Cannabinoid receptor type 2 gene is associated with comorbidity of schizophrenia and cannabis dependence and fatty acid amide hydrolase gene is associated with cannabis dependence in the Spanish population

El gen del receptor cannabinoide tipo 2 se asocia con la comorbilidad entre esquizofrenia y dependencia de cannabis y el gen de la enzima amidohidrolasa de ácidos grasos se asocia con la dependencia de cannabis en población española

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Abstract

The endocannabinoid system has been associated with various psychiatric disorders, such as schizophrenia or addictive disorders. Recent studies have found that some polymorphisms in the cannabinoid receptor type 2 (*CNR2*), cannabinoid receptor type 1 *(CNR1*) and fatty acid amide hydrolase (*FAAH*) genes could play an important role as risk factors in the etiology of these diseases. We analysed different cannabinoid gene polimorphisms from non-substance using patients diagnosed with schizophrenia (n = 379), schizophrenic patients with cannabis use disorders (n = 124), cannabis users who did not have psychoses (n = 71), and 316 controls from various Spanish hospitals and health centres. We found a statistical association between polymorphisms rs35761398 and rs12744386 in the *CNR2* gene and comorbidity of schizophrenia and cannabis dependence, as well as an association between loss of heterozygosity (overdominance) for polymorphism rs324420 in the *FAAH* gene and cannabis dependence in a Spanish population sample. The rs35761398 and rs12744386 polymorphisms in the *CNR2* gene are genetic risk factors for schizophrenia in cannabis-dependent subjects. Loss of heterozygosity for polymorphism rs324420 in the *FAAH* gene is a genetic risk factor for cannabis dependence in this population.

Key words: cannabis use disorder, schizophrenia, polymorphisms, cannabinoid receptor type 2 gene, cannabinoid receptor type 1 gene, fatty acid amide hydrolase gene.

Resumen

El sistema cannabinoide se ha asociado con varios trastornos psiquiátricos como la esquizofrenia y las adicciones. Diversos estudios han observado que algunos polimorfismos del receptor cannabinoide tipo 2 (*CNR2*), del receptor cannabinoide tipo 1 *(CNR1*) y del gen de la enzima amido hidrolasa de ácidos grasos (*FAAH*) pueden ser factores de riesgo de estos trastornos. Hemos analizado diversos polimorfismos del sistema cannabinoide en pacientes diagnosticados de esquizofrenia sin trastorno por uso de sustancias (n = 379), esquizofrenia con trastorno por uso de cannabis (n = 124), dependientes de cannabis sin psicosis asociada (n = 71) y un grupo de control (316) procedentes de diversos hospitales y centros de asistencia sanitaria españoles. Hemos encontrado una asociación entre los polimorfismos rs35761398 y rs12744386 del *CNR2* con la presencia de esquizofrenia y trastorno por uso de cannabis comórbido y una pérdida de heterocigosidad en el polimorfismo rs324420 del gen *FAAH* con la dependencia de cannabis en población española. Los polimorfismos rs35761398 y rs12744386 en *CNR2* son factores de riesgo para esquizofrenia en sujetos dependientes de cannabis. La pérdida de heterocigosidad en el polimorfismo rs324420 en el gen *FAAH* es un factor de riesgo para la dependencia de cannabis.

Palabras clave: trastorno por uso de cannabis, esquizofrenia, polimorfismos, gen del receptor cannabinoide tipo 2, gen del receptor cannabinoide tipo 1, gen de la enzima amido hidrolasa de ácidos grasos.

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Schizophrenia is a severe mental disorder with a worldwide prevalence of 0.5–1.0% and it has an enormous social and economic impact (Andreasen, 1995; Dong et al., 2019). Different epidemiologic studies have suggested that cannabis could be a risk factor for the development of schizophrenia (Marconi, Di Forti, Lewis, Murray & Vassos, 2016). Moreover, the well-known psychotropic effects of cannabinoids and the distribution of cannabinoid receptors in the brain suggest that the endocannabinoid system may be involved in schizophrenia (Fakhoury, 2017; Minichino et al., 2019) and addictive disorders (Manzanares et al., 2018; Van Hell et al., 2012). A study identified an association between early cannabis use, lower cortical thickness and high polygenic risk for psychosis in adolescents. This finding implicates processes underlying cortical maturation in mediating the link between cannabis use and proneness to schizophrenia (French et al., 2015), indicating that cannabis could potentially play a role in the development of psychosis by altering neural circuits in genetically vulnerable subjects (Aas et al., 2017; Fonseca-Pedrero, Lucas-Molina, Pérez-Albéniz, Inchausti & Ortuño-Sierra, 2019; French et al., 2015; García-Álvarez, Gomar, García-Portilla & Bobes, 2019; Parkar et al., 2011).

