Association of intron 4 VNTR polymorphism in the *NOS3* gene with rapid cycling and treatment resistance in bipolar disorder: a case-control study

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ABSTRACT

الأهداف: تقييم العلاقة بين المؤشرات السريرية للمرضى، وخاصة المحددات السريرية، ومتغير intron 4 VNTR لجين أكسيد النيتريك(*NOS3*) في مرضى اضطراب ثنائي القطب (BD).

المنهجية: تم تضمين عينة من 95 مريضًا مصابًا بمرض الاضطراب ثنائي القطب و 95 متطوعًا سليمًا في دراسة الحالات والشواهد. تم قبول المرضى على التوالي في عيادة الطب النفسي الخارجية لمدة 6 أشهر وتم تقييمهم ببعض المقاييس للمعايير السريرية. بالإضافة إلى ذلك، استخدم PCR لتحديد متغير NOS3 intron 4 VNTR.

النتائج: كان النمط الجيني *NOS3* وتوزيعات تردد الأليل للدوران السريع لمرضى BD مختلفة اختلافًا كبيرًا عن مرضى BD ذوي الدوران غير السريع ومجموعات التحكم. علاوة على ذلك، كان النمط الجيني *NOS3* وتوزيعات تردد الأليل لمرضى BD المقاومة للعلاج مختلفة بشكل كبير عن مرضى BD المستجيبين للعلاج ومجموعات التحكم. في حين أن مرضى BD الذين يحملون النمط الوراثي b/b والأليل b لديهم مخاطر أقل للدوران السريع ومقاومة العلاج، كان وجود النمط الوراثي b/a في مرضى BD أكثر عرضة لخطر الدوران السريع ومقاومة العلاج. بالإضافة إلى ذلك، كان عدد حالات الاستشفاء ودرجات مقياس تحسين الأنطباع الشامل السريري لمجموعة BD مع النمط الوراثي b/b أقل إحصائيًا من مجموعة BD ذات الأنماط الجينية b/a و a/a.

الخلاصة: نقترح أن متغير intron 4 VNTR لجين *NOS3* قد يترافق مع الدوران السريع ومقاومة العلاج في المرضى الأتراك الذين تم تشخيص إصابتهم بالاضطراب ثنائي القطب.

Objective: To evaluate the relationship between patients' clinical parameters, especially clinical specifiers, and the intron 4 VNTR variant of the endothelial nitric oxide synthase (*NOS3*) gene in bipolar disorder (BD) patients.

Methods: A sample of 95 patients with BD and 95 healthy volunteers were included in the case-control study. The patients consecutively admitted to the outpatient psychiatry clinic for 6 months and were evaluated with some scales for clinical parameters. In addition, PCR was used to determine the *NOS3* intron 4 VNTR variant.

Results: The *NOS3* genotype and allele frequency distributions of rapid cycling BD patients were significantly different from non-rapid cycling BD patients and the control groups. Furthermore, *NOS3* genotype and allele frequency distributions of treatment-resistant BD patients were significantly different from treatment-responsive BD patients and the control groups. While BD patients carrying the b/b genotype and b allele had a lower risk of rapid cycling and treatment resistance, having the b/a genotype in BD patients was at higher risk in terms of rapid cycling and treatment resistance. In addition, the number of hospitalizations and the Clinical Global Impression-Improvement Scale scores of the BD group with the b/b genotype were statistically lower than the BD group with b/a and a/a genotypes.

Conclusions: We propose that the intron 4 VNTR variant of the *NOS3* gene may be associated with rapid cycling and treatment resistance in Turkish patients diagnosed with BD.

