. The LD test for all pairs of markers in *OPRM1* and *OPRK1* showed strong LD (r^2>0.8) for rs540825 and rs510769 and for rs997917 and rs6473797, respectively. Our results indicate that these two SNPs of *OPRM1* and *OPRK1* genes were in one LD block with a defined haplotype frequency. For our study population, haplotypes of the *OPRM1, OPRD1* and *OPRK1* genes with frequencies > 3% were given in Table 5.

Haplotypes of the *OPRM1, OPRD1* and *OPRK1* genes studied in the present study were also compared in terms of plasma BUP and norBUP levels as well as total scores of SCS, COWS, BDI-II, BAI and API. No significant associations were found between plasma BUP and norBUP levels and *OPRM1, OPRD1* or *OPRK1* haplotypes ($p > 0.05$). COWS scores of the *OPRD1* CC haplotype were significantly higher than that of the TC and TT haplotypes ($p < 0.05$). In addition, API total score of the

Table 4
Comparison of individuals with OUD according to the *OPRM1* rs648893, *OPRD1* rs569356 and rs678849 genotypes in view of total scores of measurements.

SNP ID	Genetic Model	N	Median (IQR)	p-value
Substance Craving Scale				
OPRM1	Co-dominant			
rs648893	GG	13	8.41 (4.5–13.0)	$\chi 2 = 2.403$
	AG	42	6.5 (2.25–10.0)	$p = 0.301$
	AA	67	8.0 (4.0–13.0)	
	C-dominant			
	GG+AG	55	8.41 (4.0–11.0)	$U = 1698.5$,
	GG	13	8.41 (4.5–13.0)	$Z = -0.743$
				$p = 0.457$
OPRD1	Co-dominant			
rs569356	AA	97	7.00 (4.0–10.5)	$\chi 2 = 6.063$
	AG	18	8.21 (3.0–17.25)	$\mathbf{p = 0.048}$
	GG	7	14.00 (9.0–23.00)	
	A-recessive			
	AG+GG	25	9.00 (3.5–20.5)	$U = 909.5$,
	AA	97	7.00 (4.0–10.5)	$Z = -1.928$
				$\mathbf{p = 0.05}$
OPRD1	Co-dominant			
rs678849	CC	36	6.00 (0.5–12.00)	$\chi 2 = 1.371$
	CT	66	8.00 (4.75–11.25)	$p = 0.504$
	TT	20	8.71 (3.5–14.00)	
	C-dominant			
	CC+CT	102	7.5 (4.00–12.00)	$U = 909.5$,
	TT	20	8.71 (3.5–14.00)	$Z = -0.767$
				$p = 0.443$
Clinical Opiate Withdrawal Scale				
OPRM1	Co-dominant			
rs648893	GG	13	4.0 (2.0–5.0)	$\chi 2 = 0.807$
	AG	42	4.0 (1.75–6.0)	$p = 0.668$
	AA	67	3.0 (2.0–5.0)	
	C-dominant			
	GG+AG	55	4.0 (2.0–6.0)	$U = 1670.0$,
	GG	13	4.0 (2.0–5.0)	$Z = -0.892$
				$p = 0.372$
OPRD1	Co-dominant			
rs569356	AA	97	4.00 (2.00–5.00)	$\chi 2 = 6.707$
	AG	18	4.00 (2.00–5.00)	$p = 0.8035$
	GG	7	8.00 (5.00–11.00)	
	A-recessive			
	AG+GG	25	5.00 (2.5–8.5)	$U = 892.5$,
				$Z = -2.041$
				$\mathbf{p = 0.041}$
	AA	97	4.00 (2.00–5.00)	
OPRD1	Co-dominant			
rs678849	CC	36	4.00 (2.00–5.75)	$\chi 2 = 0.116$
	CT	66	3.5 (2.00–6.00)	$p = 0.944$
	TT	20	3.5 (2.00–5.00)	