CNR1, CNR2 and *FAAH* are the genes that encode some of the proteins associated with the endocannabinoid system. CB1 receptors are mainly located in the central nervous system and are abundant in the basal ganglia, hippocampus, cerebellum and cortical areas (Herkenham et al., 1991). CB2 receptors were initially found in the immune system (Galiègue et al., 1995), however, their presence has also been demonstrated in neurons and glial cells of multiple brain areas (cerebral cortex, hippocampus, amygdala, striatum, thalamus, cerebellum...) (Gong et al., 2006; Onaivi et al., 2006). Fatty Acid Amide Hydrolase (*FAAH*) is the enzyme responsible for the hydrolysis of anandamide, an endogenous ligand of this system (Deutsch, Ueda & Yamamoto, 2002).

Some studies have suggested an association between the *CNR1* gene (that encodes the CB1 receptor) and incidence of schizophrenia (Chavarría-Siles et al., 2008; Leroy et al., 2001; Martínez-Gras et al., 2006; Ujike et al., 2002) and substance use disorders, such as cannabis use disorder (Gerra et al., 2018; Hartman et al., 2009). However, evidence remains heterogeneous and controversial for both outcomes. Gouvêa et al. (2017) systematically analysed all the existing trials on *CNR1* gene variants and schizophrenia and emphasized the high heterogeneity of the results. A polymorphism consisting of nine alleles containing (AAT) 7-15 repeat sequences has been used in association studies on the *CNR1* gene and mental illness and drug abuse among different populations, with contradictory results (Ballon et al., 2006; Chavarría-Siles et al., 2008; Comings et al., 1997; Martínez-Gras et al., 2006; Tsai, Wang & Hong, 2000; Ujike et al., 2002).

In recent years, the CB2 receptor has gained attention due to its function as a modulator of neuroinflammation (Javed, Azimullah, Haque & Ojha., 2016; Kong, Li, Tuma & Ganea, 2014; Malfitano, Basu, Maresz, Bifulco & Dittel, 2014), memory processes (García-Gutiérrez et al., 2013), and reward processing, and for its role in drug addiction, and psychosis-(Onaivi, Ishiguro, Gu & Liu, 2012; Xi et al., 2011). The frequency of the CC allele of rs35761398 (R63 variant), the C allele of rs12744386, the haplotype of the CC allele of rs35761398 and the C allele of rs12744386 (CC/C) was found to be significantly increased among a Japanese population sample with schizophrenia compared with control subjects (Ishiguro et al., 2010). A significantly lower response to CB2 ligands in cultured cells transfected with the CC allele of rs35761398 compared with those transfected with the TT allele was observed, and significantly lower CB2 receptor mRNA and protein levels were found in the human brain with the C/C and C/T genotypes of rs12744386 compared with T/T genotypes (Ishiguro et al., 2010).

On the other hand, a common Single Nucleotide Polymorphism (SNP) rs324420 (C385A) in the human *FAAH* gene has been related to drug abuse, for instance, cannabis (Tyndale, Payne, Gerber & Sipe, 2007), cocaine (Patel et al., 2018) and methamphetamine (Zhang, Liu, Deng, Ma & Liu, 2020).

The aim of this study was to investigate genetic association between the *CNR1* gene $(AAT)_{7\text{-}15}$ repeat polymorphism, the *FAAH* gene SNP rs324420, the *CNR2* gene rs35761398 and rs12744386 polymorphisms, and schizophrenia and cannabis dependence in a sample of Spanish subjects.

Methods

Participants

In this study, 379 schizophrenic patients, 124 schizophrenic and cannabis use disorder (CUD) patients (Dual group), 71 CUD subjects without psychoses (cannabis group) and 316 controls who were not related to each other were analysed. Diagnoses were made according to DSM-IV-TR by clinical interview. The patients (outpatients and inpatients) were recruited from different hospitals in the Community of Madrid and Castilla-La Mancha. Cannabis users without psychosis were recruited from addiction centres or user associations in the Community of Madrid. Inclusion criteria were: being over 18 years of age, being Spanish and Caucasian, and signing the informed consent. Exclusion criteria were: being themselves or having first-degree relatives of another ethnic origin, from countries other than Spain, presence of mental disorders other than those being studied, dependence on drugs other than cannabis or tobacco, presenting comorbid organic brain pathology or other serious medical conditions and refus-

ing to participate or failing to sign the informed consent. Patients with a diagnosis of dependence on drugs other than cannabis or tobacco were excluded, although subjects were included if they used drugs but were not dependent.

