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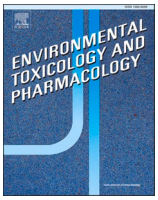

Effects of *UGT2B7* rs7662029 and rs7439366 polymorphisms on sublingual buprenorphine metabolism in heroin addicts: An improved PCR-RFLP assay for the detection of rs7662029 polymorphism

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ABSTRACT

This study aimed to determine the effects of *UGT2B7* rs7662029 and rs7439366 polymorphisms on plasma buprenorphine (BUP) concentration and different treatment responses in a sample of 109 patients with opioid use disorder (OUD) treated with sublingual BUP/naloxone. Polymorphisms were analysed by PCR-RFLP. Plasma concentrations of BUP and its metabolite norbuprenorphine were detected by LC–MS/MS. Craving, withdrawal, depression and anxiety were measured by appropriate scales. OUD patients with rs7439366 CC or rs7662029 GG genotypes had significantly lower dose-normalized (BUP/D) and dose/kg-normalized BUP (BUP/D.kg^{-1}) levels than those who were CT or AA carriers. Significant associations between *UGT2B7* rs7662029 and increased craving ($p = 0.037$) and withdrawal symptoms ($p = 0.029$) were detected. Our findings were pointing to an important role of *UGT2B7* in the metabolism of sublingual BUP/naloxone in the heroin addicts for the first time. A novel PCR-RFLP assay was developed for the determination of *UGT2B7* rs7662029 polymorphism, based on utilizing novel restriction enzyme.

1. Introduction

Opioid use disorder (OUD) is a significant public health issue affecting millions of people all over the world. It is estimated that approximately 26.8 million people live with OUD globally in 2016 and more than 100.000 people died from opioid overdoses annually. The global societal burden of this disorder is immense because of the increased risk and costs of crime along with the reduced employment and economic contribution (Strang et al., 2020). OUD is a chronic, relapsing disorder (Crist et al., 2018). Various factors such as genetic susceptibility, history of opioid experimentation, mental illness, social norms, and dysregulated stress system response contribute to the development of OUD and make patients difficult to stop opioid use (Strang et al., 2020; Clarke et al., 2014). However, there are several pharmacological interventions and non-pharmacological approaches that can help addicts reduce illicit opioid use. The aim of medication-assisted treatment is frequently to replace illicit opioids with longer acting legal opioids such as methadone and buprenorphine (BUP) which have less abuse potential (Crist et al., 2018).

BUP, developed as an analgesic in 1970, is a FDA-approved drug to treat opioid dependence by reducing withdrawal symptoms and also by blocking the effects of other opioids (Fihlman et al., 2018; Segui et al., 2020). BUP has unique and complex pharmacological properties that distinguish it from other opioids: being a partial mu-opioid receptor agonist, delta- and kappa-opioid receptor antagonist and nociceptin receptor agonist (Brown et al., 2011; Clarke et al., 2014; Davis et al., 2018). When used as a maintenance medication for OUD, it is co-formulated with naloxone to discourage parenteral administration, which can cause euphoria (Segui et al., 2020; Brown et al., 2011), and is administered sublingually since it has a very low oral bioavailability due

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to its extensive first-pass metabolism (Fihlman et al., 2018; Kreek et al., 2019). In spite of the fact that long-term opioid agonist therapy with BUP has great efficacy for the treatment of OUD compared with placebo, significant percentages of opioid addicts still fail to reduce illicit opioid use or maintain abstinence even after receiving medication for their OUD. A growing body of data suggests that genetic variations can influence variable treatment response, leaving some patients at increased risk for relapse (Segui et al., 2020; Crist et al., 2018). Variants in the genes involved in BUP metabolism (pharmacokinetics) and receptor function (pharmacodynamics) can affect the efficacy of BUP in humans. Understanding how to optimize the use of sublingual BUP with a low rate of failure or adverse outcomes is essential to reduce opioid misuse and improve treatment adherence. However, pharmacogenomic studies to date have focused predominantly on methadone and a number of relevant pharmacogenes have been identified (Segui et al., 2020). On the other hand, publications dealing with the polymorphisms affecting serum buprenorphine concentration are scarce (Ettienne et al., 2017, 2019). In these limited studies, pharmacogenetic testing seemed to have an impact on OUD management outcomes in African-American patients, which is reflected a genetic contribution to phenotypes. Thus, focus must be shift to buprenorphine pharmacogenetics through the collection of new study populations (Crist et al., 2018). Furthermore, they should be reproduced in different ethnic groups to develop robust clinical decision-making tools in the clinical setting.

BUP is extensively metabolized via N-dealkylation by cytochrome P450 (CYP450) enzymes in the liver, which yields an active metabolite, norbuprenorphine (norBUP). To a lesser extent, BUP undergoes glucuronidation mainly by UDP-glucuronosyl transferases (UGT) 2B7 and 1A1 (Fihlman et al., 2018; Brown et al., 2011). Rouguieg et al. (2010) evaluated the specific contribution of individual UGT isoforms in the metabolism of BUP and reported that UGT2B7 and UGT1A1 accounted for approximately 41% and 10% of BUP glucuronidation, respectively. To date, the relationship with *UGT2B7* gene polymorphisms with metabolism of valproic acid, lamotrigine, mycophenolic acid, tamoxifen and carbamazepine has been investigated in epileptic children, renal transplant patients and breast cancer patients (Du et al., 2016; Djebli et al., 2007; Guo et al., 2012; Genvigir et al., 2020; Ma et al., 2013; Romero-Lorca et al., 2015; Shen et al., 2016; Zhang et al., 2018). The effect of *UGT2B7* polymorphism rs7439366 at locus 802 C>T (historically termed His268Tyr) on the response to transdermal buprenorphine in patients with critical limb ischemia (Blanco et al., 2016) and in patients who underwent muscle-sparing thoracotomy (Sastre et al., 2015) was analysed. Sastre et al. (2015) found an association between the presence of the SNP 802 C>T (rs7439366) in *UGT2B7* (UGT2B7 *2/*2) and a worse analgesic response to transdermal buprenorphine. On the other hand, Blanco et al. (2016) reported that the SNP 802 C>T *UGT2B7* had no effect on response to treatment with transdermal buprenorphine. To the best of our knowledge, there has been no study examining the effects of *UGT2B7* gene polymorphisms on the metabolism of sublingual buprenorphine/naloxone in heroin addicts. Based on this background, we investigated whether the presence of the SNPs rs7439366 (historically called His268Tyr) and rs7662029 on *UGT2B7* gene are associated with serum buprenorphine and norbuprenorphine concentrations as well as different treatment responses such as craving, anxiety, withdrawal and depression in heroin addicts treated with sublingual buprenorphine/naloxone combination.

