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Significant association of mu-opioid receptor 1 haplotype with tobacco smoking in healthy control subjects but not in patients with schizophrenia and alcohol dependence

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Abstract

Tobacco smoking is highly prevalent in patients with schizophrenia and alcohol dependence. The underlying neurobiology of nicotine addiction is complex. Rewarding effects of nicotine from cigarettes are associated, among others, with mu-opioid receptors encoded by the *OPRM1* gene. The aim of the study was to evaluate the association between two *OPRM1* gene polymorphisms, rs1799971 and rs510769, and tobacco smoking in Caucasian patients with schizophrenia, alcohol dependence, and healthy control subjects. The study included 1058 Caucasians (277 patients with schizophrenia, 359 patients with alcohol dependence, and 422 healthy control subjects), subdivided according to the nicotine dependence into smokers (i.e. current smokers) and non-smokers. A significant association was found between the GC haplotype (*OPRM1* rs1799971 and rs510769) and smoking in healthy controls, but not in patients with schizophrenia and alcohol dependence. A nominal association was detected in all cases/controls, but this significance did not survive the correction for the multiple testing. This is the first study to reveal that nicotine dependence is associated with the GC haplotype of the *OPRM1* rs1799971 and rs510769 in all subjects or specifically in healthy controls. These results did not confirm the strong connection between *OPRM1* polymorphisms and nicotine dependence in schizophrenia or alcohol dependence.

Keywords: OPRM1, rs1799971, rs510769, smokers, non-smokers

1. INTRODUCTION

Nicotine addiction induced by tobacco smoking is one of the most frequent addictions. According to WHO (2012) the prevalence of daily tobacco smoking among adults in EU ranges from 10-38 %. This addiction is associated with the increased health care costs, elevated socio-economical losses, tobacco-related morbidity, mortality and shortened lifespan (Lucatch et al., 2018). Proposed risk factors for higher nicotine dependence are male gender, smoking onset at younger age and less restrictions on smoking in East European countries (Kaleta et al., 2015). Tobacco smoking is highly prevalent in patients with schizophrenia and alcohol dependence (Dome et al., 2010; Glass et al., 2006; Sagud et al., 2009). Between 50-80% of patients with alcohol dependence show high level of nicotine dependence (Meyerhoff et al., 2006). Chronic tobacco smoking in patients with alcohol dependence might be associated with larger alcohol consumption and greater problems with withdrawal, compared with non-alcoholic smokers (Meyerhoff et al., 2006). More than 60% of schizophrenic patients are current smokers (Sagud et al., 2009). Among various hypotheses aiming to explain frequent smoking in schizophrenia, a "self-medication" hypothesis was proposed (Kumari and Postma, 2005) and opposed (Manzella et al., 2015), but patients with schizophrenia usually smoke to alleviate the symptoms and improve cognitive functions (Beck et al., 2015; Lucatch et al., 2018;).

The underlying neurobiology of nicotine addiction involves different targets and interactions between nicotine and nicotinic acetylcholine receptors that affect dopaminergic, noradrenergic, serotonergic, glutamatergic, GABAergic and opioid systems (Dome et al., 2010; Lucatch et al., 2018). Rewarding effects of nicotine are associated, among others, with mu-opioid receptors (Kuwabara et al., 2014), encoded by the *OPRM1* gene. Nicotine and other addictive substances induce the release of endogenous opioids that elicit dopamine release, associated with feelings of reward and reinforcement, leading to the nicotine dependence and smoking-related behaviors (Verhagen et al., 2012). Within the numerous *OPRM1* polymorphisms (Mura et al., 2013; Sery et al., 2010) the most frequently studied is a functional rs1799971 (A/G) polymorphism consisting of the adenine to guanine substitution, that causes the replacement of asparagine at position 40 with aspartic acid (Ducat et al., 2013), and leads to N-glycosylation site modification. Reports

