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Original article

Association of CNR1 genotypes with changes in neurocognitive performance after eighteen-month treatment in patients with first-episode psychosis

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ABSTRACT

Introduction: We analyzed the association of cannabinoid receptor CNR1 genotypes with changes in neurocognitive performance in patients with first-episode psychosis (FEP) after 18 months of treatment. Our secondary aim was to analyze the association of CNR1 genotypes with changes of perceived levels of stress.

Methods: We enrolled a sample of 159 patients with FEP from two Croatian psychiatric hospitals between 2014 and 2017. Patients were assessed at baseline and after 18 months. We analyzed the associations of changes in neurocognitive test results and the perceived levels of stress with CNR1 polymorphic loci (rs7766029 and rs12720071) in 121 patients.

Results: In the analysis adjusted only for baseline neurocognitive test scores, carriers of rs7766029 CC genotype had significantly (with false discovery rate, FDR < 15%) higher improvement in verbal memory (Wechsler, Wechsler 30′) and attention (Digit span F) compared with other participants. In such analysis, rs12720071 carriers of AG genotype had significantly (FDR < 15%) higher improvement in executive functions (Block design), but lower improvement in language functions than AA carriers. In the fully adjusted analysis for age, sex, cannabis use and negative symptoms, only the association of rs7766029 genotypes with the change in the Weschler 30′ score was significant (FDR < 15%). In the analysis adjusted only for the baseline neurocognitive tests' scores, both rs7766029 and rs12720071 genotypes were significantly associated with the change in perceived levels of stress (FDR < 15%). In the fully adjusted analysis, only the association with rs7766029 genotype remained significant.

Conclusions: The rs7766029 CNR1 variants may moderate changes in neurocognitive performance as well as in perceived levels of stress of patients with FEP over time.

1. Introduction

Schizophrenia is a complex disorder with multiple etiological factors and a heterogeneous clinical presentation. In most cases, it starts in adolescence with the first acute episode (first-episode psychosis, FEP) followed by alternating periods of acute psychosis and remission. While there is still no straightforward explanation for the processes underlying this disorder, numerous reports have shown that the endocannabinoid system is involved in the development of psychosis and schizophrenia. The endocannabinoid system incorporates a group of neuromodulatory lipids, including arachidonoyl ethanol amide or anandamide, 2-arachidonoyl glycerol and their receptors, the cannabinoid type 1 receptor (CNR1) and cannabinoid type 2 receptor (CNR2) [1]. The CNR1 receptor is located in the presynaptic terminals in the

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prefrontal, frontal, temporal and hippocampal region and amygdala [2–5] and exhibits numerous interactions with various central neurotransmitters [6], while the presence of CNR2 is still under debate. However, reports consistently prove that Δ9-Tetrahydrocannabinol, the exogenous agonist of CNR1 induce psychosis and schizophrenia [7,8]. Moreover, the endocannabinoid system is involved in different aspects relevant for the development of psychosis and schizophrenia, including the reward system, emotional processing and memory and is heavily influenced by environmental stimuli, modulating the stress response [9–11], which can all contribute to vulnerability to psychosis. CNR1 is localized to chromosome 6q14–q15, a schizophrenia susceptibility locus [12]. The CNR1 gene has several functional polymorphisms and researchers have focused on the association of some of them with vulnerability to schizophrenia. In a recent review summarizing these findings, the majority of reported polymorphic loci yielded negative results [13]. However, there were several positive reports focusing on specific phenotypes or subsets of symptoms of schizophrenia and FEP. For example, Suárez-Pinilla et al. [14] reported a significant association of the CNR1 rs1049353 polymorphism with changes of caudate volume and of CNR1 rs2023239 polymorphism with changes in thalamic volume after the 3-year follow up period in a small sample of patients with FEP. Ho et al [15] reported significant associations of the CNR1 rs7766029 genotypes with white matter volumes. They also identified cannabis exposure, which is one of the risk factor for psychosis [16], as a factor moderating the link of CNR1 rs12720071 genotypes and neurocognitive performance in patients with schizophrenia, similarly to what was observed among healthy population for rs1406977 genotypes [17,18].

Based on these results, we can hypothesize that the endocannabinoid system may have an important role of in interindividual variability to treatment response/ treatment outcome. In a genome-wide association study (GWAS) focusing on neurocognition in schizophrenia, the authors suggested that CNR1 rs7766029 polymorphism could be a possible mediator of the effects of perphenazine on verbal memory [19] in patients with schizophrenia. In a previously mentioned study among patients with FEP [20], the authors reported an association of the CNR1 rs2023239 polymorphism with changes of positive and negative symptoms in FEP after three years.

As neurocognitive deficits represent the core feature of schizophrenia with possibly higher heritability and a significant influence on the treatment outcome [21] in comparison with other psychotic symptoms (e.g. neurocognitive deficits present more resistance to treatment compared with other psychotic symptoms [22,23] and predict worse long-term functional outcome after FEP [24]), we were specifically interested in the role of CNR1 receptor polymorphism on neurocognitive change over time.