2. Material and methods

2.1. Study population

The study enrolled 109 patients who had been receiving suboxone (sublingual BUP/naloxone combination) at Ankara Training and Research Hospital (AMATEM Clinic) in Ankara, Turkey. The sample was composed of outpatients admitted to the hospital for the OUD treatment. Patients were eligible for inclusion in the cohort if (i) they were at least 18 years of age, (ii) they fulfilled the criteria for opioid use disorder according to The Diagnostic and Statistical Manual of Mental Disorders (DSM–5) diagnostic criteria, (iii) they have been receiving sublingual BUP/naloxone for at least 10 days as steady-state plasma BUP concentrations are reportedly achieved 8–10 days (>5 half-lives) after initiation of therapy, (iv) they have not been receiving any drugs that might interact with the metabolism of BUP, (v) they have no acute health problems, (vi) they are willing to be treated. Exclusion criteria were as follows: (i) subjects with active drug addiction during BUP management therapy, (ii) subjects with substance use disorders other than heroin and nicotine dependence such as other opioids, alcohol and/or benzodiazepines, (iii) subjects with clinically significant comorbid psychiatric illness such as any psychotic disorders, schizophrenia, mental retardation, bipolar disorder and severe depression, (iv) subjects administered either drugs for physical diseases or psychiatric illness, (v) subjects with impaired renal or hepatic function.

Written informed consent and permission to use their information for future studies of heroin dependence and related phenotypes was obtained from each participant who were eligible for the study. A small questionnaire used to gather socio-demographic information on age, marital, education and employment status, past and present substance use, duration of heroin use, 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 the individuals. The study design was approved by the institutional ethics committee (approval no: I8–509–20 in 2020). Samplings were performed in accordance with the principles of The Declaration of Helsinki.

2.2. Sample collection and DNA isolation

Two mL of venous blood was taken from each patient into tubes with ethylenediaminetetraacetic acid (EDTA) for DNA isolation and were stored at − 20 °C. After minimum 10 days of continuous sublingual BUP/naloxone treatment, before taking daily BUP dose a 2 mL blood sample from each patient was also collected into an EDTA tube to measure the steady-state concentration.

Genomic DNA was extracted from EDTA anticoagulant peripheral whole-blood samples using the QIAamp DNA blood-kit (Qiagen, Hilden, Germany) according to the method recommended by the manufacturer.

2.3. Determination of the UGT2B7 rs7662029 and rs7439366 polymorphisms

The *UGT2B7* rs7662029 and rs7439366 polymorphisms were analysed using Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The *UGT2B7* rs7439366 polymorphism was genotyped as previously described (Zhang et al., 2014). For the determination of the *UGT2B7* rs7662029 polymorphism, forward and reverse primers of PCR assay were used designed by da Cruz (2015) (in a dissertation, not published) and a novel RFLP assay was developed in the current study.

PCR amplifications were conducted on a Techne Tc 512 PCR system in a 25 μL reaction mixture containing 200 μM of dNTPs, 10 pmol each of the forward and reverse primers, 1 U of Hot Star Taq DNA polymerase (New England Biolabs, UK), 5X PCR buffer (New England Biolabs, UK) and 50 ng of genomic DNA. The PCR cycling conditions consisted of an initial denaturation step at 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 5 min

To screen for rs7439366 polymorphism of *UGT2B7*, 264-bp fragment containing the 802 C>T polymorphic site was amplified by PCR using the following primers: forward 5'-TATCTGAGACAATGGGGAAAGC-3' and reverse 5'-GTATCTGCTTTACCCCACCCA-3'. The PCR product was then digested with *BseGI* restriction enzyme (Thermo Fisher Scientific) at 55 °C for 1 h. Digestion of the PCR product by *BseGI* yields fragments that represent the presence of the T allele (264-bp fragment) and C allele

(199- and 65-bp fragments).

For detection of the *UGT2B7* rs7662029 polymorphism, a 365 bp fragment was amplified by PCR using the following primers: forward: 5'-GGGCTCTCCAACTGATTGTT-3' and reverse: 5'- TTGTCTCTTTGCCATC-CACA – 3'. Then, 5 μL of PCR product was digested by *BspPI* (Thermo Fisher Scientific) at 55 °C for 1 h. Electrophoresis of digestion products revealed two bands corresponding to A allele (254 and 111 bp fragments) and three bands corresponding to G allele (195, 111 and 59 bp fragments). Wild type (n = 10), heterozygous (n = 10) and homozygous variant (n = 10) genotypes were verified by direct sequencing using the same forward PCR primer (Applied Biosystems 3730xl DNA analyzer, USA). Schematic illustration of the novel PCR-RFLP assay, agarose gel electrophoresis showing the RFLP product sizes and DNA sequencing of PCR products for *UGT2B7* rs7662029 polymorphism are given in Fig. 1. The undigested and digested PCR products of both polymorphisms were separated by gel electrophoresis on a 2.5% agarose gel, visualized by ethidium bromide staining under an ultraviolet illuminator, and then scanned and photographed using the Syngene Monitoring System. For agarose gel analysis of the RFLP products of *UGT2B7* rs7662029 polymorphism, 2 μL SDS (2%) was used in order to high-resolution separation of bands.

2.4. Determination of buprenorphine and norbuprenorphine in human plasma

Plasma concentrations of BUP and its metabolite norBUP were measured by Liquid Chromatography Tandem Mass Spectrometry (LC–MS/MS) using a triple quadrupole SCIEX 5500 Qtrap (AB Sciex, Darmstadt, Germany). Substances were separated using 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). The conditions of the liquid chromatography, Mass Spectrometry (MS) and Electrospray Ionization (ESI) are tabulated in Table 1.

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 its metabolite 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.

Table 1
LC-MS/MS parameters for BUP and norBUP analysis.

Conditions	Explanation	
Mobile phase A	0.1% formic acid in water	
Mobile phase B	0.1% formic acid in methanol	
Flow rate	0.5 mL/min	
Gradient	**Time (min)**	**% B**
	0.01	10
	10.0	90
	15.0	90
	15.50	10
	18.00	10
Analysis time per sample (min)	7.6	
Injection volume	10 μL	
ES Conditions	**Explanation**	
Ionization mode	Positive	
Capillary voltage (V)	2800	
Drying gas flow (L/min)	10	
Drying gas temperature (C^o)	600	
Nebulizer gas (psi)	35	
Sheath gas flow (L/min)	12	
Sheath gas temperature (C^o)	350	
Nozzle voltage (V)	0	
MS Conditions	**Explanation**	
Scan	MRM	
Time segments	1:1.8 min - diverter valve to MS	
Delta EMV (+) (V)	400	

2.5. Measurement

Patients who have been receiving sublingual BUP/naloxone were also administered Clinical Opiate Withdrawal Scale (COWS), Beck Depression Inventory-II (BDI-II), Substance Craving Scale (SCS) and Beck Anxiety Inventory (BAI) in order to investigate the effects of *UGT2B7* polymorphisms and plasma buprenorphine concentrations on depression, anxiety, craving and withdrawal.

COWS was developed to assess a patient's level of opiate withdrawal over a period of time and to quantify their level of physical dependence on opioids. This scale is a clinician-administered, pen and paper instrument and measures eleven common opiate withdrawal signs or symptoms. Canan et al. (2015) demonstrated the validity and reliability of a Turkish version of the COWS. The clinical classification of scoring results is as follows: 5–12 suggests mild withdrawal, 13–24 suggests moderate withdrawal, 25–36 suggests moderately severe withdrawal and more than 36 suggests severe withdrawal.