	C-dominant			
	CC+CT	102	4.00 (2.00–6.00)	$U = 981.5$,
	TT	20	3.5 (2.00–5.00)	$Z = -0.268$
				$p = 0.789$
Beck Depression Inventory-II				
OPRM1	Co-dominant			
rs648893	GG	13	11.93 ± 7.6	$F = 2.575$
			(0.0–22.0)	$\mathbf{p = 0.08}$
	AG	42	15.7 ± 12.19	
			(0.0–41.0)	
	AA	67	18.8 ± 10.8	
			(0.0–45.0)	
	C-dominant			
	GG+AG	55	14.82 ± 11.3	$t = -1.993$
			(0.0–41.0)	$\mathbf{p = 0.049}$
	AA	13	11.93 ± 7.6	
			(0.0–22.0)	
OPRD1	Co-dominant			
rs569356	AA	97	16.53 ± 10.79	$F = 1.247$
			(0.00–45.00)	$p = 0.291$
	AG	18	20.67 ± 10.50	
			(3.00–39.00)	
	GG	7	14.43 ± 16.98	
			(0.00–43.00)	
	A-recessive			
	AG+GG	25	18.92 ± 12.59	$t = 0.955$
			(0.00–43.00)	$p = 0.342$
	AA	97	16.53 ± 10.79	
			(0.00–45.00)	
OPRD1	Co-dominant			
rs678849	CC	36	15.42 ± 9.87	$F = 0.527$
			(0.0–36.00)	$p = 0.592$
	CT	66	17.77 ± 12.11	
			(0.0–45.00)	
	TT	20	17.40 ± 10.31	
			(0.0–36.00)	
	C-dominant			
	CC+CT	102	16.94 ± 11.34	$t = -0.167$
			(0.00–45.00)	$p = 0.868$
	TT	20	17.40 ± 10.31	
			(0.0–36.00)	
Beck Anxiety Inventory				
OPRM1	Co-dominant			
rs648893	GG	13	14.4 (8.0–15.5)	$\chi 2 = 1.156$
	AG	42	10.0 (5.75–18.25)	$p = 0.561$
	AA	67	14.0 (6.0–22.0)	
	C-dominant			
	GG+AG	55	11.0 (7.0–17.0)	$U = 1653.0$,
	GG	13	14.4 (8.0–15.5)	$Z = -0.976$
				$p = 0.329$
OPRD1	Co-dominant			
rs569356	AA	97	11.00 (6.00–18.00)	$\chi 2 = 2.606$
	AG	18	14.39 (9.25–23.75)	$p = 0.272$
	GG	7	20.00 (9.00–27.00)	
	A-recessive			
	AG+GG	25	14.40 (9.5–26.5)	$U = 961.0$,
	AA	97	11.00 (6.00–18.00)	$Z = -1.596$
				$p = 0.110$
OPRD1	Co-dominant			
rs678849	CC	36	13.00 (4.25–18.00)	$\chi 2 = 5.462$
	CT	66	14.19 (7.00–23.25)	$p = 0.06$
	TT	20	7.5 (4.5–14.3)	
	C-dominant			
	CC+CT	102	13.5 (7.00–22.25)	$U = 715.0$,
	TT	20	7.5 (4.5–14.3)	$Z = -2.111$
				$\mathbf{p = 0.035}$
Addiction Profile Index				
OPRM1	Co-dominant			
rs648893	GG	13	11.28 (11.0–14.03)	$\chi 2 = 4.795$
	AG	42	10.9 (8.6–10.03)	$p = 0.09$
	AA	67	11.83 (9.1–13.7)	
	C-dominant			
	GG+AG	55	11.06 (9.02–12.3)	$U = 1617.0$,
	GG	13	11.28 (11.0–14.03)	$Z = -1.161$
				$p = 0.246$
OPRD1	Co-dominant			
rs569356	AA	97	11.14 (9.04–13.2)	$\chi 2 = 0.142$
	AG	18	11.24 (9.3–12.9)	$p = 0.931$
	GG	7	11.07 (7.9–12.7)	
	A-recessive			
	AG+GG	25	11.22 (9.1–12.6)	$U = 1165.5$,
	AA	97	11.14 (9.04–13.2)	$Z = -0.298$
				$p = 0.766$
OPRD1	Co-dominant			
rs678849	CC	36	11.8 (9.0–13.27)	$\chi 2 = 2.893$
	CT	66	11.06 (8.7–12.8)	$p = 0.235$
	TT	20	12.4 (11.1–13.6)	
	C-dominant			
	CC+CT	102	11.07 (8.7–13.1)	$U = 794.5$,
	TT	20	12.4 (11.1–13.6)	$Z = -1.560$
				$p = 0.119$