Based on the reported association of CNR1 polymorphisms with neurocognitive performance in patients with schizophrenia and considering cannabis use, we focused specifically on CNR1 C > T (rs7766029) and CNR1 A > G (rs12720071) polymorphisms. However, in order to reduce the possible number of confounders, as suggested by Colhoun, McKeigue & Smith [25], we wanted to reduce the heterogeneity of the sample by including young patients of Croatian descendants with FEP, homogenous in terms of clinical presentation and phase of illness and exposure to therapy, and to follow them over a longer follow-up period, which allows enough time for the improvement of the majority of psychotic symptoms, including neurocognition. Secondly, even though both polymorphisms are localized in the introns/untranslated region of exon 4, they may exert their effect through the strong linkage disequilibrium (LD) with other functional variants, or by direct effect. For example, it was shown that presence of the A-allele on rs12720071 modulates the transcription factor binding site for CCAAT/enhancer-binding protein beta (C/EBPbeta) [26], with consequences on neurogenesis [27].

Our secondary objective was to study the potential association of these polymorphisms with the change of perceived levels of stress over time, taking into account the role of stress in vulnerability to schizophrenia as well as during the course of the illness [28]. More specifically: 1) the endocannabinoid system mediates the affective feature of the stress response, also reflected by the decrease in CNR1 receptors in chronic stress, with possible consequences for neurocognition [11]; 2) CNR1 genotypes (rs1049353), which were found in LD with both studied polymorphisms, were associated with emotional and cognitive response to stress among healthy subjects [29].

Thus, our main hypothesis was that CNR1 C > T (rs7766029) and CNR1 A > G (rs12720071) polymorphisms are associated with changes in neurocognitive performance of patients with FEP after 18 months of follow-up. Our secondary hypothesis was that CNR1 C > T (rs7766029) and CNR1 A > G (rs12720071) polymorphisms are associated with perceived levels of stress in patients with FEP after 18 months of follow-up.

2. Material and methods

2.1. Study design

We performed a longitudinal, prospective cohort study in two Croatian hospitals: Zagreb University Hospital Centre (ZUHC) and University Psychiatric Hospital Vrapce (UPHV) between June 2014 and June 2017 as a part of the project Biomarkers in schizophrenia- integration of complementary methods in longitudinal follow-up of FEP. Blood samples for genotyping were collected at the beginning of the study, while assessment of psychopathology and neurocognitive functioning took place at two time points: the first assessment was performed in the first few weeks of treatment, once the patients' clinical conditions allowed the performance of neurocognitive assessment (for the majority it was over the duration of the first antipsychotic treatment lasting from two to three weeks); the second assessment was performed after 18 months of follow-up. The study protocol was approved by the Ethics Committees of ZUHC and UPHV. The study was conducted in accordance with the World Medical Association Declaration of Helsinki [30]. Before the enrollment, researchers explained the study protocol to all participants and were at their disposal during the study for any additional questions. We obtained the informed signed consent form from all participants and they were free to withdraw from research any time.

2.2. Participants

Our targeted population were patients diagnosed with FEP meeting the inclusion criteria: ≥18 years of age, no history of antipsychotic use prior to admission to hospital, first episode of psychosis and fulfillment of the criteria for psychotic episode (codes F23, F29) according to the criteria of International Classification of Disorders, 10th revision (ICD-10) [31]. All participants were native Croatian speakers. Exclusion criteria were developmental disorders that can present with psychotic symptoms (intellectual disabilities, autism, pervasive developmental disorder, Asperger disorder), treatment with medications that can provoke psychosis and addictions excluding self-reported usage of cannabis. More detailed information on the participants and study protocol are described elsewhere [32]. All patients meeting those criteria who were treated at the ZUHC and UPHV for

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the first acute episode of psychosis during the study period were invited to participate in the study.

2.3. Sample size

We calculated the required sample size for the interaction of the change in neurocognitive test scores and the genotype with a targeted statistical power of 0.80, a significance level of $p < 0.05$, two repeated measurements, the minimum expected correlation between repeated measurements of $r \geq 0.60$ and the minimum standardized effect size we considered to be clinically relevant for Cohen's $d = 0.53$ ($f2 = 0.07$, partial $\eta^2 = 0.07$). Under these conditions we needed 90 respondents. Anticipating up to 5% of the data would be incorrectly collected, we estimated the initially required sample size to 95 participants. We calculated the required sample size using the G*Power version 3.1.9.4 [33].