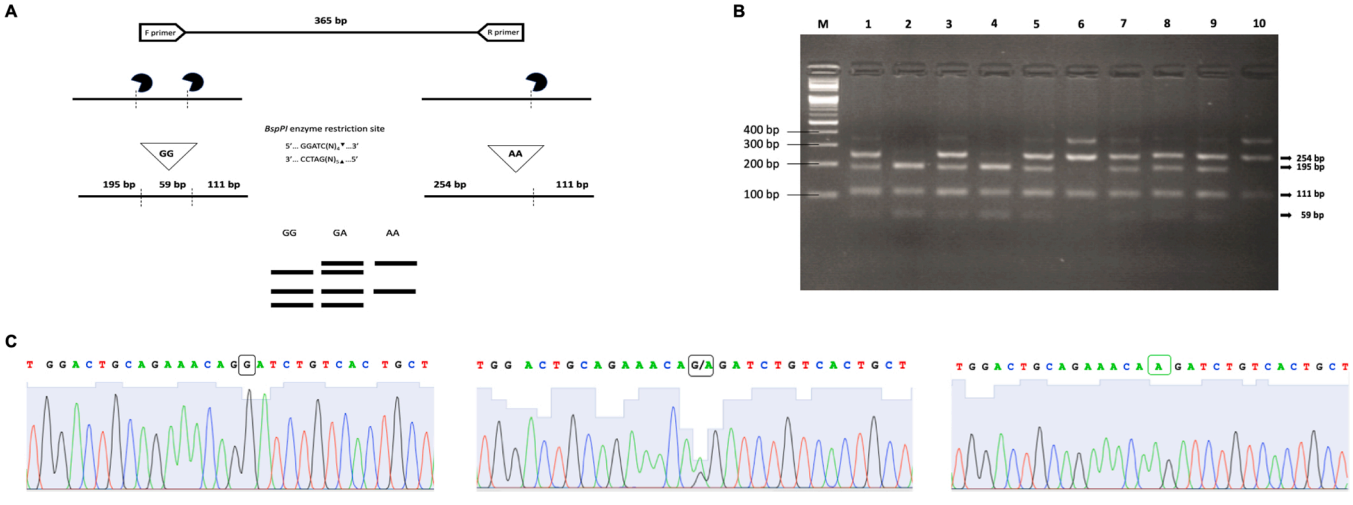

Fig. 1. (A) Schematic illustration of the novel PCR-RFLP assay (the *BspPI* recognition site and the diagrammatic representations of the restriction fragments for each *UGT2B7* rs7662029 genotypes were indicated), (B) Agarose gel electrophoresis showing the RFLP product sizes (M: 100 bp ladder; Lanes 2 and 4: homozygote wildtype (GG) (195 bp, 111 bp and 59 bp); Lanes 1,3,5,7,8 and 9: heterozygote genotype (GA) (254 bp, 195 bp, 111 bp and 59 bp); Lanes 6 and 10: homozygote mutant (AA) (254 bp and 111 bp), (C) DNA sequencing results of PCR products for *UGT2B7* rs7662029 polymorphism.

The validity and reliability of a Turkish version of the BDI was demonstrated by Hisli (1989). BDI is a Likert-type 21-item self-report tool developed to measure characteristic attitudes and symptoms of depression such as mood, suicidal ideas, fatigability, social withdrawal, punishment, guilt, indecisiveness, work difficulty, sense of failure, irritability and somatic preoccupation in psychiatric and normal populations. This scale ranges from 0 to 3 and the total score is calculated by the sum of item scores (ranging from 0 to 63).

Penn Alcohol Craving Scale (PACS) was developed to evaluate the desire for alcohol for the previous week. Evren et al. (2011) demonstrated the validity and reliability of a Turkish version of the PACS. SCS is the version of the Penn Alcohol Craving Scale (PACS) and evaluates the craving for substances other than alcohol dependence. SCS is a 5-item self-report measure scored between 0 and 6. The questions of this measure are about the frequency, intensity, and duration of craving.

BAI was developed to identify anxiety symptoms and to quantify their intensity in both adults and adolescents. It is a Likert-type 21-item self-report measure and ranges from 0 ("not at all") to 3 ("severely- I could barely stand it"). The total score is calculated by the sum of item scores (ranging from 0 to 63). The total score of 0–7 is interpreted as minimal, 8–15 as mild, 16–25 as moderate and 30–63 as severe. The validity and reliability of a Turkish version of the BAI was demonstrated by Ulusoy et al. (1998).

2.6. Statistical analyses

The Statistical Package for Social Sciences (SPSS) version 21.0 software for Windows was used for the statistical analyses. The Kolmogorov-Smirnov test was used to examine the normality of numerical variables. All data are shown as median and interquartile range (IQR) due to the non-normal distribution of numerical data. For categorical data, numbers, percentages and 95% confidence interval were given. The frequencies of the *UGT2B7* rs7439366 and rs7662029 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. 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). For each polymorphism, homozygote wild type, heterozygote and homozygote variant type were compared in view of plasma BUP concentrations, BUP/D and $BUP/D.kg^{-1}$ values, MR, measures' scores (craving, depression, anxiety and withdrawal) and characteristics of heroin use such as duration of heroin use (years), age at onset of dependence (years), quantity of heroin consumed (g/day). The patients' BUP concentrations and dose- and dose/kg-normalized plasma BUP concentrations among different *UGT2B7* functional genotypes were compared using Mann Whitney test or Kruskal-Wallis test, as appropriate. Pairwise comparisons were applied to correct type one errors resulting from multiple comparisons. The relationships between scores of measures and BUP concentrations and dose- and dose/kg-normalized plasma BUP concentration were analysed by the Spearman correlation test. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Sample demographics and characteristics

There was a total of 109 patients in the cohort, and of them, 100 were male (91.7%) and 9 were female (8.3%). The median age of the subjects was 27.0 years (Interquartile range (IQR): 25.0–32.0 years). Approximately 90% of the patients lived with a family member and 6 patients lived independently. Seventy-one patients were employed, while 38 were unemployed. Sixty-nine patients were single, while 34 were married, and six were divorced. Twenty-five (22.9%) patients had been incarcerated at least once in their lives. Comorbid conditions were characterized as substance use disorders (SUDs) and alcohol use disorder (AUD). Among the SUD, nicotine use was the most prevalent ($n = 109$), followed by alcohol use ($n = 41$), and cannabis use ($n = 11$).