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Bipolar disorder (BD) is a severe and chronic psychiatric disorder that affects approximately 1.3% of the population and is characterized by varying clinical presentations of depression and mania

in addition to manic-depressive episodes with rapid cycling and mixed features.[1,2] While the involvement of inflammatory processes, oxidative stress, genetic factors, neurotransmitters, and psychosocial factors associated with BD have been investigated, the etiology of BD has not yet been identified. [3-5]

Numerous studies have identified inflammation-related genes that may play roles in triggering BD and documented increased levels of circulating proinflammatory cytokines in different phases of BD.[6-8] T lymphocytes, macrophages, and endothelial cells secrete polypeptide molecules that support critical functions, including the proliferation of B cells, synthesis of acute-phase reactants, activation of neutrophils, and increased vascular permeability.[3] Many studies that showed an association between vascular pathologies and BD had demonstrated inflammation-related endothelial damage.[9] Goldstein et al[10] reported that BD patients are more likely to have hypertension and cardiovascular diseases and developed these disorders a decade earlier than non-BD participants.

Nitric oxide (NO) plays an essential role in various functions, including antibacterial and antitumoral functions, neurotransmission, vasodilation, and neurotoxicity related to learning and memory impairment.[11,12] In addition, a large body of evidence suggests that NO is a critical factor in many psychiatric disorders and can play both a protective and destructive role depending upon NO's production and interaction with other factors in the cell.[13,14] Nitric oxide synthase 1 (*NOS1*), *NOS2*, and *NOS3* genes encode 3 NOS enzymes that synthesize NO from L-arginine. *NOS3* gene produces endothelial NOS (eNOS), mainly involves the endothelium and maintains the basal vascular tone and cerebral blood flow; neuronal NOS (nNOS) is produced by the gene *NOS1*, and inducible NOS (iNOS) is produced by the gene *NOS2*.[12] Although different *NOS3* variants have been determined, the variable number of tandem repeats (VNTR) in intron 4, the Glu298Asp variant in exon 7, and the T786C variant in the promoter region are the most critical polymorphisms of the *NOS3* gene.[15] The VNTR in intron 4 of the *NOS3* gene is responsible for producing more than 25% of basal plasma NO.[16] Several neuropsychiatric disorders and vascular disorders such as diabetes mellitus, hypertension, QTc prolongation, and spontaneous abortion were found to be related to the polymorphisms of these *NOS* genes.[11,17]

The *NOS3* gene has been found on 7q36 and consists of 26 exons spanning approximately 21 kilobases.[18] Genome-wide scan of BD and investigation of population stratification effects on linkage provided evidence supporting susceptibility loci at 4q21, 7q36, 9p21, 12q24, 14q24, and 16p13.[19] Therefore, we hypothesized that the intron 4 VNTR variant of the *NOS3* gene might be associated with more severe and treatment-resistant forms of BD in Turkish patients. We aimed to examine the association between BD clinical information and the intron 4 VNTR variant in the *NOS3* gene by comparing genotype distributions of patients with healthy controls.

Methods. *Patient selection.* The present study was designed as a case-control study and conducted at the Bakirkoy Mazhar Osman Mental Health and Neurology Training and Research Hospital outpatient clinic, Istanbul, Turkey. A sample of 95 patients diagnosed with BD was followed for 6 months in 2018; additionally, 95 age- and sex-matched controls were included in the study. The study was approved by the Clinical Research Ethics Committee of the Istanbul Faculty of Medicine under the ethical standard for human experimentation established by the Declaration of Helsinki (10/10.01.2018).[20] Furthermore, all participants' written consent was obtained to participate in the study.

Inclusion and exclusion criteria. Inclusion criteria for the study included the following: Aged 18-65 years, a DSM-IV diagnosis of BD type I or II according to the Structured Clinical Interview for DSM-IV Axis-I Disorders (SCID-I), no history of neurological/ systemic illness, substance use, or event that might influence cognitive function. In addition, we excluded patients with a history of any comorbid Axis I disorders, neurodevelopmental disorders such as autism, mental retardation, cerebral tumor, and cerebrovascular disease.