The control population consisted of 316 individual volunteers who were not related to each other and were recruited from the Spanish population. They were health and administrative personnel from the health centres attended by the patients and companions of the patients. A clinical interview was carried out on all of them to exclude other psychiatric pathologies.

Assessment instruments

Sociodemographic variables, personal and family history and data related to substance use were obtained through a clinical interview. In addition, in that first interview psychotic symptomatology was assessed using the Positive and Negative Syndrome Scale (PANSS).

PANSS Scale (Positive and Negative Syndrome Scale). The Positive and Negative Syndrome Scale, developed by Kay, Fiszbein and Opler (1987), the Spanish version of which was created by Peralta and Cuesta (1994), is one of the most frequently used tools for assessing symptoms in schizophrenic patients. It is a clinician-administered scale that is completed based on a semi-structured interview, which takes approximately 45 minutes. In its original version, the PANSS consists of 30 items grouped into three factors: positive syndrome (which includes 7 items), negative syndrome (which also includes 7 items) and general psychopathology (which includes 16 items). In this study, in addition to using the PANSS total score, we also used the three subscales (positive, negative and general psychopathology).

Study procedure

Inpatients and outpatients treated at different mental health centres (University Hospital Fundación Alcorcón (Madrid), Ramón y Cajal Hospital (Madrid), Virgen de la Luz Hospital (Cuenca), University Hospital of Guadalajara, Nuestra Señora de La Paz Clinic (Madrid)), who met the inclusion and exclusion criteria and were willing to participate in our study, were prospectively recruited and signed an informed consent. Cannabis dependent subjects were recruited from different Drug Treatment Centres in the Community of Madrid (Majadahonda, Alcorcón, Arganzuela, Vallecas, Latina).

A total of 27 subjects were excluded either because they refused to participate in the study or to sign the informed consent.

DNA extraction and genotyping

DNA was obtained from leukocytes present in peripheral blood samples anticoagulated with EDTA, using the Sambrook Method and the DNeasy Blood & Tissue kit (Qiagen).

After extraction of DNA from peripheral blood, analysis of the different polymorphisms was carried out using PCR-based methods. The genotyping of the rs324420 polymorphism in the *FAAH* gene was performed using the SSCP (Single Strand Conformation Polymorphism) method (GeneGel Excel 12.5/24 Kit, GE Healthcare) (See Table 1 for primer sequences). The genotyping of the $(AAT)_{7\text{-}15}$ 3'UTR polymorphism was performed using a capillary electrophoresis fragment analysis technique (ABI Prism 310 Genetic Analyzer - Applied Biosystems) (See Table 1 for primer sequences). The standard GeneScan-500 LIZ, was used as a size marker (Applied Biosystems). Analysis of the results was carried out using GeneMapper 4.0 software. The genotyping of the rs35761398 and rs12744386 polymorphisms in the *CNR2* gene was performed using allelic discrimination with TaqMan probes in an iCycler Thermal Cycler (Bio-Rad) (see Table 1 for primer and TaqMan probe sequences).

Ethical concerns

Participation in this study was voluntary and all participants gave their written consent to taking part in the project. The study was approved by the Clinical Research Ethics Committee of the University Hospital Fundación Alcorcón (Madrid).

Statistical analyses

Genotype distribution was compared to the predictable value from Hardy-Weinberg equilibrium. The control and case groups were at Hardy-Weinberg equilibrium in terms of allele and genotype frequencies for the polymorphisms studied (Table 2).

Table 1. *Primers and techniques used for sample analysis*

Gene	Polymorphism	Primer 5` 3`	Techniques
CNR1	(AAT)n	A: 5' GCTGCTTCTGTTAACCCTGC 3' B: 5' ATTCCCACCTATGAGTGAGAAC 3'	Capillary electrophoresis fragment analysis (ABI Prism 310 Genetic Analyzer - Applied Biosystems)
CNR2	rs35761398	A: 5' AAGACCACACTGGCCAGGAAG 3' B: 5' CACTCTTCTGGGCCTGCTAAG 3'	Allelic discrimination with TaqMan probes in an iCycler Thermal Cycler (Bio-Rad)
			SSCP
FAAH	rs324420	A: 5' GGCCAGCCTCCTTTTATCTTATG 3' B: 5'GACGATGGAGGCCTGGCGA 3'	SSCP (Kit GeneGel Excel 12.5 / 24, GE Healthcare)

Note. Single Strand Conformation Polymorphism (SSCP).