associate *OPRM1* rs1799971 polymorphism with alcohol (Ducat et al., 2013), heroin (Haerian et al., 2013), and nicotine addiction (Domino et al., 2012; Hirasawa-Fujita et al., 2017), but there are also contradictory data (Frances et al., 2015; Kong et al., 2017; Munafo et al., 2013; Verde et al., 2011; Zhang et al., 2006). Another frequently studied polymorphism, *OPRM1* rs510769 (C/T), is located in the intronic region and can influence gene function by affecting alternative splicing (Mura et al., 2013). It was not associated with nicotine dependence in general population (Frances et al., 2015), but was found to be significantly related with smoking related phenotypes in adolescents (O'Loughlin et al., 2014). In addition, *OPRM1* haplotypes were reported to be associated with nicotine (Zhang et al., 2006), alcohol (Zhang et al., 2006), or substance dependence (Hoehe et al., 2000; Luo et al., 2003).

Since the findings from the literature are inconsistent, we aimed to evaluate, not only individual genotype association, but also a haplotype association of *OPRM1* rs1799971 and rs510769 polymorphisms with nicotine dependence (tobacco smoking) in Caucasian patients with schizophrenia, alcohol dependence and control subjects. We expected that nicotine dependence would be associated with particular *OPRM1* genotypes and haplotypes both in patients and control subjects.

2. MATERIALS AND METHODS

2.1. Experimental Subjects

This study included 1058 unrelated Caucasian subjects of Croatian origin, sampled from the same institution (University Hospital Vrapce, Zagreb, Croatia); 277 patients with schizophrenia (128 males and 149 females, 53.8 ± 1.4 years old), 359 patients with alcohol dependence (303males and 56 females, 60.1 ± 0.6 years old) and 422 healthy control subjects (373 males and 49 females, 51.1 ± 0.5 years old). The study was conducted with the approval of the Ethics Committee of the University Hospital Vrapce, Zagreb, Croatia, and in accordance with the ethical standards established by the 1975 Declaration of Helsinki. The procedures were discussed in details with the subjects, and they participated after they provided written informed consent. Inpatients with schizophrenia or alcohol dependence were diagnosed using the Structured Clinical Interview for DSM-IV (SCID) (First et al., 1995). All subjects were older than 18 years. For schizophrenia patients, exclusion criteria were diabetes, pregnancy, first-episode psychosis, and any

comorbid severe somatic or neurological disorders. Beside nicotine dependence, no other substance/alcohol dependence was present in the group of patients with schizophrenia or in healthy control subjects. Patients with alcohol dependence were admitted to the hospital due to acute intoxication or alcohol-induced withdrawal symptoms. Beside alcohol and nicotine dependence, no other co-morbid substance abuse or dependence was present**.** Alcohol-dependent patients and healthy individuals were medication-free, while patients with schizophrenia received different antipsychotics (Nedic Erjavec et al., 2017). Subjects were classified as smokers (subjects smoking ≥10 cigarettes per day, i.e. current smokers, n = 514) and non-smokers (i.e. a group of never smokers and former smokers, n = 544).

2.2. Genotyping

Blood samples were collected during routine check-ups. After DNA isolation from the peripheral blood with salting-out method (Miller et al., 1988), *OPRM1* rs1799971 and rs510769 genotypes were determined, using TaqMan™ SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) and Applied Biosystems 7300 Real-Time PCR System apparatus. The 10 μL reaction volume contained around 20 ng of DNA. Assay IDs were C_8950074_1_ for rs1799971 and C_809980_10 for rs510769. Around 10 % of randomly selected samples were genotyped twice as a quality control for genotyping assays.

2.3. Statistical analysis

Statistical evaluation was done using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). The Hardy–Weinberg equilibrium (HWE) was determined using χ^2-test (Rodriguez et al., 2009). Logistic regression analysis was used to evaluate whether sex and diagnosis are possible predictors of smoking status. Nicotine dependence and genotype and haplotype distributions were compared with χ^2-test or Fisher's exact test (Rodriguez et al., 2009), and odds ratio (*OR*) with 95% confidence interval (*CI*). Allelic (A vs. G or C vs. T) and dominant (AA vs. AG+GG or CC vs. CT + TT) analyses for both polymorphisms (Kong et al., 2017) were performed. After significant results of the χ^2-test, standardized residuals (R /i.e. R value more than 2/) (Field et al., 2012) were calculated to estimate which genotype significantly contributed to rejecting the null hypothesis. G∗Power 3 Software was used to