Diagnostic tools and scales. First, the SCID-I was used to confirm the diagnosis according to the DSM-IV-TR criteria, and the presence of any psychiatric disorder in the healthy control group was the basis for exclusion from the study.[21,22] Then, sociodemographic and clinical parameters data form was applied to BD patients. Subsequently, the Young Mania Rating Scale (YMRS) and Hamilton Depression Rating Scale (HAM-D) were administered to patients with BD to evaluate the severity of mania and depression, and the Clinical Global Impression Scale (CGI) was used to assess the severity of the disorder and response to the treatment.[23-27]

Criteria for treatment resistance and rapid cycling in BD. Sachs proposed the term "treatment-resistant bipolar disorder" to define patients who do not respond

to at least 2 antimanic or antidepressant drugs with adequate dose and duration of treatment in a specific period, such as 6 weeks for mania or depression and 6 months or three-cycle lengths for maintenance.[28] The DSM-5 defines rapid cycling BD as a pattern of presentation accompanied by four or more mood episodes in 12 months, with a typical course of mania, hypomania, or depression. The episodes must be demarcated by a complete or partial remission lasting at least two months or by switching to a mood state of the opposite polarity.[29]

DNA analyses. Blood samples for isolating DNA material were received from participants to analyze at the Istanbul Faculty of Medicine Laboratory of Medical Biology. The intron 4 VNTR *NOS3* gene variants were genotyped by polymerase chain reaction (PCR) using the following primers: (forward) 5'-AGG CCC TAT GGT AGT GCC TTT-3' and (reverse) 5'-TCT CTT AGT GCT GTG GTC AC-3'. Then, we separated the products on a 4% NuSieve GTG agarose gel and repeated the experimental process twice for each sample. We designed primers to amplify a 393 bp and/or 420 bp segment of the *NOS3* intron 4 VNTR region, including the microsatellite repeat sequence. *NOS3* intron 4 VNTR genotypic distributions were determined as 393 bp, 393, 420 bp and 420 bp for a/a, a/b, and b/b genotypes, respectively.[30]

Statistical analyses. We performed statistical analysis using IBM SPSS version 21.0 (IBM Corp. released 2012; Armonk, NY, USA). Descriptive statistics contained mean, standard deviation, frequency, and percentage. The comparison of genotype distributions of *NOS3* intron 4 VNTR variants between groups was analyzed with the Pearson chi-square test. Again, the comparisons of genotype distributions of *NOS3* intron 4 VNTR variants in BD patients in terms of rapid cycling or treatment resistance were analyzed with the Pearson chi-square test or Fisher's exact test. We also calculated the odds ratio (OR) and the 95% confidence interval (CI). The Shapiro-Wilk test evaluated the suitability of continuous variables for a normal distribution. Since the variables did not have a normal distribution, the scale scores and clinical parameters were compared using Mann-Whitney U testing. We analyzed genotype distributions in participants due to the Hardy-Weinberg Equilibrium (HWE) and accepted $p<0.05$ as a statistical significance. The power analysis was performed with the "G*power" software (version 3.0.5, http://www.psycho. uni-duesseldorf.de/abteilungen/aap/gpower3/), post hoc goodness of fit $\chi 2$ test, with an "-error" probability of 0.05. The possible presence of population stratification bias has been calculated, according to Lee and Wang, regarding intron 4 VNTR *NOS3* gene polymorphism frequencies documented for Turkish populations and incidence rate of BD in Turkey.[31-33]

Results. *NOS3 intron 4 VNTR genotyping.* Ninety-five patients with BD (56 female/39 male) were evaluated according to their clinical parameters, and the scale scores are presented in **Table 1**. According to the *NOS3* intron 4 genotype distribution, 69.5% (n=66) of the patients diagnosed with BD had the b/b genotype, 26.3% (n=25) had b/a, and 4.2% (n=4) had a/a. Eighty-one point one percent (n=77) of the healthy controls had the b/b genotype, 17.9% (n=17) had b/a, and 1.1% (n=1) had a/a. Comparing the *NOS3* genotype (b/b, b/a, a/a) and the allele frequency (b, a) distributions of patients with BD to the control group revealed a statistically significant difference between the allele frequency of the 2 groups. The BD patients had a higher frequency of the "a" allele than the control group (OR:1.892; 95% Cl: 1.033-3.463; *p*=.037) **Table 2**.