2.4. Clinical assessments

Assessment included the collection of sociodemographic data (age, education, relationship status), clinical data (assessment of illegal drug use, smoking, alcohol use, use of medication), assessment using psychopathology rating with the Positive and Negative Syndrome Scale (PANSS) [34], assessment of general functioning using the Global Assessment of Functioning scale (GAF) [35] and neurocognitive assessment with the Mini Mental Status Examination (MMSE) [36] as the measure of general cognitive impairment and a set of neurocognitive tests and subtests, divided into seven neurocognitive domains:

a) Verbal memory: Rey Auditory Verbal Learning Test (RAVLT A, RAVLT B and RAVLT A30′) [37] and Wechsler verbal paired associates (Wechsler 6 and Wechsler 30′) [38];
b) Attention: Digit span test Forwards and Backwards (Digit Span F and Digit Span B) [39];
c) Executive functions: Block design test (BD) [40], Frontal assessment battery (FAB) [41], Clock drawing test (CDT) [42], Stroop colors (STROOP 2) and Stroop word-colors (STROOP 3) [43] and Trial Making Test B (TMTB) [44];
d) Speed of processing: Stroop words (STROOP 1) [43] and Trail Making Test A (TMTA) [44].
e) Working memory: Digit symbol test (Digit symbol) [39];
f) Visuospatial abilities: Rey-Osterrieth Complex Figure Test (ROCF and ROCF 30′) [45];
g) Language functions: Semantic (category) fluency test (Semantic) [39] and Phonetic fluency test (Phonetic) [39];

All of the tests were previously used in Croatian population [46].

Perceived level of stress was assessed using the Holmes-Rahe Stress Inventory (Stress) self-report questionnaire [47], with higher scores indicating higher levels of stress. Absolute mean values and standard deviations (SD) were used in the analysis.

Assessment of cannabis use was performed during the psychiatric interview and by incorporating anamnestic and heteroanamnestic data from family members. An urine drug screening test was applied in case of uncertainties. Cannabis use was classified into

several categories: 1) no prior cannabis exposure; 2) lifetime cannabis use not meeting DSM cannabis abuse or dependence criteria; 3) cannabis abuse or dependence; 4) cannabis use (2 or 3) with lifetime use of illegal drugs, not meeting criteria for abuse or dependence.

2.5. Genotyping

In addition to data on the associations of interest described above, we investigated polymorphisms to maximally represent common genetic variants in the population. The selection of these specific polymorphisms was further based on validity (minor allele frequency $\geq$10%; information retrieved from a public database, the National Center for Biotechnology Information, dbSNP, http://www.ncbi.nlm.nih.gov/SNP/), assay availability and their potential effect on gene expression or protein function as confirmed by previous studies. Blood samples for genotyping were collected in tubes with EDTA anticoagulant (Grainer Bio-One, Kremsmünster, Austria). Genomic DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The genotyping of CNR1 (rs7766029) and CNR1 (rs12720071) was performed with TaqMan® Drug Metabolism Genotyping assays ID: C_28979971_20 and ID: C_30749291_10, by real-time PCR genotyping method on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

2.6. Study outcomes

The primary outcome was the association of CNR1 genotypes with changes in neurocognitive test results during the 18 months follow-up. The secondary outcome was the association of CNR1 genotypes with change in the perceived levels of stress. The tertiary, exploratory outcomes were: interaction of cannabis use with the association of targeted genotypes and the changes in neurocognitive test results and perceived stress after 18 months of follow-up.

2.7. Statistical analysis

We used a general linear model to test the associations of changes in neurocognitive test results, the change of perceived level of stress from baseline to the 18th month of follow-up and the perceived levels of stress with CNR1 polymorphisms. In the tests of the first and second hypotheses we entered the neurocognitive tests and perceived levels of stress as the dependent measures in the model, genotypes as the independent variable and the baseline values of neurocognitive tests and perceived levels of stress as the covariate. Age, gender, negative PANSS and self-reported cannabis use were additional covariates. All covariates were selected based on the possible effects on neurocognition based on previous reports in the literature, while negative PANSS subscale was selected among other psychopathology measures as it correlated significantly with the majority of baseline neurocognitive tests, in our previously published report [46] (Supplementary Table 1). In the analysis, "cannabis use" variable was categorized as "yes" for all participants who used cannabis more than once in life and "no" for those participants without prior exposure to cannabis. Education was not used in the analysis, as there were no relevant variations in the sample (more than 85% of participants graduated high school). Standardized effect sizes were calculated using the partial omega square statistic and Clif's delta with 95% confidence intervals. We used the Benjamini-Hochberg method with the False discovery rate (FDR) set at $\leq$15 to control the effect of multiple testing considering all the primary and secondary outcomes testing. We performed the additional exploratory analysis of correlations between baseline values of neurocognitive test results with psychopathology measures, GAF and perceived levels of stress using the Spearman rank correlation (ρ). We used analysis of variance to analyze the differences in baseline neurocognitive test and stress test results between genotypes. The significance level was set at two-tails $p < 0.05$, and all confidence intervals at 95%. Statistical analysis was performed using the programming language Python, 3.7.2 with packages Numpy 1.17.0, Scipy 1.2.1 and Statsmodels 0.10.0 [48]. A test for Hardy-Weinberg

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equilibrium (HWE) using the Markov chain method [49] and a test for linkage disequlibrium (LD) [50] was performed using the Genepop software version 4.2 [51].