determine a priori sample size (Faul et al., 2009). Due to multiple testing (2 SNPs and 3 groups of subjects), *p* value was corrected 0.05/6=0.008. For χ^2-test (with a = 0.008; with expected small to medium effect size = 0.2; power (1 − b) = 0.800), the required sample size was $n = 362$ with $df = 2$; or $n = 306$ with $df = 1$. Since this study included 1058 participants, it had adequate sample size and statistical power to detect potential significant differences. Haploview version 4.2 was used to examine the linkage disequilibrium (LD) between *OPRM1* rs1799971 and rs510769 loci, and the association of *OPRM1* rs1799971 and rs510769 with nicotine dependence (Gabriel et al., 2002). Loci are considered to be in high LD if the *D'* coefficient is > 0.80 and logarithm of odds (LOD) ≥ 2 (Barret et al., 2005; Gabriel et al., 2002).

3. RESULTS

Out of 277 subjects with schizophrenia (46% males and 54% females), 65.3% were smokers, while 34.7% were non-smokers (Table 1). In the group of 359 patients with alcohol dependence (84% males and 16% females), 59.3% were smokers, while 40.7% were non-smokers (Table 1). Among 422 healthy participants (88% males and 12% females), 28.4% were smokers and 71.6% were non-smokers (Table 1). The frequency (χ^2 test) of smokers/non-smokers ($p < 0.001$) and male and female subjects ($p < 0.001$) was significantly different between all groups (Table 1). Logistic regression analysis evaluated whether sex and diagnosis are possible predictors of smoking status ($R_{adj}^2 = 0.075$; $df = 2$; $\chi^2 = 82.76$). Sex was not significant (*coefficient* = -0.244; $p = 0.100$; *OR* = 0.78; *95% CI* = 0.59 to 1.05), while diagnosis was a significant (*coefficient* = -0.653; $p < 0.001$; *OR* = 0.52; *95% CI* = 0.45 to 0.60) predictor in this model.

In the whole sample, the genotype frequencies of the *OPRM1* rs1799971 ($\chi^2 = 0.65$; $df = 2$; $p = 0.846$) and rs510769 ($\chi^2 = 1.43$; $df = 2$; $p = 0.753$) were within the expected HWE.

To evaluate possible gender-related differences in the frequency of the *OPRM1* genotype distribution (Frances et al., 2015), all subjects were subdivided according to gender. In schizophrenia patients, there were no significant sex related differences in the genotype distribution for *OPRM1* rs510769 ($\chi^2 = 0.90$; $df = 2$; $p = 0.637$), but significant differences were found in the genotype distribution for *OPRM1* rs1799971 ($\chi^2 = 47.62$; $df = 2$; $p < 0.001$) between male and female patients with schizophrenia. No significant

differences in the *OPRM1* rs510769 (χ^2 = 1.13; *df* = 2; *p* = 0.569) and rs1799971 (χ^2 = 0.29; *df* = 2; *p* = 0.863) genotype frequencies were detected between male and female patients with alcohol dependence, or in the distribution of the rs510769 (χ^2 = 5.51; *df* = 2; *p* = 0.063) and rs179997 (χ^2 = 0.35; *df* = 2; *p* = 0.838) genotypes between male and female healthy control subjects. Therefore, for the further analyses, only patients with schizophrenia were subdivided into male and female subjects when evaluating *OPRM1* rs1799971, while other groups were not divided according to gender.

The distribution (χ^2 test) of the *OPRM1* rs510769 (*p* = 0.532) genotypes did not differ significantly between patients with schizophrenia, alcohol dependence and healthy control subjects (Table 2). As the frequency of the *OPRM1* rs1799971 genotypes was significantly different (*p* < 0.001) between patients with schizophrenia, alcohol dependence and healthy control subjects (Table 2), due to the higher frequency of AG genotype in patients with schizophrenia (R = 4.4), allelic and dominant models were additionally evaluated. Between patients with schizophrenia, alcohol dependence and healthy control subjects, there were significant differences in the frequency of *OPRM1* rs1799971 A and G alleles (*p* = 0.0003), and in the frequency of AA homozygotes compared to G allele carriers (*p* = 0.00001), due to the significant contribution of the G carriers (*R* = 4.12) or G allele (*R* = 3.38) in patients with schizophrenia. No significant differences were detected in the frequency of *OPRM1* rs510769 C and T allele carriers (*p* = 0.419), or CC genotype carriers compared to the frequency of T allele carriers (*p* = 0.150), between patients with schizophrenia, alcohol dependence, and healthy control subjects.