Comparison of NOS3 genotype and allele frequency distributions of rapid cycling BD patients with non-rapid cycling BD patients and the control groups. Comparing the *NOS3* intron 4 genotype (b/b, b/a, a/a) and allele frequency (b, a) distributions regarding the presence of clinical specifiers (atypical features, mixed features, seasonal pattern, rapid cycling, psychotic features, peripartum onset) in the BD patient group demonstrated that the *NOS3* intron 4 genotype and allele frequency distributions were significantly different between the groups of patients with BD in terms of rapid cycling. BD patients without rapid

Table 1 - Sociodemographic characteristics and the Scale Scores of BD patients.

Bipolar disorder	Mean±SD
Age	41.27±11.61
Age of onset	25.47±8.70
Duration of disease	15.93±10.71
Number of hospital	3,68±4.56
Dep. Episode	1.55±2.51
Manic episode	4.48±4.96
Total episode	6.03±5.54
HAM-D	11.36±7.47
YMRS	7.95±8.72
CGI-S	5.01±0.90
CGI-I	2.07±0.84

SD - standard deviation, hospital - hospitalization, Dep - depressive, HAM-D - Hamilton depression rating scale, YMRS - Young mania rating scale, CGI-S - clinical global impression scale-severity, CGI-I - clinical global impression scale-improvement

Table 2 - Comparison of genotype distributions of *NOS3* intron 4 VNTR variants in BD patients with the control group.

Genotypes	Bipolar disorder	Healthy control	OR	95% CI	*P*-value
NOS3	n=95 (%)	n=95 (%)			
b/b	66 (69.5)	77 (81.1)	1.880*	0.958-3.687*	.064*
b/a	25 (26.3)	17 (17.9)	0.610*	0.304-1.223*	.162*
a/a	4 (4.2)	1 (1.1)	0.242*	0.027-2.207*	.174*
Allele					
b	157 (82.6)	171 (90)			
a	33 (17.4)	19 (10)	1.892*	1.033-3.463*	.037*
	OR - odds ratio, CI - confidence interval, *Pearson chi-square				

Table 3 - Comparison of *NOS3* genotype and allele frequency distributions of rapid cycling BD patients with non-rapid cycling BD patients and the control groups.

Genotypes	Rapid cycling yes	Rapid cycling no	OR	95% CI	*P*-value
NOS3	n=20 (%)	n=75 (%)			
b/b	9 (45)	57 (76)	0.258*	0.092-0.722*	.007*
b/a	9 (45)	16 (21.3)	3.017*	1.067-8.534*	.033*
a/a	2 (10)	2 (2.7)	0.247[&]	0.032-1.871[&]	.194[&]
Allele					
b	27 (67.5)	130 (86.7)			
a	13 (32.5)	20 (13.3)	3.130*	1.389-7.049*	.004*
NOS3		Healthy control			
b/b	9 (45)	77 (81.1)	0.191*	0.069-0.530*	.001*
b/a	9 (45)	17 (17.9)	3.754[&]	1.347-10.466[&]	.016[&]
a/a	2 (10)	1 (1.1)	10.444[&]	0.899-121.379[&]	.078[&]
Allele					
b	27 (67.5)	171(90)			
a	13 (32.5)	19 (10)	0.231*	0.102-0.521*	.000*
	OR - odds ratio, CI - confidence interval, *Pearson chi-square &Fisher's Exact Test				

Table 4 - Comparison of *NOS3* genotype and allele frequency distributions of treatment resistant BD patients with treatment-responsive BD patients and the control groups.

Genotypes	Treat. Res. yes	Treat. Res. No	OR	95% CI	*P*-value
NOS3	n=25 (%)	n=70 (%)			
b/b	12 (48)	54 (77.1)	3.656	1.396-9.575*	.007*
b/a	11 (44)	14 (20)	0.318*	0.119-0.850*	.019*
a/a	2 (8)	2 (2.9)	0.338[&]	0.045-2.540[&]	.282[&]
Allele					
b	35 (55.1)	122 (87.1)			
a	15 (44.9)	18 (12.9)	2.905*	1.330-6.346*	.006*
NOS3		Healthy control			
b/b	12 (48)	77 (81.1)	0.216*	0.085-0.551*	.001*
b/a	11 (44)	17 (17.9)	3.605*	1.397-9.304*	.006*
a/a	2 (8)	1 (1.1)	8.174[&]	0.710-94.097[&]	.110[&]
Allele					
b	35 (55.1)	171 (63.9)			
a	15 (44.9)	19 (36.1)	0.259*	0.120-0.559*	.000*
	Treat - treatment; Res - resistance; OR - odds ratio; CI - confidence interval, *Pearson chi-square&Fisher's Exact Test				