3. Results

3.1. Clinical characteristics of the sample

The final sample consisted of 159 FEP who were assessed for neurocognitive functioning at baseline. Sociodemographic and clinical characteristics at baseline are presented in Table 1. During the 18-month follow-up, 30 (19%) patients were lost to follow-up (moved/changed psychiatric clinic (n = 20), refused to participate in follow-up assessment (n = 2), refused to give blood sample (n = 7), unknown (n = 9)). Patients who were lost for follow-up at the 18th month were better educated: 15/30 (50%) of them had an university degree compared with 19/121 (16%) in those with valid data at the 18th month. They had somewhat less severe positive and negative psychotic symptoms, as well as a somewhat lower total PANSS score: median (IQR) 91 (74-104) compared with 98 (86-113) in those who remained in the study. The two groups had very similar tests' results in all other tests used in this study. When compared with the baseline assessment, all psychopathology scores measured by PANSS and its subscales and GAF improved over 18 months of follow-up (Supplementary Table 2). The majority of neurocognitive test improved as well, but to a lesser extent (Supplementary Table 2). At baseline, most neurocognitive test results were correlated with negative PANSS subscale, as well as with GAF, but only exceptionally with general and positive PANSS subscale scores (Supplementary Table 1). Additionally, levels of perceived stress showed poor correlation with neurocognitive symptoms (Supplementary Table 1).

3.2. Genotyping results

For CNR1 C > T (rs7766029), the genotyping of nine blood samples was not successfully performed. Therefore, in the per-protocol population, we analyzed 112 patients for CNR1 C > T (rs7766029) and 121 CNR1 A > G (rs12720071). Frequencies of CC, CT and TT at CNR1 C > T (rs7766029) were 26, 55 and 31, respectively; frequencies of AA, AG and GG of CNR1 A > G (rs12720071) were 102, 19 and 0, respectively. No significant deviations from the expected Hardy–Weinberg proportions were observed (CNR1 C > T (rs7766029, p > 0.999; CNR1 A > G (rs12720071, p=>0.999). Test results for LD between loci CNR1 C > T (rs7766029and CNR1 A > Grs12720071 were found to be significant (p = 0.028).

3.3. Primary outcome: Association of the CNR1 C > T (rs7766029) and CNR1 A > G (rs12720071) genotypes with the changes in neurocognitive performance

3.3.1. CNR1 C > T (rs7766029)

In the analysis adjusted only for the baseline neurocognitive test scores, we found significant (FDR < 15%) associations of CNR1 C > T (rs7766029) genotypes with the changes in neurocognitive tests from different domains (Table 2).

In the analysis adjusted only for the baseline neurocognitive test scores, rs7766029 showed significant associations (FDR < 15%) with the changes of executive functions (CDT, Stroop 3), attention (Digit span F) and verbal memory (Wechsler, Wechsler 30), with CC genotype carriers achieving the highest improvement compared with the carriers of other two genotypes, except for Stroop 3. After additional adjustment for gender, age, negative symptoms and cannabis use, only the association with the change in verbal memory (Weschler 30′) was significant (FDR < 15%). Our tertiary, exploratory analysis showed that cannabis use was in significant interaction with the genotype group and change in Wechsler 30 test score (p = 0.031). Participants not using cannabis had worse initial test results and significantly better improvement, achieving similar tests results as cannabis users in the second assessment. (Table 4). In addition, rs7766029 CC genotype carriers had the worst baseline values of CDT and Wechsler 30 (Supplementary Table 3).

3.3.2. CNR1 A > G (rs12720071)

In the analysis adjusted only for the baseline neurocognitive test scores, the rs12720071 genotypes were associated with the significant (FDR < 15%) change of executive (BD) and language functions (Semantic fluency) (Table 3). However, after additional adjustment for age, sex, negative symptoms and cannabis use, none of these associations were significant. In our tertiary, exploratory analysis we found a significant interaction of

Table 1
Sociodemographic and clinical characteristics of participants by different genotypes.