Table 2. *Hardy-Weinberg equilibrium*

GENE	Polymorphism	Gender	χ^2	p-value
CNR1	(AAT)n	Women	.5389	.7638
		Men	3.2414	.1978
CNR2	rs35761398	Women	.0467	.9769
		Men	.1874	.9106
FAAH	rs324420	Women	1.5451	.4183
		Men	.1229	.9404

Note. Two degree of freedom except rs6323 and rs1799836 in man (hemizygosity), calculated for 1 degree of freedom.

The nine alleles containing $(AAT)_{7\text{-}15}$ repeat sequences were distributed in accordance with the article by Comings et al. (1997) in a group of short alleles with less than 11 AAT triplet repeats (genotype <5) and another group of long alleles ≥ 11 repeats (genotype ≥ 5). Thus, the patients and controls were subdivided into three groups according to their genotype: individuals with <5/<5, ≥ 5/≥ 5, and <5/≥ 5. These data were used as qualitative variables. The hypothesis of an association between genotypes and groups was tested using Pearson's chi-squared test and where cell sizes were equal to or smaller than 5, Fisher´s exact test was used. Bonferroni correction was applied.

A two-sided P-value test was used and P-values of < 0.05 were considered statistically significant. The analysis was carried out using OpenEpi (Open Source Epidemiologic Statistics for Public Health) online software.

Results

Sociodemographic and clinical data are summarized in tables 3 and 4. No statistically significant differences were observed in any of the polymorphic variants studied in the population analysis based on variables such as sex or age at initiation of cannabis use or psychotic symptoms.

CNR1

Regarding the $(AAT)_{7\text{-}15}$ 3'UTR polymorphism in the *CNR1* gene, we did not find statistically significant differences between the schizophrenic, dual and cannabis groups and the control population when allele and genotype frequencies were analysed (Table 5).

FAAH

Regarding the rs324420 polymorphism in the *FAAH* gene, non-statistically significant differences were found between the control population and both subjects with schizophrenia and comorbid schizophrenia and cannabis dependence when comparing genotype and allele frequencies (Table 5). Assuming an overdominance model, statistically significant differences were found between the cannabis dependent subjects and the control population (Table 6).

CNR2

Regarding the analysis of the rs35761398 polymorphism in the *CNR2* gene, when we compared the dual group with the schizophrenia and control groups, statistically significant deviations in genotype frequencies were found (Table 7). Assuming a dominant model for the less frequent allele (TT), differences in the presence of TT were statistically significant when we compared the dual group to the controls. In both cannabis users and controls, no statistically significant differences were found in genotype and allele frequencies between the rs35761398 and rs12744386 polymorphisms. There was an interaction between the rs35761398 polymorphism in the *CNR2* gene and the rs324420 polymorphism in the *FAAH* gene (Table 8).

Literature on the rs35761398 polymorphism places its origin in linkage disequilibrium within this gene. The functional linking implication of these polymorphisms in

Table 3. *General description of the sample*

	Controls N=316	Schizophrenia N=379	Schizophrenia + cannabis dependence N=124	Cannabis dependence N=71
Male (%)	42.72	60.16	87.90	70.42
Mean age at testing (p25 -p75)	31 (28 - 37)	37 (31 - 50)	29 (26 - 36)	28 (25 - 34)
Mean age at diagnosis (p25-p75)		25 (20 - 32)	25 (20 - 28)	23 (19 - 30)
Mean age of first cannabis use (p25-p75)			16 (15 - 18)	16 (15 - 17)
PANSS score: mean (95% CI)				
Positive Scale		22.6 (20.7-24.5)	21.5 (18.8-24.1)	
Negative Scale		23.8 (22.0-25.6)	20.1 (17.0-23.2)	
Global Scale		36.3 (34.2-38.3)	33.5 (31.0-35.9)	

Note. PANSS: Positive and Negative Syndrome Scale. P=percentile. CI=confidence interval. Male predominance in schizophrenia+cannabis dependence vs schizophrenia (chi^2 = 32,53, p < 0.001, OR: 4,81, IC: 95%: 2,70-8,58).

Table 4. *Percentages of drug use among groups*

	Schizophrenia and Cannabis dependence N=124	Cannabis dependence N=71	Schizophrenia N=379	Controls N=316
Cannabis (cigarette/day): mean; median	7.5; 6	6; 6	0	0
Tobacco (%)	92.1	64.3	48.4	36.8
Alcohol (%)	48.0	21.4	-	-
Cocaine (%)	32.7	10.7	-	-
Opioids (%)	9.3	0.0	-	-
Amphetamines (%)	1.3	0.0	-	-
Others (%)	12.0	0.0	-	-

Note. Percentage of use of alcohol, cocaine, opioids, amphetamines or other drugs without dependence criteria. In the subgroup of schizophrenia + cannabis dependence, 52 % of the subjects only used tobacco and cannabis.