cycling had a higher frequency of b/b genotypes and the b allele than BD patients with rapid cycling (OR: 0.258; 95% Cl: 0.092-0.722; *p*=.007; OR: 3.130; 95% Cl: 1.389-7.049; *p*=.004, respectively). Furthermore, the b/a genotype was found at a significantly higher frequency in BD patients with rapid cycling than in BD

Table 5 - Comparison of scale scores and clinical parameters regarding the *NOS3* intron 4 genotype distributions of BD patients.

Scale Scores and Clinical Parameters	(b/b) (n= 66)		(b/a, a/a) (n=29)		*P*-value
	Median (min-max)	Mean±SD	Median (min-max)	Mean±SD	
HAM-D score	9(0-31)	11.39 ±6.96	9(0-34)	11.31±8.67	.771
YMRS score	6(0-37)	7.48±8.08	1(0-35)	9.03±10.10	.575
CGI-S score	5(2-7)	4.96±0.91	5(3-6)	5.10±0.90	.297
CGI-I score	2(1-4)	1.96±0.82	2(1-4)	2.37±0.94	.037
Dep. episode	0(0-12)	1.63±2.65	0(0-9)	1.37±2.19	.604
Manic episode	2.5(0-21)	3.69±3.54	2(1-25)	6.27±6.98	.388
Total episode	4(1-23)	5.31±4.63	4(1-27)	7.65±7.03	.311
Age of onset	23.5(10-52)	25.89±9.34	24(13-44)	24.51±7.06	.647
Duration of disease	13.5(0.5-40)	14.25±9.01	18(1-40)	19.75±13.22	.081
Number of hospt.	2(0-18)	2.75±3.10	2(0-21)	5.79±6.39	.040

*Mann Whitney U test, SD - standard deviation, min - minimum, max - maximum, CGI-S - clinical global impression scale-severity, CGI-I - clinical global impression scale-improvement, HAM-D - Hamilton depression rating scale, YMRS - Young mania rating scale, dep - depressive, hospt - hospitalization

patients without rapid cycling (OR: 3.017; 95% CI: 1.067–8.534; *p*=.033) **Table 3**.

Comparing the *NOS3* intron 4 genotype and the allele frequencies of BD patients with rapid cycling to the control group showed that the *NOS3* genotype and allele frequency distributions of BD patients with rapid cycling were significantly different from the control group. The control group had a higher frequency of the b/b genotype and b allele than the BD patients with rapid cycling (OR: 0.191; 95% CI: 0.069-0.530; *p*=.001; OR: 0.231; 95% CI: 0.102-0.521; *p*<.001, respectively). In addition, the b/a genotype was found to be significantly higher in the BD patients with rapid cycling compared to the control group (OR: 3.754; 95% CI: 1.347–10.466; *p*=.016) **Table 3**.

Comparison of NOS3 genotype and allele frequency distributions of treatment-resistant BD patients with treatment-responsive BD patients and the control groups. When the *NOS3* intron 4 genotype and allele frequency distributions of the treatment-resistant BD patients were compared with the treatment-responsive BD patients, there was a statistically significant difference between the *NOS3* genotype and the allele frequency distributions of the two groups. The treatment-responsive BD patients had a higher frequency of the b/b genotype and the b allele than the treatment-resistant BD patients (OR: 3.656; 95% CI: 1.396-9.575; *p*=.007; OR: 2.905; 95% CI: 1.330-6.346; *p*=.006, respectively). In addition, the b/a genotype was found at a significantly higher frequency in the treatment-resistant BD patients compared to the treatment-responsive BD patients (OR: 0.318; 95% CI: 0.119–0.850; *p*=.019) **Table 4**.