Daily doses of BUP ranged from 2.0 mg/day to 10.0 mg/day (median 6.0 mg/day; IQR: 4.0–8.0 mg/day), equivalent to 0.02–0.16 mg/kg body weight (median 0.08 mg/kg body weight; IQR: 0.05–0.08 mg/kg body weight). According to the package insert for sublingual BUP, the FDA-approved maximum daily dose is 24 mg/day; none of the patients exceeded 24 mg/day during the treatment period. The median steady-state serum concentration of BUP was 0.19 ng/mL (IQR:0.11–0.35 ng/mL). After adjustment by dose and by dose/body weight, they were changed to 0.04 ng/mL per mg/day and to 2.58 ng/mL per mg/kg/day, respectively. The median withdrawal instances was 1 (IQR: 0–2), with a minimum of zero and a maximum of 4 instances. The median SCS score was 5 (IQR: 0–12) with a minimum of zero and a maximum of 25. In addition, there was a significant and negative correlation between craving and BUP/D, $BUP/D.kg^{-1}$ and daily BUP dose in all heroin addicts ($r = -0.320$, $p = 0.004$; $r = -0.295$, $p = 0.009$; $r = -0.284$, $p = 0.012$, respectively). Moreover, a significant and positive correlation was found between craving and anxiety ($r = 0.452$, $p = 0.001$).

3.2. The median plasma concentrations of BUP and norBUP, across UGT2B7 rs7439366 genotype subgroups

The allele and genotype frequencies, 95% confidence interval and Hardy–Weinberg equilibrium (HWE) of *UGT2B7* rs7662029 and rs7439366 polymorphisms in 109 Caucasian Turkish heroin addicts were shown in Table 2. Whereas the genotype frequencies of *UGT2B7* rs7662029 polymorphism is consistent with Hardy–Weinberg equilibrium ($p > 0.05$), *UGT2B7* rs7439366 polymorphism is significantly different from HWE ($p < 0.05$) and there were no homozygous TT individuals in the cohort. There were no statistically significant differences in age, daily drug dosage (mg/day), body height and weight, and gender distribution among both the *UGT2B7* rs7662029 and rs7439366 genotype subgroups (Table 3).

The median plasma concentrations of BUP and norBUP did not differ significantly among two *UGT2B7* rs7439366 genotype subgroups; although the median value of BUP was higher and the median norBUP plasma concentration was lower in CT genotype subgroup compared with the CC subgroup (Table 4). There was a statistically significant

Table 2
Allele and genotype frequencies of *UGT2B7* rs7662029 and rs7439366 polymorphisms observed in patients.

Allele					
rs7662029	N (218)	% Frequency (95% CI)	rs7439366	N (218)	% Frequency (95% CI)
A	104	47.8 (41.1–54.4)	C	139	63.8 (57.4–70.1)
G	114	52.2 (45.5–58.8)	T	79	36.2 (29.8–42.5)
Genotype					
rs7662029	N (109)	% Frequency (95% CI)	**rs7439366**	N (109)	% Frequency (95% CI)
AA	26	23.9 (15.9–31.9)	CC	30	27.5 (19.1–35.8)
GA	52	47.7 (38.3–57.1)	CT	79	72.5 (64.1–80.84)
GG	31	28.4 (19.9–36.8)	TT	0	NA*
HWE p-value	$\chi2 = 0.21$ $p = 0.06$		HWE p-value	$\chi2 = 35.21$ $p = 0.001$	

N:number, CI: Confidence Interval, HWE: Hardy-Weinberg Equilibrium, *NA: non-available because calculation of CI requires $n \geq 5$.

Table 3
Characteristics of the *UGT2B7* genotype subgroups with heroin addiction.

Characteristics	rs7662029			P value	rs7439366		P value
	AA	GA	GG		CC	CT	
Age (years) Median (min.-max)	28.5 (23–53)	27 (19–49)	27 (20–50)	0.134 $\chi2 = 4.010$	27 (20–50)	27 (19–53)	p = 0.463 Z = −0.735
Gender (males/females)	24/2	49/3	27/4	0.517	26/4	74/5	p = 0.235
Body height (cm) (mean±S.D.) (min.-max.)	173 ± 8.6 (150–185)	174.4 ± 7.1 (157–185)	174.9 ± 8.5 (159–194)	0.626 F= 0.471	174.5 ± 8.1 (159–194)	174.2 ± 7.8 (150–190)	p = 0.594 F= 0.286
Body weight (kg) (mean±S.D.) (min.-max.)	64 (38–150)	65 (48–157)	67 (43–100)	0.791 $\chi2 = 0.47$	67.5 (43–100)	65 (38–101)	p = 0.946 Z = −0.068
Drug dosage (mg/day) (Median, min.-max)	6 (2–8)	4 (2–10)	6 (2–8)	0.191 $\chi2 = 3.31$	6 (2–8)	4 (2–10)	p = 0.06 Z = −1.885

S.D.: standard deviation, min: minimum, max: maximum

Table 4
Comparison of heroin addicts according to the *UGT2B7* rs7439366 and rs7662029 genotypes in view of plasma BUP- and norBUP-related concentrations.

UGT2B7 rs7439366 genotypes	BUP (ng/mL)	norBUP (ng/mL)	BUP/D ng/mL per mg/day	BUP/D.kg^{-1} ng/mL per mg/kg/day	norBUP/D ng/mL per mg/day	norBUP/BUP
CC (n = 30)	0.18 (0.08–0.33)	0.46 (0.22–0.63)	0.024 (0.01–0.062)	1.31 (0.93–3.17)	0.076 (0.006–0.311)	2.3 (0.14–23.76)
CT (n = 79)	0.20 (0.11–0.37)	0.34 (0.18–0.60)	0.046 (0.021–0.10)	3.21 (1.41–6.57)	0.068 (0.003–0.444)	1.51 (0.08–24.47)
Mann-Whitney U test	U= 1018.5 p = 0.259 Z = −1.130 r = 0.11	U= 1018.5 p = 0.259 Z = −1.130 r = 0.11	U= 810.0 **p = 0.011** Z = −2.544 r = 0.24	U= 800.5 **p = 0.009** Z = −2.609 r = 0.25	U= 1148.5 p = 0.804 Z = −0.248 r = 0.02	U= 1027.0 p = 0.284 Z = −1.072 r = 0.10
***UGT2B7* rs7662029 genotypes (Co-dominant model)**						
AA (n = 26)	0.17 (0.10–0.35)	0.33 (0.19–0.64)	0.037 (0.02–0.08)	2.12 (1.22–5.07)	0.067 (0.02–0.44)	1.79 (0.2–24.47)
GA (n = 52)	0.21 (0.12–0.40)	0.33 (0.16–0.51)	0.059 (0.02–0.11)	3.30 (1.56–6.99)	0.072 (0.003–0.44)	1.34 (0.08–21.14)
GG (n = 31)	0.19 (0.08–0.33)	0.45 (0.23–0.62)	0.024 (0.01–0.07)	1.35 (0.99–4.63)	0.077 (0.006–0.311)	2.23 (0.14–23.76)
Kruskal-Wallis test	$\chi2 = 1.784$ p = 0.410	$\chi2 = 1.75$ p = 0.417	$\chi2 = 7.26$ *p = **0.027**	$\chi2 = 6.70$ * ***p = 0.035**	$\chi2 = 0.08$ p = 0.961	$\chi2 = 2.015$ p = 0.365
***UGT2B7* rs7662029 genotypes (G-Recessive model)**						
GA+AA§ (n = 78)	0.19 (0.03–1.56)	0.33 (0.03–1.78)	0.045 (0.0–0.25)	3.2 (0.0–14.4)	0.068 (0.0-0.044)	1.43 (0.08–24.5)
Mann-Whitney U test	U= 1059.5 p = 0.315 Z = −1.004	U= 1032.5 p = 0.236 Z = −1.186	U= 852.0 **p = 0.016** Z = −2.398	U= 855.5 **p = 0.018** Z = −2.375	U= 1193.5 p = 0.917 Z = −0.104	U= 1053.0 p = 0.295 Z = −1.048
***UGT2B7* rs7662029 genotypes (G-Dominant model)**						
GG+GA§§ (n = 83)	0.21 (0.04–1.8)	0.37 (0.03–1.8)	0.04 (0.0–0.3)	2.34 (0.0–14.4)	0.08 (0.03–0.44)	1.7 (0.08–23.8)
Mann-Whitney U test	U= 1013.0 p = 0.639 Z = −0.469	U= 1060.5 p = 0.895 Z = −0.132	U= 1036.5 p = 0.763 Z = −0.302	U= 1061.5 p = 0.901 Z = −0.124	U= 1049.5 p = 0.834 Z = −0.210	U= 1005.0 p = 0.599 Z = −0.526