OPRK1 CAGT haplotype (10.3) was significantly higher than that of TAAC (11.27; $p = 0.04$), CGAC (11.69; $p = 0.016$), CGAT (11.7; $p = 0.025$) and CGGC (11.27; $p = 0.05$) haplotypes. There was also a significant difference between *OPRK1* CGGC (3.0; *IQR*: 1.0–4.0) and CAGT (5.0; *IQR*: 4.0–6.0) haplotypes in view of the total score of COWS ($p = 0.038$).

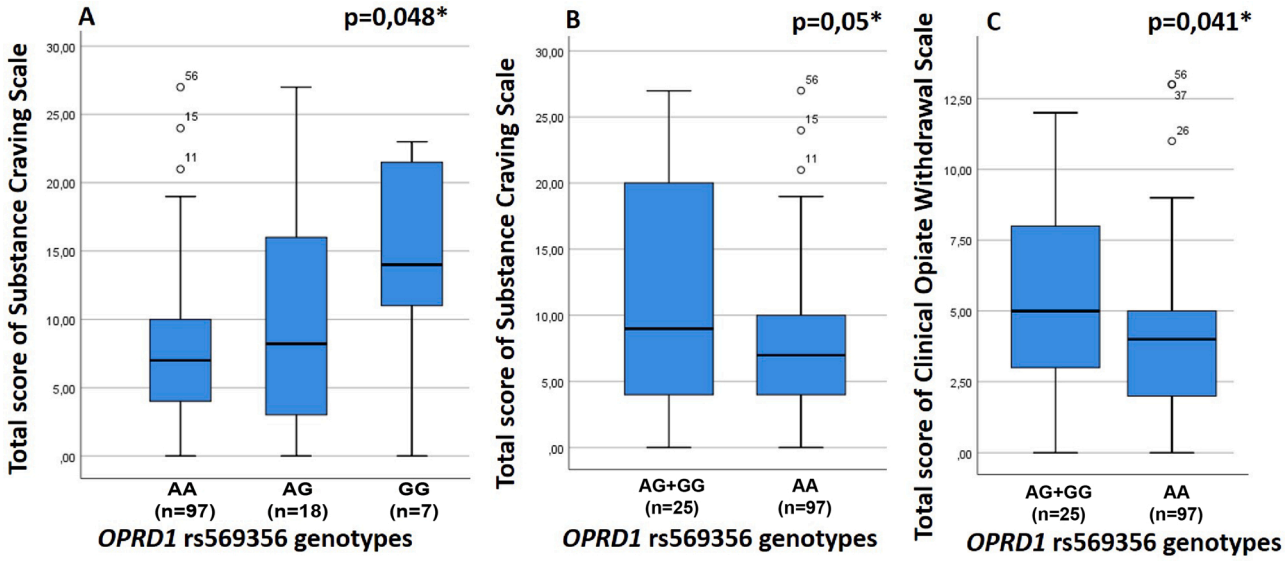

Fig. 2. *OPRD1* rs569356 genotypes and total scores of Substance Craving Scale and Clinical Opiate Withdrawal Scale. (**A**) Distribution of median total score of Substance Craving Scale in patients with TT, TC and CC, (**B**) in patients with TC+CC and TT, and (**C**) total score of Clinical Opiate Withdrawal Scale in patients with TC+CC and TT. X axis: *OPRD1* rs569356 genotypes with patient numbers; Y axis: median total scores of Substance Craving Scale (A and B), median total score of Clinical Opiate Withdrawal Scale (C).

Fig. 3. Linkage disequilibrium (D') and correlation coefficient (r^2) of gene variants in *OPRM1* (A) and *OPRK1* SNPs (B). $D' \geq 0.08$: red; $0 < D' < 1$: shades of red; $r^2 \geq 0.08$: red; $0 < r^2 < 1$: pink and shades of red.

Table 5
The frequencies of the *OPRM1, OPRD1* and *OPRK1* haplotypes according to SHEsis-Plus software.

Haplotypes			
***OPRM1* (rs540825, rs510769, rs648893)**	**n**	**% frequency**	**95% *CI***
ACG	65	26.6	20.9–32.2
TTA	57	23.3	17.9–28.7
TCA	116	47.5	41.2–53.8
***OPDR1* (rs569356, rs678849)**			
AC	96	48.7	41.7–55.7
AT	79	40.1	33.20–46.9
GC	21	10.7	6.4–15.0
***OPRK1* (rs997917, rs6473797, rs1258098, rs963549)**			
TTTC	90	33.0	27.4–38.5
CCTC	68	24.9	19.8–30.0
CCTT	37	13.6	9.5–17.7
CCCC	32	11.7	7.9–15.5
TTCC	30	11.0	7.3–14.7
CTCT	15	5.5	2.8–8.2

n: sample size, CI: Confidence interval
Figure Legends

4. Discussion

To the best of our knowledge, this is the first study showing the effect of *OPRD1* rs569356 polymorphism on plasma norBUP level and dose- and dose/kg-normalized norBUP values in individuals with OUD receiving sublingual BUP/naloxone treatment. Existing studies have indicated that *OPRD1* rs678849 was associated with opioid use disorder treatment (methadone or buprenorphine) outcome in African Americans (AA) (Crist et al., 2013 and 2019) and with the response to extended-release subcutaneous buprenorphine treatment of OUD in European Americans (EA) (Kranzler et al., 2021). Clarke et al. (2014) reported that two intronic *OPRD1* polymorphisms (rs581111 and rs529520) may predict treatment outcome only in females. Few previous studies indicated that *OPRD1* gene variations could affect the treatment responses in OUD patients by an unknown mechanism. Although the effects of buprenorphine at mu- and kappa-opioid receptors have been well-characterized, its activity on delta-opioid receptors has been less known. Negus et al. (2002) showed that buprenorphine had high affinity for, but low efficacy at delta-opioid receptors as compared to mu- or kappa- opioid receptors in rhesus monkeys. In addition, it has been suggested that opioid receptors can function as heterodimers such as MOR-DOR or KOR-DOR heterodimers and these heterodimers could increase the binding of their individual ligands (Valentino and Volkow, 2018). It has been shown that delta-opioid receptor agonists modulated the mu-opioid receptor derived analgesia in the central nervous system (Traynor and Elliott, 1993). Unlike BUP, Negus et al. (2002) suggested that BUP's major metabolite norbuprenorphine may have high efficacy at delta-opioid receptors (Negus et al., 2002). Additionally, Huang et al. (2001) showed that norBUP is a full agonist at the delta-opioid receptor distinct from BUP with no agonist activity. Thus, it seems that the mechanism action of BUP at delta-opioid receptors could be through its active metabolite norbuprenorphine. In the current study, we contributed this hypothesis by showing that *OPRD1* rs569356 variation affected the plasma norBUP concentration and the values of dose- and dose/kg-normalized norBUP.

