

# Increased serum levels of NMDA receptor antibodies in female patients with bipolar disorder

## *Bipolar bozukluk tanısı olan kadınlarda artmış serum NMDA reseptör antikorları düzeyleri*

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### SUMMARY

**Objective:** Glutamatergic/GABAergic imbalance due to autoimmune antibodies targeting N-methyl-D-aspartate receptors (NMDA-R) is considered to be one of the shared pathways between bipolar disorder (BD) and autoimmune diseases. Evidence shows female vulnerability to autoimmune disorders, and suggests a sex-specific approach in autoimmunity research in BD. We aimed to assess serum concentrations of NMDA-R antibodies and density of NMDA and GABA receptors on platelets in euthymic patients with BD in comparison to healthy individuals; and to determine the impact of sex on serum concentrations of NMDA-R antibodies and the density of NMDA and GABA receptors on platelets. **Method:** NMDA antibody IgG were detected in serum samples of 27 DSM IV euthymic patients with bipolar disorder (16 females, 11 males) and 33 healthy individuals (17 females, 16 males), using ELISA method. The densities of NMDA and GABA receptors on platelets were investigated using immunocytochemical methods. **Results:** Patients with BD presented higher serum levels of NMDA-R antibodies in comparison to healthy individuals ( $p < 0.001$ ). The densities of NMDA and GABA receptor on platelets were similar in both groups. The NMDA-R antibody levels were influenced by both diagnosis and sex ( $F = 5.813$ ,  $df = 1$ ,  $p = 0.020$ ). Tserum lithium levels showed a significant linear association with the serum NMDA-R antibody levels even adjusting for age, sex, body mass index ( $F = -56.26$ ,  $t = -2.52$ ,  $p = 0.015$ ,  $CI: -101.12/-11.40$ ). **Discussion:** Our findings support a potential role of NMDA-R antibodies in the underlying pathophysiology of BD, particularly for females.

**Key Words:** Bipolar disorder, NMDA, autoimmunity, anti-NMDA antibody, platelet, GABA, female sex

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### ÖZET

**Amaç:** N-metil-D-aspartat reseptörlerini (NMDA-R) hedef alan antikorlar nedeniyle glutamaterjik ve Gamma amino butirik asit (GABAerjik) dengenin bozulmasının bipolar bozukluk ile otoimmün hastalık arasındaki ortak yollardan biri olabileceği düşünülmektedir. Kanıtlar, kadınların otoimmün hastalıklara daha yatkın olduğunu göstermekte ve otoimmünite araştırmalarında biyolojik cinsiyete özgü bir yaklaşım desteklemektedir. Bu araştırma, bipolar bozukluğu olan ötimik hastaların serumlarında NMDA-R antikorlarının konsantrasyonlarını ve trombositlerinin yüzeylerindeki NMDA ve GABA reseptörlerinin yoğunluğunu sağlıklı kontrollerinkilerle karşılaştırmalı olarak değerlendirmeyi; ve cinsiyetin NMDA-R antikorlarının serum konsantrasyonlarına ve trombositler üzerindeki NMDA ve GABA reseptörlerinin yoğunluğuna etkisini saptamayı amaçlamaktadır. **Yöntem:** DSM IV'e göre bipolar bozukluk tanısı olan 27 ötimik hastanın (16 kadın, 11 erkek) ve 33 sağlıklı bireyin (17 kadın, 16 erkek) serum örneklerinde NMDA antikor IgG düzeyleri ELİZA yöntemi ile saptandı. NMDA ve GABA reseptörlerinin trombositler üzerindeki yoğunluğu immünohistokimyasal yöntemler kullanılarak araştırıldı. **Bulgular:** Bipolar bozukluğu olan hastaların serumlarında sağlıklı bireylerinkilere göre daha yüksek NMDA-R antikorları düzeyleri saptandı ( $p < 0.001$ ). Her iki grupta NMDA ve GABA reseptörünün trombosit yüzeylerindeki yoğunlukları benzerdi. NMDA-R antikor düzeylerinin hastalık varlığı ve cinsiyetin tarafından etkilendiği saptandı ( $F = 5.813$ ,  $df = 1$ ,  $p = 0.020$ ). Serum Lityum düzeyleri, yaş, cinsiyet, beden kitle indeksi ve sigara kullanımı açısından düzeltilmiş olarak, serum NMDA reseptör antikorları ile anlamlı negatif lineer ilişki göstermiştir ( $F = -56.26$ ,  $t = -2.52$ ,  $p = 0.015$ ,  $CI: -101.12/-11.40$ ). **Sonuç:** Bulgularımız, bipolar bozukluğun patofizyolojisinde, özellikle kadın hastalarda, NMDA-R antikorlarının olası rolünü işaret etmektedir.