To evaluate the association between tobacco smoking and *OPRM1* polymorphisms, all subjects were subdivided according to smoking status and diagnosis (Table 3). In patients with schizophrenia, the frequency (χ^2 test or Fisher exact test) of the *OPRM1* rs1799971 AA, AG and GG genotypes (*p* = 0.039) and of the AA homozygotes compared to G allele carriers (*p* = 0.021; *OR* = 0.54; *95% CI* = 0.36 to 0.48) was nominally different; but the frequency of A and G alleles (*p* = 0.155; *OR* = 0.69; *95% CI* = 0.25 to 0.34) was not significantly different between smokers and non-smokers. When patients with schizophrenia were subdivided according to gender, male smokers and non-smokers with schizophrenia had similar

frequency of the *OPRM1* rs1799971 AA, AG and GG genotypes ($p = 0.981$), A and G alleles ($p = 0.942$) and AA genotype vs. G allele carriers ($p = 0.934$). In female patients with schizophrenia, there was a nominally (χ^2 test) significant difference in the frequency of the *OPRM1* rs1799971 AA, AG and GG genotypes ($p = 0.010$), A and G alleles ($p = 0.037$), and AA homozygotes compared to G allele carriers ($p = 0.009$) between smokers and non-smokers. In patients with alcohol dependence, the frequency (χ^2 test or Fisher exact test) of the *OPRM1* rs1799971 genotypes ($p = 0.146$), A and G alleles ($p = 0.228$; *OR* = 1.35; *95% CI* = 0.16 to 0.23) and of the AA homozygotes compared to G allele carriers ($p = 0.135$; *OR* = 1.47 *95% CI* = 0.20 to 0.29) was not significantly different between smokers and non-smokers. In healthy control subjects, the distribution of the *OPRM1* rs1799971 AA, AG and GG genotypes ($p = 0.018$), A and G alleles ($p = 0.038$; *OR* = 0.61; *95% CI* = 0.16 to 0.23) and AA genotype vs. G allele carriers ($p = 0.015$; *OR* = 0.55; *95% CI* = 0.20 to 0.28) were nominally different between smokers and non-smokers, due to the presence of the GG genotype ($R = 1.7$) and the G allele ($R = 1.6$-1.9) in smokers (Table 3).

Distribution (χ^2 test or Fisher exact test) of *OPRM1* rs510769 CC, CT and TT genotypes ($p = 0.961$), C and T alleles ($p = 0.907$), and the CC vs. T allele carriers ($p = 0.898$) was not significantly different between smokers and non-smokers with schizophrenia (Table 3). In patients with alcohol dependence, the frequency of *OPRM1* rs510769 CC, CT and TT genotypes ($p = 0.418$), C and T alleles ($p = 0.923$), and the CC vs. T allele carriers ($p = 0.915$) did not differ significantly between smokers and non-smokers (Table 3). No significant differences were found in the frequency of the *OPRM1* rs510769 CC, CT and TT genotypes ($p = 0.891$), C and T alleles ($p = 0.760$), and the CC vs. T allele carriers ($p = 0.662$) between healthy control smokers and non-smokers (Table 3).

Haploview software (version 4.2) evaluated the possible haplotype association of *OPRM1* rs1799971 and rs510769 with smoking status, and LD plot revealed that *OPRM1* rs510769 and rs1799971 loci were highly linked ($D' = 0.86$). When subdivided into carriers of the AT, AC and GC haplotypes, either in all patients, or in patients with schizophrenia, alcohol dependence and healthy subjects, GC haplotype was the least common haplotype. The frequency of the GC haplotypes between all smokers and all non-smokers was nominally significant ($p = 0.0139$), and significantly different ($p = 0.0057$) between healthy control smokers

and non-smokers. There was a non-significant trend for the higher frequency of the GC haplotypes in smokers compared to non-smokers with schizophrenia (p = 0.0627) or alcohol dependence (p = 0.071). These findings confirmed that GC haplotype was strongly associated with nicotine dependence, and that this significance was most pronounced in all subjects (cases/controls) and significant in healthy controls (Table 4).