* Pairwise comparisons of *UGT2B7* rs7662029 subgroups for BUP/D: group GG was significantly different to group GA (p = 0.022). Groups AA:GG and AA:GA were not significantly different (p = 0.719 and p = 0.659, respectively).* * Pairwise comparisons of *UGT2B7* rs7662029 subgroups for BUP/D.kg^{-1}: group GA was significantly different to group GG (p = 0.029). Groups GG:AA and AA:GA were not significantly different (p = 0.605 and p = 0.911, respectively). §compared to GG, §§compared to AA

difference between rs7439366 genotype subgroups with regard to median dose normalized BUP (BUP/D) and dose/kg-normalized BUP (BUP/D.kg^{-1}) values as determined by Mann-Whitney U test. BUP/D and BUP/D.kg^{-1} values were significantly higher in CT genotype subgroup as compared to CC subgroup (Fig. 2). In addition to that, the median norBUP/BUP value representing the metabolic activity of BUP to norBUP conversion was lower in CT genotype subgroup compared to CC subgroup, although the difference observed between *UGT2B7* rs7439366 genotype subgroups was not statistically significant (p = 0.28).

3.3. The median plasma concentrations of BUP and norBUP, across UGT2B7 rs7662029 genotype subgroups

In Table 4, the effects of *UGT2B7* rs7662029 polymorphisms on median BUP and norBUP plasma concentrations, BUP/D, norBUP/D, BUP/D.kg^{-1} and norBUP/BUP values were also given. The median BUP and norBUP plasma concentrations did not differ significantly among the rs7662029 genotype subgroups (p > 0.05). A Kruskal-Wallis H test showed that there was a statistically significant difference in BUP/D between the rs7662029 genotype subgroups, with a median BUP/D of 0.037 ng/mL per mg/day for AA genotype, 0.059 ng/mL per mg/day for GA genotype and 0.024 ng/mL per mg/day for GG genotype. In addition, there was also a statistically significant difference between

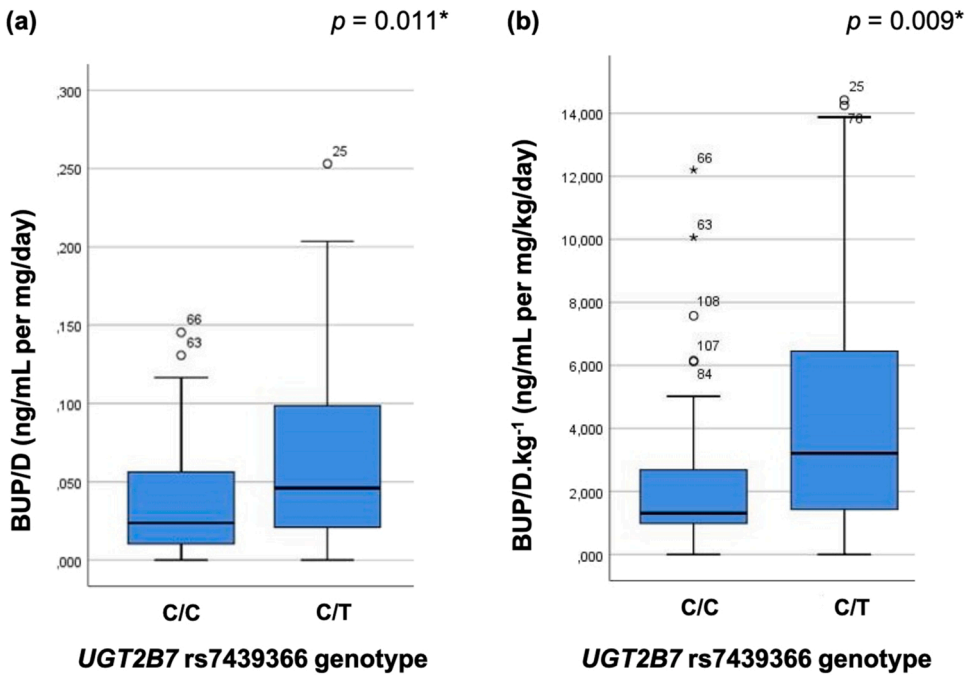

Fig. 2. *UGT2B7* rs7439366 genotypes and dose-normalized and dose/kg- normalized BUP concentrations. (**a**) Distribution of median dose normalized concentration of BUP and (**b**) dose/kg- normalized BUP concentrations in patients with CC and CT. Mann–Whitney U test confirmed that *UGT2B7* wild type genotype had increased metabolism of BUP (p = 0.011 and p = 0.009, respectively).

rs7662029 genotype subgroups with regard to BUP/D.kg^{-1} values as determined by Kruskal-Wallis test. Pairwise comparison analyses revealed that rs7662029 GG subgroup was significantly different to GA subgroup. Subgroups AA:GG and AA:GA were not significantly different (Table 4, Fig. 3). The median norBUP/D was lowest in rs7662029 AA subgroup although the difference observed between rs7662029 genotype subgroups was not statistically significant. No statistically significant difference for norBUP/BUP ratio was found between rs7662029 genotype subgroups ($\chi 2 = 2.015$, P = 0.365) although this ratio was higher in GG genotype subgroup compared to wild type and heterozygote genotype subgroups.