OPRD1 gene is the first human opioid receptor gene to be cloned and is located at chromosome 1 (1p36.1-p34.3) (Uhl et al., 1994). *OPRD1* gene, having a conserved coding sequence, has only two polymorphisms in the coding sequence (T921C in exon 3 and G80T in exon 1) (Gelernter and Kranzler, 2000; Mayer et al., 1997). Beside these two exonic polymorphisms, rs569356 was the only identified variant in the promoter region of the *OPRD1* and is 1968 bp upstream to the transcription start site. In a study by Zhang et al. (2010) examining the functional significance of the *OPRD1* rs569356, the minor G allele was found to be associated with greater expression levels of luciferase as compared to the major A allele. Additionally, Zhang and co-workers suggested that this SNP is situated in the binding site of the transcription factors according to the results of the electrophoretic mobility shift assay. In parallel line with Zhang's findings, statistically significant differences in plasma norBUP levels (GG:AA, $p = 0.027$; GG:AG, $p = 0.017$), in norBUP/D value (GG:AG, $p = 0.05$), and in norBUP/D.kg^{-1} value (GG:AA, $p = 0.04$; GG:AG, $p = 0.043$) were found between *OPRD1* rs 569356 genotype subgroups in the present study. Based on the findings of Zhang and ours, it is feasible to suggest that the increased binding of norBUP to delta-opioid receptors due to the increased expression levels of delta-opioid receptors in OUD patients with minor G allele as compared to major A allele could result in decreased circulating norBUP level of OUD patients with GG. However, more research is necessary to determine this effect of *OPRD1* rs569356 variant on BUP pharmacology. Furthermore, significant associations between *OPRD1* rs569356 and opioid craving and withdrawal were found in the present study ($p < 0.05$). OUD patients with at least one G allele (AG+GG) had higher craving and withdrawal as compared to those with A allele due to most likely lower plasma norBUP concentration. Additionally, the median daily amount of heroin consumed per day was significantly higher in OUD patients with *OPRD1* rs569356 AG+GG genotypes ($\tilde{x}$=3,6 g/day; *IQR*: 3.0–5.0) than in those with AA genotype ($\tilde{x}$=3 g/day; *IQR*: 2.0–4.25) before treatment with buprenorphine ($p = 0.045$). It might be speculated that greater expression levels of delta-opioid receptors due to the minor G allele could cause the increased use of heroin to see the desired euphoric effect.

No significant associations were found between rs678849, the other *OPRD1* SNP examined in the current study, and plasma BUP and norBUP concentrations ($p > 0.05$), which was not consistent with previous studies showing the effect of *OPRD1* rs678849 on buprenorphine treatment responses in AA and EA (Crist et al., 2013 and 2019; Kranzler et al., 2021). On the other hand, it was found that there were differences between *OPRD1* rs678849 genotypes (CC+CT vs. TT) in view of dose- and dose/kg-normalized norBUP values (0.29 vs. 0.36 and 19.43 vs. 22.6, respectively), suggesting the effect of this polymorphism on BUP pharmacology via its major metabolite. Crist and et al., (2013, 2019) reported that individuals receiving buprenorphine treatment with CT+TT were less likely to have positive opioid drug screens as compared to CC genotype. However, they did not measure the plasma BUP or norBUP levels. Crist's studies and our findings could indicate that C allele could be a risk factor for opioid craving due to the low levels of norBUP in the plasma as compared to T allele. Thus, opioid-positive urine tests were common in individuals with OUD having at least one C allele while they were in buprenorphine treatment. Gelernter et al. (2007) also supported this speculation by showing that increased risk of relapse were associated with the *OPRD1* rs678849 CC genotype relative to TT genotype. Furthermore, the *OPRD1* rs678849 CC+CT genotypes significantly had a higher anxiety total score than TT genotypes in the current study. Anxiety could be associated with the desire to use substances having euphoric properties. The involvement of the delta opioid receptor system in the regulation of anxiety was shown in studies on delta opioid receptor knockout mice exhibiting anxiogenic-like phenotype (Filliol et al., 2000; Perrine et al., 2006). Altogether, all existing studies including ours reported that rs678849 is associated with buprenorphine treatment efficacy in different populations by an unknown mechanism. It is thought that *OPRD1* rs678849, an intronic polymorphism, could affect alternative splicing (Piltonen et al., 2019). Therefore, this genetic variation could be a good candidate pharmacogenetic marker.

We further investigated the interaction between *OPRD1* rs569356 and rs678849 in the intron and observed a non-linkage disequilibrium between them using SHesis-Plus software (r^2<0,8), which is consistent with Nelson et al. (2012). In the current study, *OPRD1* haplotypes with frequencies > 3% were C-C, T-C and T-T. No significant difference between these haplotypes in terms of plasma BUP and norBUP

concentrations was found. On the other hand, there was a significant association between *OPRD1* haplotypes and the intensity of opioid withdrawal ($p < 0.05$). The *OPRD1* rs569356 and rs678849C-C haplotypes significantly had a higher intensity of opioid withdrawal symptoms relative to T-C and T-T haplotypes in the current study while C allele of these 2 intronic polymorphisms had no effect on COWS total score alone, showing that haplotypes of gene polymorphisms could affect the gene function regardless of the one-by-one polymorphisms. Gong et al. (2020) showed that there is a positive relationship between craving and withdrawal symptoms in individuals with OUD receiving methadone treatment. Additionally, craving and withdrawal symptoms are factors that may increase the risk of relapse in individuals with OUD. Therefore, our findings regarding the effects of haplotypes on opioid withdrawal could be useful for treatment failures of individuals who experience greater opioid withdrawal due to their *OPRD1* rs569356 and rs678849C-C haplotypes.