These results revealed that, besides the G allele of the *OPRM1* rs1799971, the C allele of the *OPRM1* rs510769 in combination with G allele was associated with nicotine dependence.

4. DISCUSSION

The present study revealed that: 1) the GC haplotype of the *OPRM1* rs1799971 and rs510769 polymorphisms was significantly associated with smoking in healthy controls, and nominally in all subjects, while this haplotype association remained at the trend-level in patients with schizophrenia and alcohol dependence; 2) the presence of the one or two G alleles of the *OPRM1* rs1799971 showed nominally significant association with nicotine dependence in patients with schizophrenia (especially in female patients) and in healthy control subjects, but not in patients with alcohol dependence; but due to the multiple testing correction, these differences did not remain significant; 3) *OPRM1* rs510769 polymorphism was not related to smoking in patients with schizophrenia, alcohol dependence and in healthy control subjects.

This is a first study showing a significant association between the GC haplotype of the *OPRM1* rs1799971 and rs510769 polymorphisms and smoking in all participants, especially in healthy controls, while a trend was detected in patients with schizophrenia or alcohol dependence. Although this GC haplotype block was the rarest among other *OPRM1* haplotypes, it was more frequently found in healthy smokers compared to non-smokers. The G allele of the *OPRM1* rs1799971 in combination with the C allele of the *OPRM1* rs510769 in a haplotype block was significantly associated with nicotine dependence, suggesting that GC haplotype conferred to an increased risk for nicotine dependence. However, these findings disagree with no association between smoking initiation or nicotine dependence and *OPRM1* rs1799971 and rs510769 genotypes or haplotypes in the population-based study of the individuals from the European ancestry (Zhang et al., 2006). The reasons for these discrepancies might be explained by the different study design

and different diagnoses, since present study included cases/controls from the same center, and the same ethnic (Croatian) origin, while Zhang et al. (2006) used population-based Virginia twin study of the European ancestry. Present study evaluated only 2 SNPs, out of which rs1799971 was individually, and in a haplotype block, associated with smoking, while the other study (Zhang et al., 2006), the frequency of other *OPRM1* haplotype combinations (i.e. rs2075572, rs10485057 and rs10485058) significantly differed between smokers and non-smokers, and the association between smoking initiation and rs9479757, rs2075572 and rs10485057 was detected. However, rs1799971 was in high LD with core haplotype markers associated with smoking initiation and nicotine dependence (Zhang et al., 2006). Therefore, inconsistent data might be explained with the high LD of rs1799971 with additional known or unknown causative SNPs (Zhang et al., 2006), ethnic or racial differences, number of subjects, differences in the characteristics of the analyzed cohorts, gender, age, different diagnostic entities and heterogeneity in the definition of the smoking-related phenotypes or different definition of significance (Mura et al., 2013; Verhagen et al., 2012).

The present results showed that *OPRM1* rs1799971 G allele is more common (only on a trend level) in Caucasian smokers compared to non-smokers, in line with previous data (Domino et al., 2012; Hirasawa-Fujita et al., 2017). More G alleles in smokers is expected due to a higher pleasurable nicotine effects associated with the G allele (Bernardi et al., 2016; Schuck et al., 2014), lower nicotine metabolite cotinine concentration in plasma of G allele carriers compared to the A allele carriers (Bernardi et al., 2016), and a higher number of cigarettes smoked per day in G allele compared to AA genotype carriers in patients with schizophrenia of mixed racial origin (Hirasawa-Fujita et al., 2017). Literature data suggest that the G allele might be associated with smoking related phenotypes (Chen et al., 2013; Fang et al., 2014; Vink et al., 2009). GWAS revealed that *OPRM1* is associated with smoking initiation (Chen et al., 2013). We have found slightly higher frequency of the G vs. A allele, or the G allele vs. AA genotype carriers in smokers compared to non-smokers in patients with schizophrenia and healthy control subjects. These results were not significant due to multiple testing correction. No significant associations were detected in patients with alcohol dependence. Collectively, our data agree with a lack of strong, significant association between