**Anahtar Sözcükler:** bipolar bozukluk, NMDA, otoimmünite NMDA antikorları, GABA, kadın cinsiyet

## INTRODUCTION

Bipolar Disorder (BD) is a chronic, severe mental illness, which has been repeatedly associated with high comorbidity with various types of autoimmune diseases (1-6). Certain types of autoimmune diseases, such as thyroiditis (7), systemic lupus erythematosus (8-10) and multiple sclerosis (11,12), share some similar symptoms (i.e. affective symptoms, cognitive dysfunction) with mood episodes of BD. Even though several shared pathophysiological pathways (e.g. glutamatergic insults, immune dysfunction) between BD and autoimmune diseases have been implicated in the increased comorbidity with such diseases in patients with BD (13-15), the underlying nature of the relationship between BD and autoimmune comorbidities has not yet been clearly defined.

Alterations in both of glutamate and Gamma amino butyric acid (GABA) mechanisms, one of the prominent disturbances related to neurobiology of BD, may have possible associations with autoimmunity in BD (16-19). Glutamate is the main excitatory, whereas GABA is the main inhibitory neurotransmitter in the central nervous system (17). Several studies suggest significant abnormalities in concentrations and functions of the glutamatergic and GABAergic receptors in BD (22-27). Despite growing evidence on the importance of glutamatergic / GABAergic dysregulation and immune dysfunctions in the neurobiology of BD (25), there is limited data on the role of the antibodies targeting glutamatergic system in the pathophysiology of BD.

Autoimmune antibodies against NMDA receptors can cause reversible and selective decreases in NMDA receptors' surface density by a mechanism of cross-linking and internalization, that correlates with the levels of NMDA receptor antibodies (28-32). Previous evidence demonstrated increased NMDA-R antibodies in serum or cerebrospinal fluid samples of patients with a variety of disorders, such as encephalitis, epilepsy, systemic lupus erythematosus, as well as psychotic and affective disorders (33-39). Furthermore, a meta-analysis study reported that patients with a range of psychiatric conditions, including BD had a three times

greater likelihood to have increased serum levels of NMDA-R antibodies in comparison to healthy individuals (40). This evidence may indicate the possible role of the NMDA-R antibodies in the glutamatergic / GABAergic disturbances, as well as abnormal glutamatergic / GABAergic receptor functionality in BD (35).

Epidemiological data shows that several autoimmune disorders, such as systemic lupus erythematosus, Sjögren's syndrome and thyroiditis, are more prevalent in females (41-43). Notably, NMDA-R encephalitis, an autoimmune disease in which autoimmune antibodies attack NMDA receptors at central neuronal synapses, is one of the diseases showing most prominent degrees of sex bias, and predominantly seen in young females (30,31). It is well known that several sex differences also exist in the presentation and clinical course of BD; and females with BD are more prone to develop depressive and mixed episodes, BD type II, seasonal variations and rapid cycling BD (44-48), as well as comorbid obesity (49), autoimmune thyroid diseases (50-53) and migraine (54,55). Recent evidence show several sex specific differences in several parameters, including oxidative stress (56), cognition (57,58) and brain structures between sexes in BD (59,60). Further investigation is needed to understand the possible associations between sex specific autoimmune mechanisms and glutamatergic/GABAergic dysfunction in BD (44).