OPRM1 rs648893 variation showed an association with increased intensity of depression ($p = 0.049$), with a mean BDI-II score of 18.8 $\pm$ 10.8 for TT genotype and 14.82 $\pm$ 11.3 for CC+CT genotype. This finding suggested that *OPRM1* rs648893 TT genotype may be a risk factor for drug-seeking behavior in OUD patients due to the increased intensity of depressive symptoms. Mu-opioid receptor is distributed in the brain, mainly in globus pallidus, thalamus and caudate putamen. It has high affinity for both endogenous (beta-endorphin and enkephalin) and exogenous (morphine, heroin and buprenorphine) ligands. It is responsible for opioidergic effects such as euphoria, analgesia and opioid withdrawal. *OPRM1* gene encoding mu-opioid receptor has more than 250 single nucleotide polymorphisms (Ding et al., 2013). Among these, *OPRM1* rs648893 is an intronic polymorphism (Zhang et al., 2006). Intronic sequences could be involved in alternative DNA splicing and a total of ten human *OPRM1* splice variants have been identified so far (Bare et al., 1994; Pan et al., 2005). Although these splice variants producing mu-opioid receptor subtypes contained exon 1, 2 and 3 as in original human *OPRM1*, they have different amino acid sequences due to the splicing downstream from exon 3 (Bare et al., 1994). However, their potency and efficacy were different among splice variants according to the adenylyl cyclase and [35 S]GTPgammaS binding assays, suggesting that this differentiation could explain the wide range of opioid responses (Pan et al., 2005). In the current study, the significantly increased depressive symptoms in OUD patients with TT as compared to CC genotype could be the result of varying activation patterns of *hMOR-1* splice variants. If this relationship between splice variants and drug efficacies could be proven, different effectiveness of the maintenance treatments among opioid patients could be explained, which might provide new insights into the treatment strategies.

In the current study, the influence of four different *OPRK1* polymorphism (rs997917, rs12548098, rs6473797 and rs963549) on plasma BUP and norBUP concentrations as well as the total score of SCS, COWS, BDI-II, BAI and API was also analyzed. According to the single locus analyses, no significant effect was found ($p < 0.05$). Haplotype analysis with SHesis-Plus software showed that *OPRK1* haplotypes with frequencies > 3% were CTCT, TTTC, CCTC, CCTT, CCCC and TTCC in our population. There was not a significant difference between these six haplotypes in view of plasma BUP and norBUP concentrations and total score of measurements ($p > 0.05$). However, when these haplotypes were compared in pairs, individuals with CTCT had significantly lower total score of API as compared to those with TTTC, CCTC, CCTT, CCCC or TTCC. Additionally, a significant difference was found between *OPRK1* CCCC and CTCT haplotypes in view of the intensity of opioid withdrawal. To the best of our knowledge, this is the first report showing the candidate *OPRK1* rs997917-rs12548098-rs6473797-rs963549 haplotype was associated with the severity of addiction or opioid withdrawal. Only one of four SNPs (rs6473797) was previously reported to be significantly associated with the severity of alcohol craving in a Korean population (Park et al., 2020). Contrary to our results for the *OPRK1* haplotypes, Karpyak et al. (2013) detected no evidence for associations of *OPRK1* haplotype containing rs6473799–rs7836120–rs6985606–rs963549–rs1691894 SNPs with negative craving, alcohol dependence or depression related phenotypes. We further investigated the interaction between these *OPRK1* polymorphisms and observed a strong linkage disequilibrium between rs997917 and rs6473797 using SHesis-Plus software (r^2>0,8), which is consistent with Yuferov et al. (2022) and non-consistent with Albonaim et al. (2017). These inconsistent findings indicated that more studies in larger sample size are required with other populations.

In conclusion, the current study indicated for the first time a prominent effect of the *OPRD1* rs569356 gene variation on sublingual buprenorphine pharmacology due to its major metabolite norbuprenorphine. Our finding regarding the effect of *OPRD1* haplotype containing rs569356-rs678849 SNPs on opioid withdrawal could be useful for treatment failures of individuals who experience greater opioid withdrawal during the maintenance treatment. Future genome-wide association studies of *OPRD1* rs569356 comprising different population groups will require to verify the contribution of this variation on BUP pharmacology, and thus, to identify the patients with OUD who are less likely to respond to treatment. Additionally, to the best of our knowledge, this is the first report showing the candidate *OPRK1* rs997917-rs12548098-rs6473797-rs963549 haplotype was associated with the severity of addiction or opioid withdrawal. For *OPRM1*, single SNP and candidate haplotype analyses investigating the association with plasma BUP and nor BUP concentrations and the severity of depression, anxiety, craving, withdrawal and addiction showed no significance.

Author contributions

DKA designed and directed the study. DKA and SÖK conducted the genetic analysis and prepared the manuscript. MD, BO and GZİ collected venous blood samples and demographic data of all subjects. CB conducted the LC/MS-MS analysis. All authors contributed to and have approved the final manuscript.

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CRediT authorship contribution statement

Dilek Kaya-Akyüzlü: Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. **Selin Ozkan-Kotiloglu:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Mustafa Danışman:** Resources. **Ceylan Bal:** Methodology. **Begüm Oğur:** Resources. **Gamze Zengin ispir:** Resources.

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 The Scientific and Technical Research Council of Turkey (TUBITAK).

Data Availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2023.104143.

References

Albonaim, A., Fazel, H., Sharafshah, A., Omarmeli, V., Rezaei, S., Ajamian, F., Keshavarz, P., 2017. Association of OPRK1 gene polymorphisms with opioid dependence in addicted men undergoing methadone treatment in an Iranian population. J. Addict. Dis. 36, 227–235. https://doi.org/10.1080/10550887.2017.1361724.

Bare, L., Mansson, E., Yang, D., 1994. Expression of two variants of the human mu opioid receptor mRNA in SK-N-SH cells and human brain. FEBS Lett. 354, 213–216. https://doi.org/10.1016/0014-5793(94)01129-x.