smoking and *OPRM1* rs1799971 observed in Caucasian (Frances et al., 2015; Munafo et al., 2013; Verde et al., 2011; Zhang et al., 2006) or Chinese (Fang et al., 2014) or Spanish (Verde et al., 2011) subjects, or between *OPRM1* rs1799971 and smoking related phenotypes (Verhagen et al., 2012). This absence of significant association could not be explained by the different distribution, since genotype frequency of the *OPRM1* rs1799971 in all our subjects was 71.3% for AA homozygotes, 26.7% for AG heterozygotes and 2.0% for GG homozygotes, which corresponds to the estimated *OPRM1* rs1799971 genotype frequency (70.2% for AA homozygotes, 27.2% for AG heterozygotes and 2.6% for GG homozygotes) in European population (1000 Genomes Project Consortium et al., 2015). Our present findings do not support the conclusion that *OPRM1* rs1799971 polymorphism is associated with smoking-related phenotypes (Ray et al., 2011). However, it was strongly associated with smoking in combination with *OPRM1* rs510769 polymorphism. Other *OPRM1* haplotype blocks with other alleles near this locus might also increase risk to other smoking-related phenotypes such as smoking initiation (Verhagen et al., 2012; Zhang et al., 2006). The mechanism by which the presence of the *OPRM1* rs1799971 G allele in a haplotype block might be associated with smoking is not yet clear. The study with [^{11}C]carfentanil PET imaging suggested that *OPRM1* rs1799971 G allele is associated with lower mu opioid receptor availability, leading to differences in the subjective reward effects of nicotine in different brain regions (Ray et al., 2011). Due to the reduction in mu opioid receptors in these brain regions, lower mRNA levels for these receptors were detected in the *post-mortem* brain in G allele carriers (Munafo et al., 2007).

In line with previous data (Frances et al., 2015; Munafo et al., 2007; Ray et al., 2006; Saccone et al., 2007; Zhang et al., 2005) significant gender related difference in the *OPRM1* rs1799971 genotype distribution was detected in patients with schizophrenia. In addition, *OPRM1* rs1799971 genotype and allele frequency differed significantly between patients with schizophrenia, alcohol dependence and healthy controls, due to the more frequent G allele presence in schizophrenia. These findings correspond to positive association between *OPRM1* rs1799971 polymorphism and schizophrenia in Han Chinese (Ding et al., 2013) or Caucasian i.e. Czech (Sery et al., 2010) subjects.

In line with other Caucasian data (Cupic et al., 2013), present study did not detect any significant association between *OPRM1* rs1799971 and *OPRM1* rs510769 polymorphisms and alcohol dependence. Racial (Hirasawa-Fujita et al., 2017) and ethnic (Sery et al., 2010) differences, population stratification, small sample sizes, false positives in case/control studies, might also affect *OPRM1* rs1799971 and rs510769 genotype distribution. In agreement with the lack of significant association observed between *OPRM1* rs510769 polymorphism and smoking initiation, nicotine dependence (Zhang et al., 2006) or smoking (Frances et al., 2015), our results confirmed that *OPRM1* rs510769 polymorphism was not related to smoking. This result differs from reports showing a significant association between *OPRM1* rs510769 polymorphism and nicotine dependence in ever smokers (Munafo et al., 2007), or with smoking-related phenotypes in adolescents (O'Loughlin et al., 2014). The *OPRM1* rs510769 genotype frequency in our study was similar to the estimated *OPRM1* rs510769 genotype frequency (57.9% for CC homozygotes, 36.4% for CT heterozygotes and 5.8% for TT homozygotes) in European population (1000 Genomes Project Consortium et al., 2015). Although in our study this individual SNP was not related to smoking, in a haplotype block, with *OPRM1* rs1799971 polymorphism, it was significantly associated with smoking. In healthy controls, the GC haplotype was strongly associated with nicotine dependence. This relationship might be explained by the significant association of nicotine metabolite cotinine in plasma and *OPRM1* rs510769 polymorphism (Schuck et al., 2014). Therefore, *OPRM1* haplotype combinations, and not individual SNPs, might be more strongly associated with smoking related phenotypes (Zhang et al., 2006). Some limitations and strengths need to be acknowledged. Our study comprised of only Caucasian subjects, but it might be considered as a replication of our previous study that included US patients with schizophrenia and bipolar disorder of different ethnic origin (Caucasians, African-American and others) (Hirasawa-Fujita et al., 2017), revealing that the present study results could be generalized. We included only two SNPs of the *OPRM1* gene, so additional SNPs or GWAS should be performed to find/confirm a significant association between risk alleles, haplotype blocks and smoking.