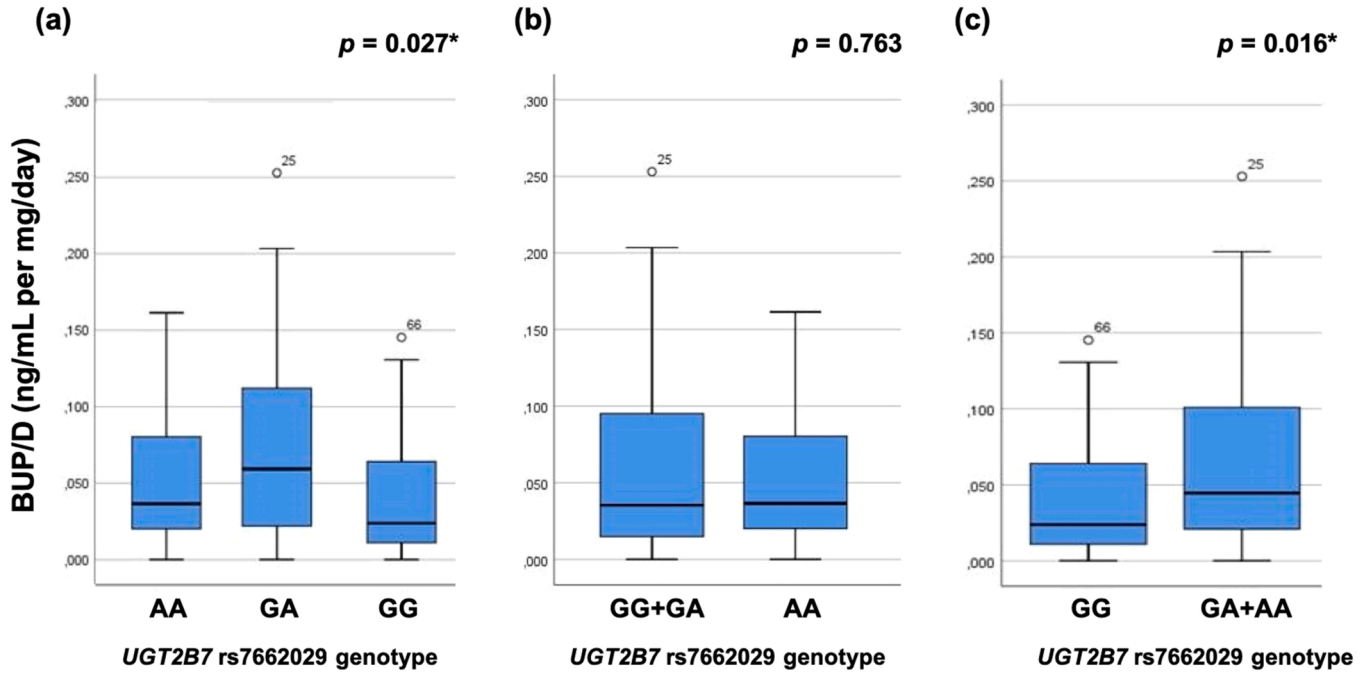

Fig. 3. (a) The Kruskal–Wallis test revealed a significant difference in dose normalized BUP concentrations (BUP/D) between each genotype group (AA, GA, and GG) for the rs7662029 polymorphism (p = 0.027). (b) The Mann–Whitney U test revealed no significant difference in BUP/D between the GG+GA group and AA group (p = 0.763). (c) This difference was significant between GA+AA group and GG group (p = 0.016). This might suggest that A allele of the rs7662029 polymorphism decreased the UGT2B7's activity of glucuronidation of BUP to glucuronide conjugates.

3.4. Opioid craving and withdrawal, depression and anxiety, across *UGT2B7* rs7662029 and rs7439366 genotypes

Table 5 showed scores of craving (SCS), withdrawal (COWS), depression (BDI-II) and anxiety (BAI) by *UGT2B7* rs7662029 and rs7439366 genotypes in heroin addicts. Median SCS, COWS, BDI-II and BAI scores did not differ significantly between two *UGT2B7* rs7439366 genotype subgroups. However, scores of SCS and BAI were higher and BDI-II score was lower in CC genotype subgroup compared to CT.

Kruskal-Wallis H test showed that there was a statistically significant difference between the rs7662029 genotype subgroups in median SCS score, with a median SCS score of 9.00 for AA genotype, 3.00 for GA genotype and 6.00 for GG genotype (Table 5). Pairwise comparison analyses revealed that rs7662029 AA subgroup was significantly different to GA subgroup. The median BDI-II score was highest in rs7662029 AA subgroup although the difference observed between rs7662029 genotype subgroups was not statistically significant. The effects of *UGT2B7* rs7662029 and rs7439366 polymorphisms on characteristics of heroin use such as duration of heroin use, age at onset of dependence, quantity of heroin consumed (g/day) were also analysed and there were no noticeable differences between *UGT2B7* rs7662029 and rs7439366 variant and wild type subgroups (data was not shown).

4. Discussion

To our knowledge, this is the first study investigating the relationship between *UGT2B7* genotypes and BUP metabolism in heroin addicts treated with BUP/naloxone combination. Glucuronidation is a very important metabolic pathway of BUP and norBUP. Based on a study with 63Ni electroncapture gas chromatographic assay, BUP was excreted mainly glucuronide conjugated metabolites (Fihlman et al., 2018) and 70% of a buprenorphine dose was glucuronides (Brown et al., 2011).

UGTs are glycoproteins that glucuronidate many endogenous compounds, some nonsteroidal anti-inflammatory drugs and clinically important opioids such as morphine and buprenorphine in humans (Wiener et al., 2004; Sastre et al., 2015). UGTs, a superfamily of enzymes, are divided into 3 families (UGT1, UGT2 and UGT8) that have been shown to be expressed in humans (Gall et al., 1999). In a study by Rouguieg et al. (2010), the contribution of UGT2B7 in the metabolism of BUP was found higher compared to that of UGT1A1 (41% vs. 10%). The *UGT2B7* gene encoding the UGT2B7 enzyme is localized in the chromosomal 4q13 region and some polymorphisms affecting the UGT2B7 enzyme activity were described so far (Bendaly et al., 2004; Wiener et al., 2004; Blevins-Primeau et al., 2009; Gall et al., 1999). However, little is known on the relationship between sublingual buprenorphine metabolism and the genetic polymorphisms of *UGT2B7*. Thus, whether individual differences at two polymorphic sites (rs7439366 and rs7662029) in the *UGT2B7* gene could affect the buprenorphine metabolism and treatment responses remains to be determined.

In the present study, *UGT2B7* rs7439366 TT individuals were not observed in the cohort and it was found that this polymorphism is significantly different from HWE. The expected frequency of TT genotype should be 14.3%. Although there were no homozygous TT individuals, most of the individuals (n = 79, 72.5%) had heterozygote genotype (CT). On the basis of this data, the allele frequency of C allele was 0.64 and the frequency for T allele was 0.36. In parallel line with our finding, Senturk-Ciftci et al. (2017) reported the *UGT2B7* CC, CT, and TT genotype frequencies as 24 (36.9%), 36 (55.4%), and 5 (7.7%), respectively, in Turkish renal transplants patients. These two studies indicated that the frequency of *UGT2B7* rs7439366 T allele was low in Turkish population unlike the other Caucasian populations where allele frequencies for C and T are almost similar (Bhasker et al., 2000). The reason of the deviation from the HWE for *UGT2B7* rs7439366 polymorphism may be due to the small sample sizes of Turkish studies, which causes random error in allele frequencies. Thus, it seems that further studies with more Turkish individuals are needed in order to clarify this deviation from HWE.