Brown, S.M., Holtzman, M., Kim, T., Kharasch, E.D., 2011. Buprenorphine metabolites, buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide, are biologically active. Anesthesiology 115, 1251–1260. https://doi.org/10.1097/ALN.0b013e318238fea0.

Canan, F., Kuloğlu, M., Guven, M., Gecici, O., 2015. Reliability and Validity of the Turkish Version of the Clinical Opiate Withdrawal Scale (COWS). Bull. Clin. Psychopharmacol. 25, 267–271. https://doi.org/10.5455/bcp.20150404070711.

Clarke, T.K., Crist, R.C., Ang, A., Ambrose-Lanci, L.M., Lohoff, F.W., Saxon, A.J., Ling, W., Hillhouse, M.P., Bruce, R.D., Woody, G., Berrettini, W.H., 2014. Genetic variation in OPRD1 and the response to treatment for opioid dependence with buprenorphine in European American females. Pharm. J. 14, 303–308. https://doi.org/10.1038/tpj.2013.30.

Crist, R.C., Clarke, T.K., Ang, A., Ambrose-Lanci, L.M., Lohoff, F.W., Saxon, A.J., Ling, W., Hillhouse, M.P., Douglas Bruce, R., Woody, G., Berrettini, W.H., 2013. An intronic variant in OPRD1 predicts treatment outcome for opioid dependence in African-Americans. Neuropsychopharmacol 38, 2003–2010. https://doi.org/10.1038/npp.2013.99.

Crist, R.C., Doyle, G.A., Nelson, E.C., Degenhardt, L., Martin, N.G., Montgomery, G.W., Saxon, A.J., Ling, W., Berrettini, W.H., 2018. A polymorphism in the OPRM1 3′-untranslated region is associated with methadone efficacy in treating opioid dependence. Pharm. J. 18, 173–179. https://doi.org/10.1038/tpj.2016.89.

Crist, R.C., Phillips, K.A., Furnari, M.A., Moran, L.M., Doyle, G.A., McNicholas, L.F., Cornish, J.W., Kampman, K.M., Preston, K.L., Berrettini, W.H., 2019. Replication of the pharmacogenetic effect of rs678849 on buprenorphine efficacy in African-Americans with opioid use disorder. Pharm. J. 19, 260–268. https://doi.org/10.1038/s41397-018-0065-x.

Ding, S., Chen, B., Zheng, Y., Lu, Q., Liu, L., Zhuge, Q.C., 2013. Association study of OPRM1 polymorphisms with Schizophrenia in Han Chinese population. BMC Psychiatry 13, 107. https://doi.org/10.1186/1471-244X-13-107.

Ettienne, E.B., Chapman, E., Maneno, M., Ofoegbu, A., Wilson, B., Settles-Reaves, B., Clarke, M., Dunston, G., Rosenblatt, K., 2017. Pharmacogenomics-guided policy in opioid use disorder (OUD) management: An ethnically-diverse case-based approach. Addict. Behav. Rep. 6, 8–14. https://doi.org/10.1016/j.abrep.2017.05.011.

Ettienne, E.B., Ofoegbu, A., Maneno, M.K., Briggs, J., Ezeude, G., Williams, S., Walker, C., Chapman, E., 2019. Pharmacogenomics and Opioid Use Disorder: Clinical Decision Support in an African American Cohort. J. Natl. Med. Assoc. 111, 674–681. https://doi.org/10.1016/j.jnma.2019.09.006.

Evren, C., Gürol, D.T., Ögel, K., 2011. Reliability and validity of the Penn Alcohol Craving Scale (PACS) Revised Version for substance craving in male substance dependent inpatients. Turk. J. Psychiatry 22, 70–85.

Ferrari, D., Manca, M., Premaschi, S., Banfi, G., Locatelli, M., 2018. Toxicological investigation in blood samples from suspected impaired driving cases in the Milan area: Possible loss of evidence due to late blood sampling. Forensic Sci. Int. 288, 211–217. https://doi.org/10.1016/j.forsciint.2018.04.038.

Fihlman, M., Hemmilä, T., Hagelberg, N.M., Backman, J.T., Laitila, J., Laine, K., Neuvonen, P.J., Olkkola, K.T., Saari, T.I., 2018. Voriconazole Greatly Increases the Exposure to Oral Buprenorphine. Eur. J. Clin. Pharm. 74, 1615–1622. https://doi.org/10.1007/s00228-018-2548-8.

Filliol, D., Ghozland, S., Chluba, J., Martin, M., Matthes, H.W., Simonin, F., Befort, K., Gaveriaux-Ruff, C., Dierich, A., LeMeur, M., Valverde, O., Maldonado, R., Kieffer, B. L., 2000. Mice deficient for delta-and mu-opioid receptors exhibit opposing alterations of emotional responses. Nat. Genet. 25, 195–200. https://doi.org/10.1038/76061.

Food and Drug Administation, 2002. Center for Drug Evaluation and Research.

Gelernter, J., Kranzler, H.R., 2000. Variant detection at the delta opioid receptor (OPRD1) locus and population genetics of a novel variant affecting protein sequence. Hum. Genet. 107, 86–88. https://doi.org/10.1007/s004390000340.

Gelernter, J., Gueorguieva, R., Kranzler, H.R., Zhang, H., Cramer, J., Rosenheck, R., Krystal, J.H., Cooperative Study #425 Study Group, V.A., 2007. Opioid receptor gene (OPRM1, OPRK1, and OPRD1) variants and response to naltrexone treatment for alcohol dependence: results from the VA Cooperative Study. Alcohol. Clin. Exp. Res. 31, 555–563. https://doi.org/10.1111/j.1530-0277.2007.00339.x.