	CNR1 C > T (rs7766029)						CNR1 A > G (rs12720071)			
	CC		CT		TT		AA		AG	
	n = 26		n = 55		n = 31		n = 102		n = 19	
Male gender	18	(69.2)	36	(65.5)	18	(58.1)	66	(64.7)	12	(63.2)
Age (years), median (IQR)	24	(22-30)	23	(22-29)	25	(21-33)	24	(22-31)	24	(21-29)
Education										
Primary school	1	(3.9)	5	(9.1)	1	(3.2)	7	(6.9)	1	(5.3)
Secondary school	15	(57.7)	29	(52.7)	21	(67.7)	60	(58.8)	9	(47.4)
College/University	10	(38.5)	21	(38.2)	9	(29.0)	35	(34.3)	9	(47.4)
Smoking cigarettes	13	(50.0)	32	(58.2)	20	(64.5)	63	(61.8)	10	(52.6)
Cannabis use	11	(42.3)	32	(58.2)	16	(51.6)	54	(52.9)	11	(57.9)
Alcohol misuse	1	(3.9)	3	(5.5)	2	(6.5)	7	(6.9)	0	(0.0)
Positive and Negative Syndrome Scale, mean (SD)										
Positive symptoms	26	(6.8)	26	(7.0)	27	(5.6)	27	(6.7)	27	(7.6)
Negative symptoms	25	(6.2)	24	(6.7)	24	(7.4)	24	(6.4)	26	(7.4)
General symptoms	50	(8.7)	49	(10.2)	48	(10.1)	49	(8.9)	51	(13.2)
Total score	100	(17.6)	99	(20.6)	99	(20.0)	99	(18.3)	104	(24.8)
GAF, median (IQR)	25	(15-20)	25	(20-30)	20	(20-30)	25	(20-30)	20	(15-25)

Data presented as number and (percentage), unless stated otherwise.
Abbreviations: IQR=interquartile range; SD=standard deviation; GAF=Global Assessment of Functioning scale.

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Table 2
Statistically significant association of CNR1 C > T (rs7766029) genotypes with the changes of test results.

Genotype	Baseline		At 18th month		Δ	(95% CI)	ω2	Unadjusted coefficient	p	Adjusted coefficient	p
CDT											
CC (n = 26)	8.5	(2.31)	9.6	(0.68)	1.1	(0.23, 1.93)	0.79	−0.24	0.030*	−0.21	0.063
TC (n = 55)	9.3	(0.89)	9.7	(0.71)	0.4	(0.20, 0.59)	−0.32				
TT (n = 31)	9.6	(1.16)	9.6	(1.13)	0.1	(-0.25, 0.38)	−0.18				
Digit span F											
CC (n = 26)	11.0	(1.4)	10.8	(1.74)	−0.3	(-0.75, 0.21)	−0.04	−0.31	0.050*	−0.29	0.039
TC (n = 55)	11.1	(1.43)	11.3	(1.25)	0.3	(-0.07, 0.58)	−0.08				
TT (n = 31)	11.1	(1.77)	11.9	(2.05)	0.8	(0.03, 1.65)	0.40				
Stress											
CC (n = 26)	120.4	(101.34)	60.2	(65.37)	−60.1	(-103.35, -16.88)	−0.11	−30.75	0.003*	−27.19	0.008
TC (n = 55)	119.4	(127.76)	84.1	(84.78)	−34.4	(-72.52, 3.67)	0.54				
TT (n = 31)	181.6	(134.42)	118.9	(119.04)	−62.6	(-123.66, -1.57)	−0.12				
STROOP 3											
CC (n = 26)	160.5	(66.82)	135.9	(55.39)	−24.7	(-46.91, -2.39)	0.16	10.07	0.043	7.91	0.099
TC (n = 55)	149.6	(59.63)	123.5	(48.5)	−26.8	(-40.19, -13.39)	0.23				
TT (n = 31)	136.8	(47.41)	119.1	(28.75)	−17.7	(-29.93, -5.56)	−0.07				
Weschler											
CC (n = 26)	38.8	(7.47)	43.4	(2.08)	4.6	(1.85, 7.38)	0.23	−1.09	0.022*	−0.93	0.048
TC (n = 55)	41.7	(6.64)	44.4	(2.63)	2.7	(1.19, 4.16)	0.13				
TT (n = 31)	41.7	(5.45)	45.0	(2.88)	3.4	(1.60, 5.11)	−0.04				
Weschler 30'											
CC (n = 26)	6.3	(1.53)	7.2	(0.73)	0.9	(0.39, 1.45)	0.32	−0.32	0.003*	−0.28	0.007*
TC (n = 55)	7.2	(1.05)	7.	(0.93)	0.3	(0.08, 0.62)	−0.09				
TT (n = 31)	7.3	(1.44)	7.5	(0.88)	0.2	(-0.37, 0.76)	0.04				

Test results are presented as mean (standard deviation).
Abbreviations: Δ = mean of absolute changes; CI = confidence interval; General linear model unadjusted coefficient and p-values and adjusted for age, cannabis use, gender and negative symptoms; ω^2 = Partial omega squared; CDT = Clock Drawing Test; Digit span F = Digit span Forwards; Stress = the Holmes-Rahe Stress Inventory; Stroop 3 = Stroop word-colours; Wechsler = Wechsler verbal paired associates; Wechsler 30′ = Wechsler verbal paired associates after 30 min.
* statistically significant outcomes at 15% false discovery rate calculated using Benjamini-Hochberg method.

Table 3
Statistically significant association of CNR1 A > G (rs12720071) genotypes with the changes of tests' results.