Table 5. *Distribution of genotype and allele frequencies among subgroups*

Polymorphism (gene)	Genotype/Allele		Controls N (%)	Schizophrenia N (%)	Schizophrenia + cannabis dependence N (%)	Cannabis dependence N (%)
(AAT)n (CNR1)	Genotype	LL	171 (54.11)	190 (50.13)	58 (46.77)	33 (57.89)
		LS	116 (36.71)	145 (38.26)	52 (41.94)	20 (35.09)
		SS	29 (9.18)	44 (11.61)	14 (11.29)	4 (7.02)
	Allele	L	458 (72.47)	525 (69.26)	168 (67.74)	86 (75.44)
		S	174 (27.53)	233 (30.74)	80 (32.26)	28 (24.56)
rs35761398 (CNR2)	Genotype	CC/CC	101 (31.96)	116 (30.61)	24 (19.35)	21 (29.58)
		CC/TT	152 (48.10)	181 (47.76)	70 (56.45)	34 (47.89)
		TT/TT	63 (19.94)	82 (21.64)	30 (24.19)	16 (22.54)
	Allele	CC	354 (56.01)	413 (54.49)	118 (47.58)	76 (53.52)
		TT	278 (43.99)	345 (45.51)	130 (52.42)	66 (46.48)
rs324420 (FAAH)	Genotype	CC	202 (63.92)	254 (67.02)	90 (72.58)	53 (75.71)
		CA	104 (32.91)	107 (28.23)	31 (25.00)	11 (15.71)
		AA	10 (3.16)	18 (4.75)	3 (2.42)	6 (8.57)
	Allele	C	508 (80.38)	615 (81.13)	211 (85.08)	117 (83.57)
		A	124 (19.62)	143 (18.87)	37 (14.92)	23 (16.43)

Note. L=long allele. S=short allele.

Table 6. *Association results for frequency contrast between cannabis dependent group and controls/schizophrenia + cannabis dependence groups*

		Polymorphism	Model-Adjustment procedure	χ^2	D.F.	p-value	ODDS-R (95% IC)
Cannabis dependent	Schizophrenia + cannabis dependence	rs35761398 (CNR2)	Codominant	2.0365	2	.3612	
			Alelles	.9904	1	.3196	1.23 [.82; 1.86]
			Dominant	2.0075	1	.1565	1.63 [.83; 3.23]
		rs324420 (FAAH)	Codominant	5.4918	2	.0642	
			Alelles	.1559	1	.6930	.89 [.51; 1.57]
			Heterocygosis	2.2743	1	.1315	.56 [.26; 1.20]
	Controls	(AAT)n (CNR1)	Codominant	.3618	2	.8345	
			Alelles	.3177	1	.5730	.88 [.56; 1.38]
		rs35761398 (CNR2)	Codominant	.4757	2	.7883	
			Alelles	.4793	1	.4887	1.14 [.79; 1.64]
		rs324420 (FAAH)	Codominant	1.9360	2	.0042	2.74 [1.45; 5.31]
			Alelles	.7574	1	.3842	.81 [.49; 1.31]
			Heterocygosis	8.1024	1	.0044	2.63 [1.33; 5.22]

receptor action and the descriptions of infrequent haplotypes in other populations was the reason for genotyping this second polymorphism in the *CNR2* gene, in order to determine whether those haplotypes were present in the sample included in this study. They were genotyped in both the control group and the dual diagnosed patients. The haplotype linkage between the rs12744386 and rs35761398 polymorphisms in the population studied was 100%, with the following haplotypes found to be present: C - CC y T - TT. Alternative haplotypes, that is, T - CC and C - TT, were not found in the population included in this study.

Discussion

Different epidemiological studies have suggested that cannabis use could be a risk factor for the development of schizophrenia (Marconi et al., 2016). However, only a small proportion of cannabis users develop psychosis, which can be partially explained by genetic factors. Converging evidence from animal and human studies suggests that the endocannabinoid system (ECS) is involved in the pathophysiology of psychosis (Fakhoury, 2017; Minichino et al., 2019; Rodriguez-Muñoz, Sánchez-Blázquez, Callado, Meana & Garzón-Niño, 2017). Thus, logical candidate genes

Table 7. *Association results for frequency contrast in rs35761398 between dual group and controls/schizophrenia groups*

Gene	Polymorphism	Model	Groups		χ^2	P value.	ODDS-R (95% IC)
CNR2	rs35761398	Dominant (CC/TT+TT/TT)	Dual	Controls	6.9595	.0083	1.96 [1.18; 3.24]
				Schizophrenia	5.8892	.0152	1.84 [1.12; 3.02]