In our analysis, BUP/naloxone-treated heroin addicts with the *UGT2B7* rs7439366 CT genotype had significantly higher plasma BUP/D and BUP/D.kg^{-1} concentrations compared to carriers with CC genotype. Although the difference was not statistically significant, addicts with *UGT2B7* rs7439366 CT took lower doses of medicine compared to those with *UGT2B7* CC (4 mg/day vs. 6 mg/day). It might be speculated that higher portion of BUP/D and BUP/D.kg^{-1} concentrations in plasma may result from more slowly elimination of drug due to the *UGT2B7* rs7439366 variation related with decreased metabolic activity. Because of a decreased buprenorphine metabolism, it would be expected that heroin addicts carrying at least one T allele (CT or TT) *UGT2B7* rs7439366 take lower daily drug dosage due to higher exposure to BUP and its metabolite norBUP during the treatment with this drug compared to those carrying CC genotype. This is in line with many previous studies suggesting that homozygous TT genotype carriers displayed reduced glucuronidating activity (Wiener et al., 2004; Blevins-Primeau et al., 2009; Barbier et al., 2000; Gall et al., 1999; Romero-Lorca et al., 2015; Luo et al., 2020; Wang et al., 2011; Parmar et al., 2011). On the other hand, Coffman et al. (1998), Thibaudeau et al. (2006), Sawyer et al. (2003), Girard et al. (2003), Sastre et al. (2015) and Yang et al. (2017) reported that the polymorphism of *UGT2B7* at codon 268 was associated with higher enzymatic activities. In addition, there have been studies reported no influence of this polymorphism on *UGT2B7* protein (Court et al., 2003; Peterkin et al., 2007; Bernard et al., 2006; Blanco et al., 2016). Therefore, the results of studies examining the functional effect of *UGT2B7* rs7439366 on drug metabolism have been conflicting.

In previous in vitro studies with cell lines such as the non-UGT-expressing HK293 cell line as well as cell lines overexpressing wild-type or variant UGTs, a significant decrease in glucuronidation activity for the *UGT2B7* rs7439366 TT genotype was found. This functional role of this polymorphism was shown for various substrates including tobacco carcinogen metabolites, tamoxifen, procarcinogenic metabolites of benzo(a)pyrene (BaP), steroid hormones (androgens and estrogens) (Bendaly et al., 2004; Wiener et al., 2004; Blevins-Primeau et al., 2009; Gall et al., 1999). These findings indicated that the enzymatic activity of UGT2B7 decreases due to *UGT2B7* rs7439366 polymorphism regardless of its substrates. On the other hand, Coffman et al. (1998)

Table 5
Total scores of SCS, COWS, BDI-II and BAI of heroin addicts according to *UGT2B7* rs7439366 and rs7662029 genotypes.

UGT2B7 rs7439366 genotypes	SCS score	COWS score	BDI-II score	BAI score
CC (n = 30)	6 (0–23)	1 (0–4)	15 (1–55)	13 (0–63)
CT (n = 79)	4 (0–25)	1 (0–4)	18 (1–48)	9 (0–52)
Mann-Whitney U test	U= 988.5 p = 0.178 Z = −1.346 r = 0.12	U= 1093.0 p = 0.698 Z = −0.388 r = 0.04	U= 1067.5 p = 0.728 Z = −0.347 r = 0.03	U= 955.5 p= 0.141 Z = −1.473 r = 0.14
***UGT2B7* rs7662029 genotypes**				
AA (n = 26)	9 (0–25)	1 (0–4)	22 (1–40)	12 (0–49)
GA (n = 52)	3 (0–23)	0 (0–4)	16 (2–48)	8 (0–52)
GG (n = 31)	6 (0–3)	1 (0–4)	16 (1–55)	12 (0–63)
Kruskal-Wallis test	χ2 = 8.34 ***p = 0.015**	χ2 = 6.89 ****p = 0.032**	χ2 = 0.513 p = 0.774	χ2 = 3.417 p = 0.181

* Pairwise comparisons of *UGT2B7* rs7662029 subgroups for SCS score: group GA was significantly different to group AA (p = 0.037). Groups AA:GA and AA:GG were not significantly different (p = 0.077 and p = 1.00, respectively).

** Pairwise comparisons of *UGT2B7* rs7662029 subgroups for COWS score: group GA was significantly different to group GG (p = 0.029). Groups GA:AA and AA:GG were not significantly different (p = 0.553 and p = 0.712, respectively).

suggested that the change in amino acid sequence due to the base change have a minimal influence on enzyme activity. They hypothesized that UGT2B7 may select its substrates according to the amino terminal half of the protein and the His268Tyr amino acid substitution changes the affinity for buprenorphine. Furthermore, Wang et al. (2011) suggested that codon 268 is located within the substrate binding domain and amino acid residue at position 268 could be a key region of UGT2B7 protein. The other hypothesis for the functional effect of this polymorphism is that another promoter polymorphism (at position −79) linked together with *UGT2B7* TT and decreases the transcription rate of the UGT2B7 enzyme (Duguay et al., 2004). Our findings showing the statistically significant differences in dose-normalized BUP and dose/kg-normalized BUP concentrations between *UGT2B7* rs7439366 genotype subgroups seemed to support the studies showing a decrease in enzyme activity due to the amino acid substitution, which are inconsistent with observations reported for transdermal buprenorphine (Sastre et al., 2015; Blanco et al., 2016). Hitherto, only Sastre and co-workers (2013) and Blanco and co-workers (2016) determined the effect of *UGT2B7* rs7439366 polymorphism on buprenorphine metabolism. While Sastre et al. suggested an increased activity for the T allele; Blanco et al. reported no effect of this polymorphism on BUP metabolism. On the contrary to these previous reports, in the present study, we examined the effect of this polymorphism on sublingual BUP metabolism and suggested a higher enzymatic activity for the *UGT2B7* rs7439366 CC genotype. These conflicting results for the inter-individual variability in BUP metabolism indicated that further investigations are needed to improve our knowledge of the effects of *UGT2B7* rs7439366 on BUP metabolism.