Advantages of this study are the large Caucasian cases/controls, sampled from the same center, diagnosed with SCID by the same psychiatrists, and a strict Bonferroni correction applied for the genotype and

haplotype analyses. The needed sample size and statistical power were calculated in advance and ensured that the study included enough subjects and power to detect significant differences.

This study revealed a link between the G allele of the *OPRM1* rs1799971 polymorphism and tobacco smoking. A novel association was detected between the *OPRM1* rs1799971-rs510769 GC haplotype and smoking in healthy control subjects.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Ethics approval was given by the Ethics Committee of the University Hospital Vrapce. The authors confirm that all procedures contributing to this study comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. All the experiments were undertaken with the understanding and written informed consent for each subject.

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DECLARATION OF INTEREST

Authors declare no competing interests.

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Table 1. Distribution of sex and smoking in patients with schizophrenia, alcohol dependence and healthy control subjects

	Sex		
	Male	**Female**	**χ^2- test**
	N (%)	**N (%)**	
Patients with schizophrenia N = 277	128 (46.2)	149 (53.8)	$\chi^2 = 184.14$; df = 2; **p < 0.001***
Patients with alcohol dependence N = 359	303 (84.4)	56 (15.6)	
Healthy control subjects N = 422	373 (88.4)	49 (11.6)	
	Smoking		
	Smokers	**Non-smokers**	**χ^2- test**
	N (%)	**N (%)**	
Patients with schizophrenia N = 277	181 (65.3)	96 (34.7)	$\chi^2 = 116.32$; df = 2; **p < 0.001***
Patients with alcohol dependence N = 359	213 (59.3)	146 (40.7)	
Healthy control subjects N = 422	120 (28.4)	302 (71.6)	

* significant difference after Bonferroni correction

Table 2. Genotype frequencies of *OPRM1* rs1799971 and rs510769 polymorphisms in patients with schizophrenia, alcoholism and healthy control subjects

***OPRM1* rs1799971**				
Genotype	**AA**	**AG**	**GG**	**χ^2- test**
	N (%)	**N (%)**	**N (%)**	
Patients with schizophrenia N = 277	161 (58.1)	112 (40.5)	4 (1.4)	$\chi^2 = 40.97$; df = 4; **p < 0.001***
Patients with alcohol dependence N = 359	271 (75.5)	84 (23.4)	4 (1.1)	
Healthy control subjects N = 422	323 (76.2)	86 (20.5)	13 (3.3)	
***OPRM1* rs510769**				
Genotype	**CC**	**CT**	**TT**	**χ^2- test**
	N (%)	**N (%)**	**N (%)**	
Patients with schizophrenia N = 277	161 (58.1)	98 (35.4)	18 (6.5)	$\chi^2 = 3.16$; df = 4; p = 0.532
Patients with alcohol dependence N = 359	193 (53.8)	139 (38.7)	27 (7.5)	
Healthy control subjects N = 422	253 (59.9)	142 (33.7)	27 (6.4)	

OPRM1: mu-opioid receptor 1 gene; * significant difference after Bonferroni correction

Table 3. Genotype frequencies of *OPRM1* rs1799971 and rs510769 polymorphisms in patients with schizophrenia, alcohol dependence and healthy control subjects subdivided into smokers and non-smokers