Table 8. *Association analysis between CNR2 and FAAH genes*

Genotype rs35761398 – rs324420	Controls (a) N (%)	Schizophrenia + Cannabis dependence (b) N (%)	Ratio (%a /% b)	χ^2	p- value	ODDS-R (95% CI)
CC/CC – CC	51 (16.14)	16 (12.90)	.7995	.7224	.3953	.77 [.42; 1.41]
CC/CC – A carrier	50 (15.82)	8 (6.45)	.4077	6.8337	.0089 (1)	.37 [.17; .80]
TT carrier – CC	151 (47.78)	74 (59.68)	1.2489	5.0407	.0248	1.62 [1.06; 2.46]
TT carrier – A carrier	64 (20.25)	26 (20.97)	1.0353	.0279	.8672	1.04 [.63; 1.74]
				9,031	.0289 (*)	

Nota. (1) Bonferroni correction implies that significant values are those p-values below 0.0125. (*) $X^2 = 9{,}031$; GL= 3; p = 0,0289.

that could influence the likelihood of developing psychosis include *CNR1*, *CNR2*, and *FAAH*.

CNR1

We found no evidence of association between the *CNR1* microsatellite and schizophrenia, which is consistent with findings in other studies (Ballon et al., 2006; Dawson, 1995; Seifert, Ossege, Emrich, Schneider & Stuhrmann, 2007; Tsai et al., 2000). An association has been found between the hebephrenic subtype of schizophrenia and the AAT-repeat polymorphism in a Japanese population and in a family-based association study on a Costa Rican population (Chavarría-Siles et al., 2008; Ujike et al., 2002). Our sample of schizophrenic subjects mainly included paranoid-type patients (data not shown), as hebephrenic-type schizophrenia is very uncommon in the Spanish population. The Ujike and Chavarría-Siles studies did not find significant differences in the frequency of genotype or alleles between subjects with paranoid-type schizophrenia and controls.

While no association has been found here between schizophrenia and other different *CNR1* polymorphisms (Leroy et al., 2001; Zammit et al., 2007), another Spanish group (Martínez-Gras et al., 2006) did find significant differences for this polymorphism between 113 patients and 111 healthy controls. Allele 4 was more frequent in controls, suggesting a protective effect against schizophrenia development. The sample was more heterogeneous, including comorbid substance abuse in the schizophrenic group, which could explain the discrepancies with our findings. On the other hand, the frequency of the (AAT)12 repeat allele was increased in schizophrenic cocaine dependent subjects in an African-Caribbean population (Ballon et al., 2006).

Although we did not find an association between this polymorphism and schizophrenia, many other data suggest that the CB1 receptors could play a key role in its pathogenesis, or it could be linked with some of the phenotypes related to this disease. In this regard, an association has been described between some of the *CNR1* gene polymorphisms and the psychomimetic effects of cannabis in a healthy population (Krebs, Morvan, Jay, Gaillard, & Kebir, 2014), the cognitive function of psychotic first-episodes (Rojnic et al., 2019) and the pharmacogenetic response in psychosis (Hamdani et al., 2008). In addition, there have been changes described in *CNR1* gene expression (Tao et al., 2020) and in the methylation of the DNA of the *CRN1*

gene in schizophrenia-(D'Addario et al., 2017), as well as reduced availability of CB1 receptors in different brain areas in psychotic first-episodes (Borgan et al., 2019).

Comings et al. (1997) found that this polymorphism was significantly associated with a number of different types of drug dependence and intravenous drug use. In accordance with our findings, other authors did not find an association between the AAT polymorphism and substance abuse (Covault, Gelernter & Kranzler, 2001; Heller, Schneider, Seifert, Cimander & Stuhrmann, 2001; Li et al., 2000). Comparison of allele distributions among different ethnic groups showed marked genetic variation among populations (Comings et al., 1997; Li et al., 2000; Ujike et al., 2002). It is important to note that, in our sample, all patients were Caucasian.

FAAH

When comparing schizophrenic patients with or without cannabis use disorder to controls, no significant differences were found with regard to allele frequencies or genotype distribution of the *FAAH* gene. This is consistent with the findings of Morita et al. (2005) in relation to a Japanese population, and the recently published results of Hindocha et al. (2020), which also failed to find a significant association between the rs324420 genotype and psychotic experiences in cannabis users. Bioque et al. (2019) analysed the genotypes of 321 patients with first-episode psychosis. A total of 15 *CNR1, CNR2* and *FAAH SNPs* were analysed, but they only found statistical significance in the case of the rs2295633 polymorphism of the *FAAH* gene. Homozygote carriers of the T allele who were cannabis users had a greater likelihood of presenting a psychotic episode than users of cannabis without this genotype. Sufficient statistical significance was not found with regard to the rs324420 polymorphism. Watts et al. (2020) recently found that lower levels of *FAAH* were associated with more severe psychotic symptoms. These results were independent of cannabis exposure.