Gerra, G., Somaini, L., Leonardi, C., Cortese, E., Maremmani, I., Matteo Manfredini, M., Donnini, C., 2014. Association between gene variants and response to buprenorphine maintenance treatment. Psychiatry Res 215, 202–207. https://doi.org/10.1016/j.psychres.2013.11.001.

Gong, H., Zhang, Y., Ren, Q., Zhou, Z., Zhou, H., Sun, X., Zhang, C., Voon, V., Zhao, M., Yu, S., 2020. The Chinese version of obsessive compulsive drug use scale: validation in outpatient methadone maintenance treatment program. BMC Psychiatry 20, 465. https://doi.org/10.1186/s12888-020-02843-2.

Hisli, N., 1989. Reliability and validity of Beck Depression Inventory among university students. Turk. Psikol. Derg. 7, 3–13.

Huang, P., Kehner, G.B., Cowan, A., Liu-Chen, L.Y., 2001. Comparison of pharmacological activities of buprenorphine and norbuprenorphine: norbuprenorphine is a potent opioid agonist. J. Pharmacol. Exp. Ther. 297, 688–695.

Imai, H., Morita, M., Morita, H., Ohyama, T., Tanaka, S., Uchida, S., Namiki, N., Uemura, N., Ohashi, K., 2020. The μ-opioid receptor gene polymorphism 118A>G weakens the pharmacological action of buprenorphine. Int. J. Clin. Pharmacol. Ther. 58, 626–633. https://doi.org/10.5414/CP203755.

Karpyak, V.M., Winham, S.J., Preuss, U.W., Zill, P., Cunningham, J.M., Walker, D.L., Lewis, K.A., Geske, J.R., Colby, C.L., Abulseoud, O.A., Hall-Flavin, D.K., Loukianova, L.L., Schneekloth, T.D., Frye, M.A., Bazov, I., Heit, J.A., Bakalkin, G., Mrazek, D.A., Biernacka, J.M., 2013. Association of the PDYN gene with alcohol dependence and propensity to drinl negative emontional states. Int. J. Neuropsychopharmacol. 16, 975–985. https://doi.org/10.1017/S1461145712001137.

Kaya-Akyüzlü, D., Özkan-Kotiloğlu, S., Bal, C., Yalçın-Şahiner, Ş., Avcıoğlu, G., Danışman, M., 2022a. Effects of UGT2B7 rs7662029 and rs7439366 polymorphisms on sublingual buprenorphine metabolism in heroin addicts: An improved PCR-RFLP assay for the detection of rs7662029 polymorphism. Environ. Toxicol. Pharmacol. 94, 103902 https://doi.org/10.1016/j.etap.2022.103902.

Kaya-Akyüzlü, D., Özkan-Kotiloğlu, S., Bal, C., Avcıoğlu, G., Yalçın-Şahiner, Ş., Şahiner, İ.V., 2022b. Sublingual buprenorphine/naloxone treatment is not affected by OPRM1 A118G and BDNF Va66Met polymorphisms, but alters the plasma beta-endorphin and BDNF levels in individuals with opioid use disorder. Environ. Toxicol. Pharmacol. 95, 103979 https://doi.org/10.1016/j.etap.2022.103979.

Kranzler, H.R., Lynch, K.G., Crist, R.C., Hartwell, E., Moigne, A.L., Laffont, C.M., Andorn, A.C., 2021. A Delta-Opioid Receptor Gene Polymorphism Moderates the Therapeutic Response to Extended-Release Buprenorphine in Opioid Use Disorder. Int. J. Neuropsychopharmacol. 15, 89–96. https://doi.org/10.1093/ijnp/pyaa069.

Kreek, M.J., Reed, B., Butelman, E.R., 2019. Current status of opioid addiction treatment and related preclinical research. Sci. Adv. 5, eaax9140. https://doi.org/10.1126/sciadv.aax9140.

Leander, J.D., 1987. Buprenorphine has potent kappa opioid receptor antagonist activity. Neuropharmacology 26, 1445–1447. https://doi.org/10.1016/0028-3908(87)90112-2.

Li, Z., Zhang, Z., He, Z., Tang, W., Li, T., Zeng, Z., He, L., Shi, Y., 2009. A partition-ligation-combination-subdivision EM algorithm for haplotype inference with multiallelic markers: update of the SHEsis (http://analysis.bio-x.cn). Cell. Res. 19, 519–523. https://doi.org/10.1038/cr.2009.33.

Martin, W.R., Eades, C.G., Thompson, J.A., Huppler, R.E., Gilbert, P.E., 1976. The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog. J. Pharmacol. Exp. Ther. 197, 517–532.

Mayer, P., Rochlitz, H., Rauch, E., Rommelspacher, H., Hasse, H.E., Schmidt, S., Höllt, V., 1997. Association between a delta opioid receptor gene polymorphism and heroin dependence in man. Neuroreport 8, 2547–2550. https://doi.org/10.1097/00001756-199707280-00025.

Negus, S.S., Bidlack, J.M., Mello, N.K., Furness, M.S., Rice, K.C., Brandt, M.R., 2002. Delta opioid antagonist efects of buprenorphine in rhesus monkeys. Behav. Pharmacol. 13, 557–570. https://doi.org/10.1097/00008877-200211000-00005.