Baseline	Baseline		At 18th month		Δ	(95% CI)	ω2	Unadjusted coefficient	p	Adjusted coefficient	p
BD											
AA (n = 102)	53.4	(11.35)	55.4	(11.57)	2.0	(-0.50, 4.43)	0.11	−5.22	0.015*	−4.89	0.017
AG (n = 19)	45.9	(16.39)	52.5	(13.56)	6.6	(0.11, 13.04)	0.27				
Semantic											
AA (n = 102)	44.7	(10.6)	48.2	(12.17)	3.5	(1.62, 5.46)	0.32	−4.63	0.021*	−3.13	0.102
AG (n = 19)	41.1	(11.1)	42.6	(10.28)	1.5	(-3.38, 6.44)	0.09				
Stress											
AA (n = 102)	149.3	(132.75)	93.7	(96.42)	−55.1	(-84.27, -25.93)	−0.07	−48.67	0.014*	−42.27	0.031
AG (n = 19)	83.7	(79.44)	62.5	(69.19)	−21.3	(-67.89, 25.36)	0.43				

Test results are presented as mean (standard deviation).
Abbreviations: Δ = mean of absolute changes; CI = confidence interval; General linear model unadjusted coefficient and p-values and adjusted for age, cannabis use, sex and negative symptoms; ω^2 = Partial omega square; BD = Block design test; Semantic = Semantic fluency test; Stress = Holmes-Rahe Stress Inventory.
* Statistically significant outcomes at 15% false discovery rate calculated using Benjamini-Hochberg method.

rs12720071 genotypes and negative symptoms (p = 0.031) and cannabis use (p < 0.001) on BD change. Additionally, the rs12720071 AG carriers showed worse baseline values of BD (Supplementary Table 3).

3.4. Secondary outcome: Association of the CNR1 C > T (rs7766029) and CNR1 A > G (rs12720071) genotype with the change in the perceived stress

In the analysis adjusted only for the baseline neurocognitive test scores, the rs7766029 CC genotype carriers and rs12720071 AG carriers reported the lowest and significant (FDR < 15%) level of change in the perceived stress. In the fully adjusted analysis, only the association with the rs7766029 remained significant with FDR < 15%. Our tertiary, exploratory analysis showed a significant interaction of cannabis use and both genotypes on the change of perceived stress (Table 5). We did not find statistically significant associations of genotypes with any other baseline test values or changes. Results of the associations of genotypes with all baseline values of all applied tests and changes of tests after 18 months of follow-up are given in Supplementary Table 1.

4. Discussion

4.1. Association of CNR1 genotypes and changes in neurocognitive performance after eighteen months of follow-up

In the present study we found significant associations of rs7766029 CNR1 genotype with verbal memory and attention. CC genotype carriers achieved the highest improvement in all tests compared with the other two genotypes. However, in the fully adjusted analysis, only the association with the change in verbal memory (Weschler 30′) was significant, but cannabis use was in significant interaction with the genotype group and change in Wechsler 30 test scores.

Notably, the rs7766029 CC genotype carriers had the worst baseline values of CDT and Wechsler 30′, and the rs12720071 AG carriers showed worse baseline values of BD suggesting the CC

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Table 4
Association of CNR1 C > T (rs7766029) genotypes with changes in Wechsler 30′ test scores including the interaction with cannabis use.

	Baseline		At 18th month		Δ	(95% CI)	ω2
Cannabis users							
CC (n = 11)	7.6	(0.35)	7.6	(0.35)	0.0	(-0.46, 0.46)	0.03
CT (n = 32)	7.3	(9.94)	7.6	(0.74)	0.2	(-0.18, 0.56)	0.18
TT (n = 16)	7.6	(0.64)	7.6	(0.69)	−0.1	(-0.67, 0.54)	0.12
Cannabis non-users							
CC (n = 15)	5.7	(1.43)	7.2	(0.74)	1.6	(0.92, 2.28)	0.13
CT (n = 23)	6.4	(1.15)	7.6	(0.59)	0.6	(0.18, 0.95)	0.10
TT (n = 15)	7.0	(1.72)	7.5	(0.68)	0.5	(-0.51, 1.44)	0.27

Wechsler test results are presented as mean (standard deviation).
Abbreviations: Δ = mean of absolute changes; CI = confidence interval; ω^2= Partial omega square.

genotype and AG as the risk genotypes. These results are partially concordant with reports by Ho et al. [52], who found that patients with rs7766029 (C allele) had lower white matter volumes than those with the T allele, although with unknown effect on neurocognition, and that carriers of rs12720071 polymorphism (G allele) had lower white matter and worse results on processing speed/attention and problem-solving tests than those with the A allele, indicating worse results on executive functioning. Concordantly, in a case control study CNR1 rs7766029 polymorphisms were found showing trends of the C allele toward an association with schizophrenia. Bearing in mind that the purpose of the analysis of differences in baseline values according to genotypes was only exploratory, these funding are only suggestive.