In the present study, median BUP/D and BUP/D.kg^{-1} values were significantly lower in rs7662029 GG genotype subgroup compared to AA and AG genotype subgroups. Furthermore, it should be noted that heterozygote GA individuals have the highest median BUP/D and BUP/D.kg^{-1} values. This indicates that UGT2B7's activity of glucuronidation of BUP to glucuronide conjugates is significantly higher in wild type allele carriers. In addition, addicts with rs7662029 GG genotype take more doses of medicine (6 mg/day) compared to those with A variant allele (AA+GA) (4 mg/day). Furthermore, the difference between variant and heterozygote groups regarding to the median score of craving was statistically significant. AA genotype subgroup's craving score was higher compared to subgroup having at least one G allele due to most likely higher BUP/D values. According to this data, heroin addicts with AA and GA genotypes seemed to possess lower levels of enzymatic activity and eliminate buprenorphine more slowly. The functional effects of SNP rs7662029 on *UGT2B7* gene expression have so far been unclear due to the limited studies published. Tian et al. (2012) examined the impact of 12 SNPs including rs7662029 in *UGT2B7* gene on methadone treatment and suggested that these SNPs may play important roles in opiate withdrawal symptoms. Cilião et al. (2017) observed an association between this polymorphism and the incidence of graft rejection episodes in 246 Brazilian patients with kidney transplant and suggested rs7662029 SNP is functional due to in strong linkage disequilibrium (LD) with the polymorphism rs7438135. Hu et al., 2014 demonstrated this LD between the polymorphisms rs7662029 and rs7438135 that of G allele showed a decrease (50%) in promoter activity of the gene. According to Hu's report, the 23 prevalent polymorphisms including rs7662029 and rs7438135 in the 4-kb *UGT2B7* promoter are linked together. However, the rs7662029 polymorphism is an intronic variant in *UGT2B7* gene and could not have an effect on promoter activity of the *UGT2B7* gene. Holthe et al. (2003) sequenced six exons and the 5′ regulatory sequences of the human *UGT2B7* gene. According to the updated version of the nucleotide sequence of the *UGT2B7* gene proximal promoter, at position − 327 (as mentioned in Hu et al., 2014), there is not a single base change from guanine to adenine. Therefore, we hypothesized that the effect of *UGT2B7* rs7662029 might not be due to its LD with other variants in the 4-kb *UGT2B7* promoter region. Thus, whether this intronic SNP is in LD with other functional SNP(s) or whether it exerts a direct effect on *UGT2B7* gene expression has still been an open question. Further investigation is warranted to explore the mechanisms by which rs7662029 variants exert their effects on gene expression and, also, enzyme activity.

In the present study, the effects of *UGT2B7* gene polymorphisms on depressive symptoms, craving, anxiety, withdrawal measured by the Beck Depression Inventory-II, Substance Craving Scale, Beck Anxiety Inventory and Clinical Opiate Withdrawal Scale, respectively, were also analysed. There was not a statistically significant difference between *UGT2B7* rs7439366 genotype subgroups with regard to median scores of BDI-II, COWS, SCS and BAI. However, the median score of BDI-II was higher in heroin addicts with CT genotype than those with CC genotype. As mentioned above, heroin addicts with CT genotype took less daily BUP dose due to higher median BUP/D and BUP/D.kg^{-1} values compared to those with CC genotype. According to these data, it could be expected that the increased depressive symptoms might be due to low levels of daily BUP dosage in heroin addicts with CT genotype compared to those with CC genotype. It is well known that BUP has an antidepressant effect, which is related to its antagonistic activity at kappa-opioid receptors (Segui et al., 2020; Falcon et al., 2016). In addition, craving and anxiety were found lower in heroin addicts with CT genotype most probably due to higher BUP/D and BUP/D.kg^{-1} values compared to heroin addicts with CC genotype. Decreased craving might be related to higher plasma BUP/D values and reduced anxiety in the present study. Buprenorphine target opioid receptors and exert its effect by reducing opioid cravings (Segui et al., 2020). A significant and negative correlation between craving and BUP/D, BUP/D.kg^{-1} and daily BUP dose indicated that craving is affected by plasma BUP concentration. Moreover, it could be suggested that decreased craving could lead a reduction in anxiety in heroin addicts due to the finding showing a significant and positive correlation between craving and anxiety.

Unlike to *UGT2B7* rs7439366 polymorphism, there was a statistically significant difference between *UGT2B7* rs7662029 genotype subgroups with regard to median scores of SCS and COWS. It should be noted that heterozygote GA individuals have the lowest median SCS score. Pairwise comparisons of *UGT2B7* rs7662029 subgroups for SCS score showed that group GA had significantly lower craving score than group AA ($p = 0.037$). This significant difference seemed to be due to higher plasma BUP/D and BUP/D.kg^{-1} concentrations. Interestingly, score of COWS was significantly different between *UGT2B7* rs7662029 AA and GA genotype subgroups although heroin addicts with these genotypes treated with suboxone at least 10 days. It would be expected that these addicts would not have withdrawal symptoms. Our findings concerning withdrawal may indicate that this difference was due to genetic variation in *UGT2B7* gene affecting the plasma BUP levels.

PCR-RFLP is still a common, inexpensive, rapid and sensitive method to determine the single nucleotide polymorphisms, although various assays such as TaqMan assay, fluorescently labelled probes technique and DNA sequencing have been developed recently (Feng et al., 2016). However, for the polymorphism site rs7662029 of *UGT2B7* gene, we could not find an appropriate restriction enzyme to recognize it in literature. Thus, in our opinion, this lack of RFLP assay limits the studies concerning the effect of *UGT2B7* rs7662029 variants on drug metabolism via UGT2B7 enzyme. There has been no published PCR-RFLP method designed to analyse *UGT2B7* rs7662029 gene so far. However, we found a dissertation by da Cruz (2015) in Portuguese that designed PCR forward and reverse primers. Then, for the present study, we found a new restriction enzyme using http://www.insilicase.com/Web/RFLP.aspx website. This improved PCR-RFLP assay was successfully performed to human blood samples and the reliability of the presented assay was verified by direct sequencing. Thus, it can be used in research laboratories with a limited budget especially in developing countries.

A limitation of our study is that it has been conducted on a limited number of women participants because the prevalence of heroin use was lower in females compared to males in Turkey due to social and economic reasons. A study with more samples from female heroin addicts

needs to be conducted in the future in order to assess the effect of gender and confirm our findings. Despite this limitation, our study brings to attention an inter-individual variability in plasma buprenorphine concentrations and responses due to the *UGT2B7* gene polymorphism. In the near future, we hope that our findings will contribute to personalized OUD treatment and will guide dosing decisions instead of empirical and trial-and-error dose selection, which will improve outcomes in maintaining the treatment of heroin addiction.

5. Conclusions

The current study indicated for the first time a prominent effect of the *UGT2B7* gene variations on sublingual buprenorphine metabolism and treatment responses. Furthermore, a novel, reproducible and practical PCR-RFLP assay was developed for the determination of *UGT2B7* rs7662029 polymorphism, based on utilizing novel restriction enzyme.

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CRediT authorship contribution statement

Dilek Kaya-Akyüzlü: Conceptualization, Methodology, Formal analysis, Investigation; Writing – review & editing. **Selin Özkan-Kotiloğlu:** Conceptualization, Methodology, Investigation; Writing – review & editing. **Ceylan Bal:** Methodology **Şafak Yalçın-Şahiner:** Resources. **Gamze Avcıoğlu:** Methodology. **Mustafa Danışman:** Resources.

Author contributions

DKA designed and directed the study. DKA and SÖK conducted the genetic analysis and prepared the manuscript. ŞYŞ and MD collected venous blood samples and demographic data of all subjects. CB performed measurement of plasma buprenorphine and norbuprenorphine levels with LC-MS/MS. GA contributed to laboratory analysis under the supervision of CB.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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