The primary objective of this study was to assess concentrations of NMDA-R antibodies in serum samples, and densities of NMDA and GABA receptors on surface of platelets in patients with BD in comparison to healthy individuals. A secondary aim was to highlight the potential impact of sex on serum concentrations of NMDA-R antibodies, densities of NMDA and GABA receptors on the surface of platelets in patients and controls.

## METHOD

### Participants

Patients with BD type I who had been euthymic for at least six months (n=35) were recruited from the Mood Disorder Unit of the Dokuz Eylül

University. Patients who had been euthymic for at least six months were selected in order to override potential confounding effect of an acute episode or possible residual effects of a previous episode on the findings.

Healthy individuals (HI) (n=33) with no known medical problems, no family history of major psychiatric or neurological disorders, mental retardation, cancer, cardiovascular disease or diabetes mellitus, who volunteered for the study through announcements, were recruited. As semi-structured interview for DSM-5 has not been available yet, the Structural Clinical Interview for DSM-IV interview was used to confirm psychiatric conditions of the healthy individuals and patients (62). Symptomatic severity was assessed using Young Mania Rating Scale (YMRS) (63), Hamilton Depression Scale-17 (HAMD-17) (64), Clinical Global Impression (CGI) (65) and Global Assessment of Functionality (GAF) (66) scales.

The exclusion criteria for the patients were as follows: having any comorbid axis I psychiatric diagnosis, acute infection, having any significant problem in routine blood and urine tests, neurological disorders, history of head trauma, chronic medical condition (e.g.,hypertension, diabetes mellitus), substance use (excluding tobacco), neurodegenerative diseases, epilepsy or previous brain surgery, auditory or visual impairment, and being pregnant or breastfeeding. According to these criteria, two patients were excluded due to diabetes mellitus comorbidity, one patient due to ankylosing spondylitis comorbidity, two patients were excluded because of depressive symptoms (HAMD-17 score >7), and one due to manic symptoms (YMRS > 7). An additional two patients were excluded due to hemolysis, which made it impossible to study their samples. 27 patients with BD and 33 healthy individuals were included in the study. The study was approved by the local ethics committee of the Dokuz Eylul University. All participants provided written informed consent.

#### Collection and preparation of the blood samples

An experienced physician obtained the blood samples between 10-12 am after an overnight fast

by patients.

Serum samples were kept at -80° C until ELISA tests for NMDA antibody levels. Platelet rich plasma (PRP) samples were obtained from 10 ml blood samples in citrate-tubes by centrifuging 10 minutes at 200g. After adding DMSO (10%), the PRP samples were kept at -80° C until further analyses of NMDA and GABA receptors on platelets.

#### ELISA tests

Human NMDA-R antibodies (IgG) in serum were investigated by the quantitative double antigen sandwich enzyme linked immunosorbent assay (Human NMDA antibody (IgG) ELISA kit , Sunredbio, Shanghai, catalogue no: 201-12-2139) according to manufacturing instructions. Briefly, human NMDA-R antibodies (IgG) were antigen labelled with biotin and combined with streptavidin-HRP. Labeled antigens formed immune complex after incubation with NMDA-R antibodies (IgG) in serum samples. Following a wash period to remove any unbound reagent, a substrate solution was added to the wells and color developed in proportion to the amount of human NMDA antibody (IgG) bound. The color development was stopped and the intensity of the color was measured at 450 nm with-in 15 minutes.

The assays were performed in duplicate and different plate areas were identified in 96-well plates and tested blind to detect any variations. The sensitivity of the kit was 1,582 pg/mL and the assay limits of the method were between 2 and 600 pg/mL. Inter-assay coefficient variation (CV) was ~10 %. A five point concentration calibration curve, ranging from 20 to 320 pg/mL, was used for quantification of human NMDA-R antibodies (IgG). The absorbance of the samples lower than the absorbance of the lowest calibrator was interpreted as  $\leq 20$  pg/mL to obtain analytical safety.