While the rs12720071 genotypes were seemingly associated with the change in executive functions, after full adjustment the associations were not significant. It is thus possible that the observed trend in the associations of the rs12720071 genotypes with neurocognitive performance actually reflect the linkage disequilibrium with rs7766029 or with another polymorphic loci, such as CNR1 rs1049353 [15] which was reported by Suárez-Pinilla et al. [14] as having a significant association with changes of caudate volume after a 3-year period in a sample of patients with FEP. Interestingly, the rs1049353 polymorphism has also been suggested as being overrepresented with its G variant in patients with schizophrenia abusing cannabis [53]. This could also suggest that the observed association of neurocognitive changes with rs12720071 genotypes or even the rs7766029 genotype may reflect the effects of synonymous rs1049353 polymorphism, especially considering the observed interaction with cannabis use in our sample.

Interestingly, in 2013 Onwuameze et al. [54] reported significant CNR1 rs12720071 genotype interaction effects with cannabis use on total cerebral, frontal and parietal white matter volumes, but not on temporal white matter volumes. More specifically, they reported that rs12720071 G allele carriers with heavy marijuana use had significantly smaller white matter volumes than their A homozygote counterparts. This effect was not seen among those who did not use cannabis. While we observed the interaction of cannabis use with the association of rs12720071 genotypes and changes in the Block design test results, the association was not statistically significant after full adjustment. However, we found significant interaction of cannabis use with verbal memory domain changes and rs7766029 genotypes, in the sense that among cannabis users the effects of genotype on baseline scores and improvement of verbal memory was lost, but it remained in the non-user group. Similarly, Suárez-Pinilla et al. reported an association of rs1535255 polymorphism with white matter volumes at baseline and after 3 years only among patients who were not cannabis smokers, while the association in the group of cannabis non-users was lost [14]. It is possible that cannabis may exert moderating effects on the risk genotypes in different directions, probably under the influence of numerous other effects, such as the frequency of use and the use of concomitant medication.

The explanation of why the risk alleles may be associated with better improvement is not straightforward. The risk genotype is supposedly associated with lower activity of the receptor, which possibly contributes to a genetic make-up of lesser vulnerability to environmental stimuli. In a mice model, significant increase in CNR1 expression and a consistent reduction in DNA methylation at specific CpG sites of gene promoter was found in the prefrontal cortex, induced by prenatal methylazoxymethanol acetate exposure, indicating the responsivity of CNR1 to stress. However, the role of CNR1 on the overall vulnerability to stress seems very complex and may seem contradictory, depending on the system they influence [55]. For example, although initial studies of *cnr1* knockout mice demonstrated increased injury following stroke, indicating that activation of the *cnr1* was neuroprotective, later studies of selective antagonists of *cnr1* also demonstrated a protective effect, and surprisingly the double knockout animals had improved outcomes [56]. It is thus also possible that the carriers of the CC rs7766029 genotype are more vulnerable to different environmental stressors, reflected in initial test scores, but also possibly more receptive to general stress-reducing factors, such as treatment in general.

4.2. The role of levels of perceived stress

Interestingly, the overall highest perceived levels of self-reported stress were found among those abusing cannabis, but in particularly if

Table 5
Association of CNR1 C > T (rs7766029) and CNR1 A > G (rs12720071) with changes in Stress Holmes-Rahe Stress Inventory scores including the interaction with cannabis use.

	Baseline		At 18th month		Δ	(95% CI)	ω2
CNR1 C > T (rs7766029)							
Cannabis users							
CC (n = 11)	150.2	(107.78)	79.1	(52.34)	−64.6	(-134.38, 511)	0.05
CT (n = 32)	134.7	(140.71)	89.9	(81.40)	−42.0	(-94.42, 10.42)	0.30
TT (n = 16)	235.1	(124.06)	131.0	(105.05)	−104.1	(-178.18, -29.95)	0.14
Cannabis non-users							
CC (n = 15)	116.2	(89.73)	54.4	(72.62)	−56.8	(-113.64, 0.04)	0.07
CT (n = 23)	103.4	(104.55)	80.0	(91.58)	−23.4	(-78.66, 31.84)	0.15
TT (n = 15)	124.5	(120.89)	106.1	(131.13)	−18.4	(-114.24, 77.44)	0.21
CNR1 A > G (rs12720071)							
Cannabis users							
AA (n = 54)	175.5	(144.66)	101.1	(87.94)	−72.9	(-113.72, -30.99)	0.44
AG (n = 11)	119.0	(86.88)	92.3	(82.55)	−20.3	(-99.37, 58.83)	0.07
Cannabis non-users							
AA (n = 48)	124.8	(110.29)	90.5	(108.38)	−33.2	(-79.27, 12.82)	0.45
AG (n = 8)	50.1	(44.61)	27.5	(21.72)	−22.6	(-54.00, 8.75)	0.00

Stress Holmes-Rahe Stress Inventory test results are presented as mean (standard deviation).
Abbreviations: Δ = mean of absolute changes; CI = confidence interval; ω^2 = Partial omega square.