		***OPRM1* rs1799971**			
Genotype		**AA**	**AG**	**GG**	**χ^2- test**
		N (%)	**N (%)**	**N (%)**	
Patients with	**Smokers**	96 (53.0)	83 (45.9)	2 (1.1)	$\chi^2 = 6.51$; df = 2;
schizophrenia	N = 181				**p = 0.039***
N = 277	**Non-smokers**	65 (67.7)	29 (30.2)	2 (2.1)	
	N = 96				
Patients with alcohol	**Smokers**	167 (78.4)	45 (21.1)	1 (0.5)	$\chi^2 = 3.85$; df = 2;
dependence	N = 213				p = 0.146
N = 359	**Non-smokers**	104 (71.2)	39 (26.7)	3 (2.1)	
	N = 146				
Healthy control	**Smokers**	82 (68.3)	31 (25.8)	7 (5.8)	$\chi^2 = 8.03$; df = 2;
subjects	N = 120				**p = 0.018***
N = 422	**Non-smokers**	241 (79.8)	55 (18.2)	6 (2.0)	
	N = 302				
		***OPRM1* rs510769**			
Genotype		**CC**	**CT**	**TT**	**χ^2- test**
		N (%)	**N (%)**	**N (%)**	
Patients with	**Smokers**	106 (58.6)	63 (34.8)	12 (6.6)	$\chi^2 = 0.08$; df = 2;
schizophrenia	N = 181				p = 0.961
N = 277	**Non-smokers**	55 (57.3)	35 (36.5)	6 (6.3)	
	N = 96				
	Smokers	114 (53.5)	86 (40.4)	13 (6.1)	

Patients with alcohol	N = 213				$\chi^2 = 1.74$; df = 2;
dependence	**Non-smokers**	79 (54.1)	53 (36.3)	14 (9.6)	p = 0.418
N = 359	N = 146				
Healthy control	**Smokers**	74 (61.7)	39 (32.5)	7 (5.8)	$\chi^2 = 0.23$; df = 2;
subjects	N = 120				p = 0.891
N = 422	**Non-smokers**	179 (59.3)	103 (34.1)	20 (6.6)	
	N = 302				

OPRM1: mu-opioid receptor 1 gene; * not significant after Bonferroni correction

Table 4. The frequencies of *OPRM1* rs1799971 and rs510769 haplotypes in all subjects, as well as in patients with schizophrenia, alcohol dependence and healthy control subjects, subdivided into smokers and non-smokers

OPRM1 **Haplotype**	**Experimental Subjects**			**χ^2- test** **(non-smokers vs. smokers)**
	All subjects	**Smokers**	**Non-smokers**	
	N = 1058	N = 514	N = 544	
AT (%)	24.2	23.8	24.5	$\chi^2 = 0.13$; p = 0.714
AC (%)	60.5	58.7	62.1	$\chi^2 = 2.56$; p = 0.110
GC (%)	14.8	61.2	12.9	$\chi^2 = 6.05$; **p = 0.014***
	Schizophrenic patients	**Smokers**	**Non-smokers**	
	N = 277	N = 181	N = 96	
AT (%)	24.2	24.3	24.5	$\chi2 = 0.01$; p = 0.907
AC (%)	54.2	51.9	58.3	$\chi2 = 2.07$; p = 0.150
GC (%)	21.7	24.0	17.2	$\chi2 = 3.47$; p = 0.063
	Alcohol-dependent patients	**Smokers**	**Non-smokers**	
	N = 359	N = 213	N = 146	
AT (%)	26.6	25.9	27.5	$\chi2 = 0.23$; p = 0.631
AC (%)	60.6	63.1	57.1	$\chi2 = 2.60$; p = 0.107
GC (%)	12.5	10.6	15.2	$\chi2 = 3.27$; p = 0.071
	Healthy subjects	**Smokers**	**Non-smokers**	
	N = 422	N = 120	N = 302	
AT (%)	22.4	20.8	23.1	$\chi2 = 0.51$; p = 0.475
AC (%)	64.3	60.5	65.8	$\chi2 = 2.17$; p = 0.141
GC (%)	12.5	17.5	10.5	$\chi2 = 7.65$; **p = 0.006****

OPRM1: mu-opioid receptor 1 gene; * not significant after Bonferroni correction; ** significant difference after Bonferroni correction