Ögel, K., Evren, C., Karadağ, F., Gürol, D.T., 2012. The development, validity, and reliability of the Addiction Profile Index (API). Turk. Psikiyatr. Derg. 23, 263–275.

Pan, L., Xu, J., Yu, R., Xu, M.M., Pan, Y.X., Pasternak, G.W., 2005. Identification and characterization of six new alternatively spliced variants of the human mu opioid receptor gene. Oprm. Neurosci. 133, 209–220. https://doi.org/10.1016/j.neuroscience.2004.12.033.

Panlilio, L.V., Stull, S.W., Kowalczyk, W.J., Phillips, K.A., Schroeder, J.R., Bertz, J.W., Vahabzadeh, M., Lin, J.L., Mezghanni, M., Nunes, E.V., Epstein, D.H., Preston, K.L., 2019. Stress, craving and mood as predictors of early dropout from opioid agonist therapy. Drug. Alcohol. Depend. 202, 200–208. https://doi.org/10.1016/j.drugalcdep.2019.05.026.

Park, C.I., Hwang, S.S., Kim, H.W., Kang, J.I., Lee, S.H., Kim, S.J., 2020. Association of opioid receptor gene polymorphisms with drinking severity and impulsivity related to alcohol use disorder in a Korean population. CNS Neurosci. Ther. 26, 30–38. https://doi.org/10.1111/cns.13138.

Perrine, S.A., Hoshaw, B.A., Unterwald, E.M., 2006. Delta opioid receptor ligands modulate anxiety-like behaviors in the rat. Br. J. Pharm. 147, 864–872. https://doi.org/10.1038/sj.bjp.0706686.

Piltonen, M., Parisien, M., Grégoire, S., Chabot-Doré, A.J., Jafarnejad, S.M., Bérubé, P., Djambazian, H., Sladek, R., Geneau, G., Willett, P., Stone, L.S., Shabalina, S.A., Diatchenko, L., 2019. Alternative Splicing of the Delta-Opioid Receptor Gene Suggests Existence of New Functional Isoforms. Mol. Neurobiol. 56, 2855–2869.

Randesi, M., Rotrosen, J., Nunes, E.V., Lee, J.D., Novo, P., Levran, O., Ott, J., Pavlicova, M., Scodes, J., Kreek, M.J., 2020. Variants of opioid genes and response to treatment of opioid use disorder with buprenorphine-naloxone versus extended-release naltrexone in Caucasians. Am. J. Drug Alcohol Abus. 46, 761–768. https://doi.org/10.1007/s12035-018-1253.

Seguí, H.A., Melin, K., Quiñones, D.S., Duconge, J., 2020. A review of the pharmacogenomics of buprenorphine for the treatment of opioid use disorder. J. Transl. Genet. Genom, 4, 263–277 https://10.20517/jtgg.2020.35.

Shi, Y.Y., He, L., 2005. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Res 15, 97–98. https://doi.org/10.1038/sj.cr.7290272.

Taqi, M.M., Faisal, M., Zaman, H., 2019. OPRM1 A118G Polymorphisms and Its Role in Opioid Addiction: Implication on Severity and Treatment Approaches. Pharmagenomics. Pers. Med 12, 361–368. https://doi.org/10.2147/PGPM.S198654.

Traynor, J.R., Elliott, J., 1993. Delta-Opioid receptor subtypes and cross-talk with mu-receptors. Trends Pharmacol. Sci. 14, 84–86. https://doi.org/10.1016/0165-6174(93)90068-u.

Uhl, G.R., Childers, S., Pasternak, G., 1994. An opiate-receptor gene family Reunion. Trends Neurosci. 17, 89–93. https://doi.org/10.1016/0166-2236(94)90110-4.

Ulusoy, M., Şahin, N.H., Erkmen, H., 1998. Turkish version of the Beck Anxiety Inventory: psychometric properties. J. Cogn. Psychother. 12, 163–172.
Valentino, R.J., Volkow, N.D., 2018. Untangling the complexity of opioid receptor function. Neuropsychopharmacology 43, 2514–2520. https://doi.org/10.1038/s41386-018-0225-3.
Wang, S., 2019. Historical Review: Opiate Addiction and Opioid Receptors. Cell Transpl. 28, 233–238. https://doi.org/10.1177/0963689718811060.
Yiannakopoulou, E., 2015. Pharmacogenomics and Opioid Analgesics: Clinical Implications. Int. J. Genom., 368979 https://doi.org/10.1155/2015/368979.
Yuferov, V., Butelman, E.R., Randesi, M., Ott, J., Kreek, M.J., 2022. Analyses of polymorphisms of intron 2 of OPRK1 (kappa-opioid receptor gene) in association with opioid and cocaine dependence diagnoses in an African-American population. Neurosci. Lett. 768, 136364 https://doi.org/10.1016/j.neulet.2021.136364.
Zhang, H., Gelernter, J., Gruen, J.R., Kranzler, H.R., Herman, A.I., Simen, A.A., 2010. Functional impact of a single-nucleotide polymorphism in the OPRD1 promoter region. J. Hum. Genet 55, 278–284. https://doi.org/10.1038/jhg.2010.22.
Zhang, L., Kendler, K.S., Chen, X., 2006. The mu-opioid receptor gene and smoking initiation and nicotine dependence. Behav. Brain Funct. 2, 28. https://doi.org/10.1186/1744-9081-2-28.