We found an association between the rs324420 polymorphism in the *FAAH* gene and cannabis dependence. The presence of fewer heterozygotes in the rs324420 *FAAH* polymorphism was associated with cannabis dependence, which leads us to hypothesise that the heterozygous genotype confers some protection against this dependence, in accordance with an overdominance model. Heterozygosity could thus be a balance between the demands of flexibility and stability in the neural pathways involved. Just as in our research, other authors have found an association between the AA or CC homozygotes of this polymorphism and substance use disorders (Flanagan, Gerber, Cadet, Beutler & Sipe, 2006; Sipe et al., 2002), and different clinical manifestations in cannabis users (Haughey, Marshall, Schacht, Louis & Hutchison, 2008; Schacht, Selling & Hutchison, 2009).

FAAH is the critical regulator of the endogenous levels of anandamide (Fezza, De Simone, Amadio & Maccarrone, 2008). The *FAAH* polymorphism rs324420 predicts a substitution of proline at position 129 of the protein by a threonine residue (P129T), resulting in a protein that is more susceptible to proteolytic degradation (Sipe, Chiang, Gerber, Beutler & Cravatt, 2002). Thus, *FAAH 385A* is associated with lower enzymatic activity. FAAH knockout mice have shown altered cannabis tolerance and dependence (Falenski et al., 2010) suggesting that altered FAAH activity may modify endocannabinoid signalling in reward-controlling areas and contribute to addictive vulnerability (Van Hell et al., 2012).

Previous literature about the relationship between the rs324420 polymorphism and drug use patterns seems to be extremely heterogeneous and complex (Hindocha et al., 2019; Melroy-Greif, Wilhelmsen & Ehlers, 2016; Tyndale et al., 2007). Lower levels of FAAH have been identified in healthy A-carriers of rs324420 (Boileau et al., 2015). Reduced brain FAAH binding has been found in cannabis users compared to controls. In addition, lower binding has been associated with abstinence, impulsivity and increased cannabinoid blood levels (Boileau et al., 2016). In the cannabis user sample of the Hindocha et al. (2019) study, A carriers showed a greater bias towards appetitive stimuli in comparison with CC carriers. Hariri et al. (2009) found that there was an association in carriers of *FAAH 385A* with possible increased endocannabinoid signalling, and that there was increased reward-related ventral striatal reactivity and more impulsivity in comparison with C385 homozygotes. On the contrary, Filbey, Schacht, Myers, Chavez & Hutchison (2010) identified higher activation in reward areas in C-allele carriers in a sample of regular marijuana users.

Our findings should be interpreted with caution because significance levels for codominant and overdominance models were similar and the study population number was limited (the AA genotype was only present in few cases and the cannabis dependent control subgroups were small).

CNR2

Different studies have shown that CB2 receptors are present in neural progenitor cells, neurons and glial cells. In addition, CB2 receptor function has not only been linked to neurological disorders involving neuroinflammation but also to neuropsychiatric disorders like drug addiction, psychosis, depression, and eating disorders (Onaivi et al., 2012).

Nonetheless, we observed an association between the polymorphisms rs35761398 and rs12744386 in *CNR2* and comorbid schizophrenia and cannabis dependence. We found that the high function genotype of *CNR2* was associated with schizophrenia, but only in cannabis dependent

subjects. We tested several models of inheritance and found that statistical association was enhanced when a dominant model for the TT allele was assumed. To our knowledge, this is the first study performed on a Caucasian population. Ethnicity should be taken into consideration when interpreting our results because differential allelic distributions have been described in previous scientific literature on other ethnic groups. For instance, an association has been reported between schizophrenia and the low function haplotype in a Japanese population sample (Ishiguro et al., 2010) and other different *CNR2* polymorphisms in Chinese samples (Tong et al., 2013), whereas in Korean samples, no association was found (Bae et al., 2014).