When the *NOS3* intron 4 genotype and the allele frequency distributions of the treatment-resistant BD patients were compared with the control group, the *NOS3* genotype and allele frequency distributions of the treatment-resistant BD patients were significantly different from the control group. The control group had a higher frequency of the b/b genotype and the b allele than the treatment-resistant BD patients (OR: 0.216; 95% CI: 0.085-0.551; *p*=.001; OR: 0.259; 95% CI: 0.120-0.559; *p*<.001, respectively). Additionally, the b/a genotype was significantly higher in the treatment-resistant BD patients compared to the control group (OR: 3.605; 95% CI: 1.397–9.304; *p*=.006) **Table 4**.

Comparison of scale scores and clinical parameters regarding the NOS3 intron 4 genotype and the allele frequencies of BD patients. Comparing the scale scores (HAM-D, YMRS, CGI-S, CGI-I) and clinical parameters (age of onset, duration of disorder, number of hospitalizations, number of manic episodes, depressive episodes, and total episodes) regarding the *NOS3* genotypes of BD patients, the number of hospitalizations and CGI-I scores were significantly different between the genotype groups (b/b vs. b/a and a/a). In addition, the number of hospitalizations and CGI-I scores of the b/b group were statistically lower than the b/a and a/a group (*p*=.040; *p*=.037, respectively) **Table 5**.

Discussion. In this study, we found that the BD patients carrying the *NOS3* intron 4 b/b genotype and b allele had a significantly lower frequency of rapid cycling and treatment resistance. Similarly, the number of hospitalizations and the CGI-I scores of the BD group with the b/b genotype were statistically lower than the BD group with b/a and a/a genotypes. Currently, progress in biotechnology, neuroimaging,

and molecular genetics have contributed to new perspectives on the etiopathogenesis of BD. Numerous studies have identified genes that may be involved in triggering BD. Alves et al[34] conducted a literature review of 129 articles and reported that 79 genes are associated with BD. The 5 genes that are the most mentioned in the literature are calcium voltage-gated channel subunit alpha1 C (CACNA1C), ankyrin 3 (ANK3), disrupted in schizophrenia 1 (DISC1), D-amino acid oxidase (DAOA), and tryptophan hydroxylase 2 (TPH2).[34] Several oxidative stress markers have also been hypothesized to be associated with the pathophysiology of BD. Many research studies have reported oxidative damage to deoxyribonucleic acid (DNA), ribonucleic acid (RNA), lipids, and proteins. A meta-analysis by Brown et al[35] reported DNA or RNA damage as well as increased NO and lipid peroxidation levels in BD. The BD studies have demonstrated that NO levels are enhanced during different mood episodes, especially in depressive episodes.[36] Therefore, it seems likely that significantly higher concentrations of plasma nitrite in BD patients might be related to acute changes in regional eNOS activity. Reif et al[16] also reported that the *NOS3* genotype might be a modest genetic risk factor for developing BD.

Rapid cycling, a well-recognized specifier, is reported to exist in 5–15% of patients with BD. Rapid cycling BD patients seem to have higher rates of medical comorbidity, suicidal risk, treatment-resistant, and family history for BD, as well as increased susceptibility to DNA damage or mRNA hypo-transcription compared to BD patients without rapid cycling.[37] In the present study, BD patients carrying the *NOS3* intron 4 b/b genotype and b allele had a lower risk of rapid cycling, while BD patients with the b/a genotype had a higher risk of rapid cycling. Kirov et al. found that the frequency of the Met allele in the catechol-O-methyltransferase *(COMT)* gene was significantly higher in a rapid cycling BD group than in a non-rapid cycling group.[38] In contrast, their following study did not detect a statistically significant difference in the rate of low-activity monoamine oxidase-A *(MAO-A)* alleles between ultra-rapid cycling and non-rapid cycling BD patients.[39]

Several critical studies have investigated the relationship between brain-derived neurotrophic factor *(BDNF)* gene polymorphisms and rapid cycling BD. For example, Muller et al[40] showed a higher frequency of the Val allele of BDNF in rapid cycling BD compared to non-rapid cycling BD. Liu et al[41] demonstrated a relationship between the *BDNF* gene polymorphism (rs7127507) and rapid cycling BD. Similarly, Munkholm et al[42] reported that rapid cycling BD patients had

increased BDNF plasma levels compared to healthy controls. Recently, they also reported an association between increased DNA damage and impaired repair mechanisms in rapid cycling BD patients compared to healthy controls.[43] Our study contributes to these studies by demonstrating a relationship between the *NOS3* intron 4 gene and rapid cycling BD for the first time.