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they were the carriers of the TT rs7766029 and AA rs12720071 genotypes. This could be explained by different subgroups of patients who develop psychosis, one with stronger (or different) biological vulnerability and the other with more vulnerability to environmental stressors, possibly associated with a distinct biological make-up. This may be in line with contradictory findings in the literature on the neurocognitive status of patients with schizophrenia who use cannabis versus the ones who do not [57–59].

Moreover, there was a significant interaction of self-reported cannabis use and self-perceived level of stress with both genotypes on the changes in neurocognition, with the carriers of the TT rs7766029 genotype and AA rs12720071 genotype who used cannabis showing the greatest decrease in the perceived levels of stress, although the later association was not statistically significant at FDR. Interestingly, Wirtz et al. [29], reported an association between rs1049353 polymorphism and activation of prefrontal cortex when viewing negative pictures after stress (more in AA/AG then the GG genotype) and showed that memory performance correlated with amygdala and hippocampus activity and connectivity in stressed carriers of AA/AG but not the GG genotype, all suggesting more vulnerability/stronger affective response to negative stressors in the carriers of the A allele. On the other hand, the G allele was suggested as a risk variant in schizophrenia and cannabis abuse [14,53]. Following the same logic in our results, one possible explanation may be that the carriers of risk alleles, the CC rs7766029 and AG rs12720071 genotype, perceive their emotions less intensely, perhaps also due to negative symptoms of illness, and thus rated their perceived level of stress as lower compared with carriers of other variants, but are in fact more susceptible to FEP with a different genetic background (for example, non-affective psychosis vs. affective psychosis). However, as both of the studied genotypes were found in LD with the rs1049353 polymorphism, our results may also reflect the association of the rs1049353 polymorphism with the levels of perceived stress [29]. Again, this could suggest a moderating effect of cannabis use on the overall treatment response/progression of illness.

The levels of stress were measured by a self-assessment scale only, without verification of any of the biological indicators (such as cortisol levels), which does not have to be congruent. However, subjective perception may modulate behavior more than objective stress indicators (persons with higher levels of perceived stress may be more prone to engage in activities to decrease stress, e.g. self-medication with the use of cannabis).

Among those who did not abuse cannabis, the direction of associations of the CNR1 rs7766029 genotypes and the changes in perceived stress was concordant the one observed with changes in neurocognitive performance: the carriers of the rs7766029 CC genotypes showed the greatest decrease of perceived stress levels, as well as the best improvement of neurocognitive performance. This could suggest that among those who develop FEP without prior exposure to cannabis, vulnerability to stress may affect neurocognitive performance in a more direct manner, again suggesting their different biological vulnerability/pathway of development of FEP compared to those who developed FEP with prior exposure to cannabis.

4.3. Limitations of the study

The study was performed in a naturalistic setting and the medication and treatment was given according to clinical judgments of the treating physician. As all patients were treated with multiple methods and different medication over the studied period, we assumed that the improvement in psychopathology and general functioning, as well as in neurocognition is the response to overall treatment. In general, during the study period all patients were treated with treatment that is usual practice in the included hospitals, which implies that the treatment usually begins with hospital treatment, with the focus on pharmacotherapy indicated by the treating psychiatrists over 3–4 weeks. After hospital discharge, treatment is continued by the same psychiatrists through outpatient treatment comprising short outpatient visits (30–45 minutes) usually once a month or with an addition of multimodal psychosocial programs through daily hospital setting, if agreed on by the patients. Because of this diversity and complexity, it is very difficult to analyze the effect of genotypes on specific treatment approaches.

By choosing neurocognitive symptoms as a measure of treatment response, as they show stronger genetic association compared with other psychotic symptoms, as well as a relative resistance to treatment across different study populations, regardless of the type of pharmacological treatment, we hope to have reduced the number of confounding factors. Furthermore, although we included the data on cannabis use in the analysis, the data were obtained by psychiatric interview only, without verification with cannabis testing in most cases. Another limitation could be the relatively low sample size for pharmacogenetic studies, and it should be noted that some of the findings may by falsely negative.

4.4. Conclusions

To the best of our knowledge, this is the first time an association of CNR1 polymorphisms and change of neurocognition has been found in a sample of previously untreated patients with FEP followed during 18 months of treatment. Moreover, we detected significant interactions of self-reported cannabis use with genotypes resulting in changes in neurocognitive test results as well as in perceived levels of stress possibly indicating a moderating effect of cannabis on the course of illness, associated or influenced by the perception of being under stress. Even though our results are promising, it is critically important to validate these findings in larger cohorts of patients and over a longer follow-up design to firmly establish the potential of these polymorphisms as pharmacogenetic markers of neurocognitive change, especially considering the role of cannabis use.

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Declaration of Competing Interest

None.

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