Banaszkiewicz, Biala & Kruk-Slomka (2020) conducted a review on schizophrenia-like symptoms induced via CB2 receptor modulation in animal models, suggesting a key function in schizophrenia. Schizophrenia-related behaviours were observed in mice with deletion of CB2 receptors (Ortega-Alvaro, Aracil-Fernández, García-Gutiérrez, Navarrete & Manzanares, 2011). It is suggested that a lack of the CB2 receptor might impair neural development, thus inducing relevant alterations in several brain areas, based on findings supporting a pro-neurogenic role of the CB2 receptor in the control of fundamental neural cell processes (Galve-Roperh, Aguado, Palazuelos & Guzman, 2008). These results seem contrary to our data as they relate psychosis to lower function of CB2 receptors. Our data would support an alternative explanation: that excessive activity of these receptors could facilitate this psychotic phenotype. Therefore, cannabis use could disrupt neuronal differentiation during adolescence and provoke psychoses in vulnerable subjects through a mechanism involving CB2.

On the other hand, inflammatory and immunological processes interfering with brain development are discussed as a cause of schizophrenia, and the CB2 receptor is a main component of these processes (Sahu et al., 2019). Glia are implicated in schizophrenia pathogenesis, and the CB2 receptor is relevant (De Almeida & Martins-de-Souza, 2018). It has been hypothesised that an increased number of activated microglial cells in patients with schizophrenia contribute to disease pathogenesis (Juckel et al., 2011).

Furthermore, in Alzheimer´s disease, CB2 receptors are abundantly and selectively expressed in neuritic plaque-associated astrocytes and microglia, respectively (Benito et al., 2003), and activation of CB2 receptors expressed by immune cells is likely to reduce their antiviral response, thus favouring the CNS entry of infected monocytes with simian immunodeficiency virus (Benito et al., 2005).

Thus, overactivation of the CB2 receptor could be a psychosis vulnerability factor, and cannabis use could provoke psychosis in these vulnerable subjects. It has been found that THC inhibits the chemotactic response of microglia through activation of the CB2 receptor (Cabral, Raborn, Griffin, Dennis & Marciano-Cabral, 2008). Furthermore, cannabis use in the context of specific cannabinoid receptor genotypes may contribute to white matter abnormalities, which could in turn increase schizophrenia risk (Ho, Wassink, Ziebell & Andreasen, 2011). White matter alterations are relevant in schizophrenia, and adolescent cannabis use has specific effects on these abnormalities (Peters, Blaas & de Haan, 2010).

In addition, the expression of *CNR2* gene transcripts in animals treated with drugs of abuse is increased in comparison with controls (Ishiguro et al., 2007). Therefore, cannabis use could modify *CNR2* transcription, and in subjects with highly activated CB2 receptors, could contribute to psychotic symptoms through an unknown mechanism. It has also been reported that clinical remission from schizophrenia is accompanied by significant decreases in CNR2 mRNA levels in mononuclear peripheral blood cells (De Marchi et al., 2003).

The limitations of this study are related to the nature of association studies and therefore, our results must be interpreted with some caution. The strength of our results is limited by a small sample size, particularly in the cannabis dependent group. It would be preferable to replicate our genetic studies in independent samples, since this could clarify the possible role of the *CNR1, CNR2,* and *FAAH* gene variants. Secondly, the possibility of a result occurring by chance cannot be ruled out, although we did apply Bonferroni correction. These results should therefore be confirmed among a larger population. Likewise, it would have been useful to administer the PANNS scale to the controls and in particular to the CUD subjects, in order to rule out clinical psychotic symptoms. Urine tests for detecting drugs were not carried out on the control group so it is possible that some subjects were using cannabis. Drug testing was conducted on the non-cannabis using schizophrenia group.

Despite these limitations, we believe our study has identified a major genetic protective factor against cannabis-associated psychosis in the Spanish population, which deserves greater attention in future investigations. That research should also examine whether specific phenotypic characteristics, such as symptom profile, age at onset, and treatment response, are associated with the *CNR2* polymorphism. No previous reports on these polymorphisms in cannabinoid-associated psychosis are available. The mutation detected by this polymorphism results in a change in the amino acid sequence of the protein, so direct functional consequences are expected. Cannabis dependent subjects with the TT genotype exhibited a significantly higher risk of psychosis. These findings suggest that dysfunction of the endocannabinoid system due to genetic mutation may constitute a risk factor for cannabis-associated psychosis.

Apart from genetic variants, it would also be advisable to include epigenetic variables in future research. Changes in DNA methylation in the promotor region of *CNR1* genes

in schizophrenic patients have been described (D'Addario et al., 2017; Tao et al., 2020).

Finally, we did not find a relationship between *CNR1* and *FAAH* variants and psychosis. Overall, our findings suggest that *FAAH* variants are associated with cannabis dependence but not with schizophrenia in Spanish patients, which would imply that differences in endocannabinoid function could play a part in the pathophysiology of this illness. Confirmation of our findings among other populations and independent samples would be useful for the design of pharmacological strategies focused on prophylaxis and treatment of these patients.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

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