When we defined treatment resistance in BD according to Sachs's criteria mentioned in the method section, we found that the *NOS3* intron 4 genotype or allele frequency distributions were significantly different between the BD groups in terms of treatment response. Comparing the clinical parameters and scale scores regarding the *NOS3* intron 4 distributions in BD patients, the number of hospitalizations and the CGI-I scores of the BD group with the b/b genotype were significantly lower than the BD group with b/a and a/a genotypes. As previous evidence has reported, the number of hospitalizations is thought to be associated with the treatment response in BD.[44] Additionally, the CGI-I is used to evaluate the improved or worsened condition of the patient by comparing a baseline state before the treatment.[45] Therefore, we can speculate that the Turkish BD patients with the b/b genotype and b allele have a lower risk of developing treatment resistance. A literature review on BD and treatment resistance identified studies on patient responses to lithium treatment. One of these studies showed that the lithium response might be associated with the *BDNF* Val66Met polymorphism and serum BDNF levels.[46] A short allele of the serotonin-transporter-linked polymorphic region *(5-HTTLPR)* has also been related to both unipolar and bipolar affective disorders and inadequate antidepressant response.[47] In another study, Rybakowski et al[48] found that the lithium response has been associated with the dopaminergic receptor D1 *(DRD1)* gene polymorphism. Lithium may also affect the cyclic adenosine monophosphate (cAMP) pathway, which is an intracellular signaling system; Mamdani et al. reported a relationship between the lithium response for BD and 2 polymorphisms of the cAMP-responsive element-binding protein 1 *(CREB1)* gene located at chromosome 2q32–34.[49] Few studies in the literature have examined the response to non-lithium mood stabilizers in patients with BD. One study identified a relationship between -116C/G polymorphism of X-box binding protein 1 *(XBP1)* and treatment response to prophylactic valproate in BD.[50] In our study, when all mood stabilizers were evaluated together, a significant difference was found between treatment-resistant and treatment-responsive groups regarding *NOS3* intron 4 polymorphism. In addition, 70% of patients diagnosed

with rapid cycling BD (n=14/20) were also resistant to treatment with any mood stabilizer. This result is also crucial for showing the association between treatment resistance and rapid cycling in BD.

Several limitations should be considered in the present study. First, the small sample size of BD patients was a limitation in the current study; this research question should be studied with a larger group of participants to verify the outcomes. Secondly, in our study, the VNTR polymorphic region of the *NOS3* in intron 4 was examined, but it was not possible to know how 27-bp repeat polymorphism would link with other single nucleotide polymorphisms of *NOS3* gene (-786T/C and 894G/T polymorphisms). Therefore, considering the multi-genetic nature of BD, our study's third limitation is that it was about single nucleotide polymorphism. Lastly, we did not measure nitrite or nitrate levels that may have established a direct correlation between the investigated polymorphism.

In conclusion, we found that a VNTR variant in the *NOS3* intron 4 gene was associated with rapid cycling and treatment resistance in Turkish patients diagnosed with BD. Confirming the current results with further coding region variants in other populations who live in more extensive geographical regions will contribute to a better understanding of the relationship between *NOS3* intron 4 gene polymorphism, rapid cycling, and treatment resistance in BD. The genetics and pharmacogenetics of NOS and other genes involved in NO metabolism have not yet been sufficiently investigated in BD. Increasing sample sizes and accounting for ethnic origin are necessary for high-level evidence. We believe that augmenting understanding of eNOS signaling pathways and developing new technologies will help identify eNOS as a primary target for treating BD.

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