#### Immunocytochemical process

The PRP samples from 45 of the participants (28 patients with BD, 17 HI) were selected after optimization processes for immunocytochemical

assessment (15 participants did not provide PRP samples), and analyzed using immunocytochemical methods to investigate the profiles of NMDA and GABA receptors. Anti-NMDAR2A (clone ab78483, 1:100, Abcam) and Anti-GABA antibodies (clone ab86186, 1:100, Abcam) were applied on cytospin preparations of PRP samples which were fixed in 95% alcohol before immunostaining. Immunohistochemistry conditions were optimized for each individual antibody using manufacturers' recommendations. The immunostaining density was scored as 0: negative, 1: low density, 2: moderate density, 3: high density.

### Statistical analyses

We identified and quantified the levels of NMDA antibody in serum samples, density of NMDA and GABA receptor on platelets samples from patients and healthy individuals.

Skewness and kurtosis calculations were used to test normality for continuous variables. NMDA antibody levels were log transformed to provide normal distribution. Group differences were evaluated with independent samples t-test. Chi-Square test was used to examine categorical data. The impact of sex, smoking and BD diagnosis on the NMDA-R antibody levels was determined using three-way univariate analysis which included diagnosis, sex and smoking status as fixed factors and age as a covariate.

Pearson correlation tests were applied to explore the correlations between clinical variables and dependent variables. Linear regression analysis were applied to confirm the impacts of clinical variables on dependent variables. The significantly correlated variables and possible confounders (i.e. age, sex, body mass index and smoking status) were included in linear regression analyses. The IBM SPSS Statistics 25.0 (Chicago IL, USA) for Windows was used for data analysis. A p-value < 0.05 was assumed to correspond to statistically significant difference between means.

## RESULTS

Sociodemographic and clinical characteristics of the BD patients and healthy individuals are described in Table 1. There were no significant differences between the BD patients and healthy individuals with respect to variables of sex, smoking status and body mass index. The patient group ( $40.74 \pm 8.24$ ) was significantly older than the healthy group ( $34.97 \pm 8.29$ ) ( $F=0.008$ ,  $df=58$ ,  $p=0.009$ ).

Patient group was consisted of 16 females and 11 males with BD type I. The mean duration of illness was  $14.74 \pm 6.67$  years, and the duration of current euthymia was  $35.35 \pm 36.17$  weeks. All patients were on psychotropic medications; four patients were receiving a single medication: one, an antidepressant, two, valproate, and one, lithium carbonate. Three patients were on lithium carbonate and valproate combination, five on two mood-stabilizers in combination with a second generation-antipsychotic, eleven on a mood stabilizer in combination with a second generation antipsychotic, and five on a mood-stabilizer in combination with a second generation-antipsychotic and an antidepressant. The mean duration of mood-stabilizer use was  $108.26 \pm 64.28$  weeks, and the mean duration of antipsychotic use was  $68.22 \pm 63.23$  weeks.

### The levels of NMDA-R antibodies

Patients with BD had significantly higher serum NMDA antibody levels ( $39.24 \pm 20.11$  pg/mL) in comparison to healthy individuals ( $23.96 \pm 11.73$  pg/mL) ( $t=4.363$ ,  $df=58$ ,  $p<0.001$ ) (Fig.1). The number of subjects with NMDA antibody levels below 20 pg/mL ( $n=35$ ; 58.3%) was significantly lower in patients with BD ( $n=7$ ; 25.9%) compared to that of healthy controls ( $n=28$ ; 84.4%) ( $p<0.001$ ).

Further comparisons showed that there was no significant difference between smokers and non-smokers with respect to serum NMDA antibody levels ( $p = 0.458$ ). Females with BD ( $46.06 \pm 22.01$  pg/mL) had significantly higher serum levels of NMDA antibody than males with BD ( $29.33 \pm 11.94$  pg/mL) ( $t=2.478$ ,  $df=25$ ,  $p=0.020$ ), whereas

**Table 1.** Demographic and clinical characteristics of the participants

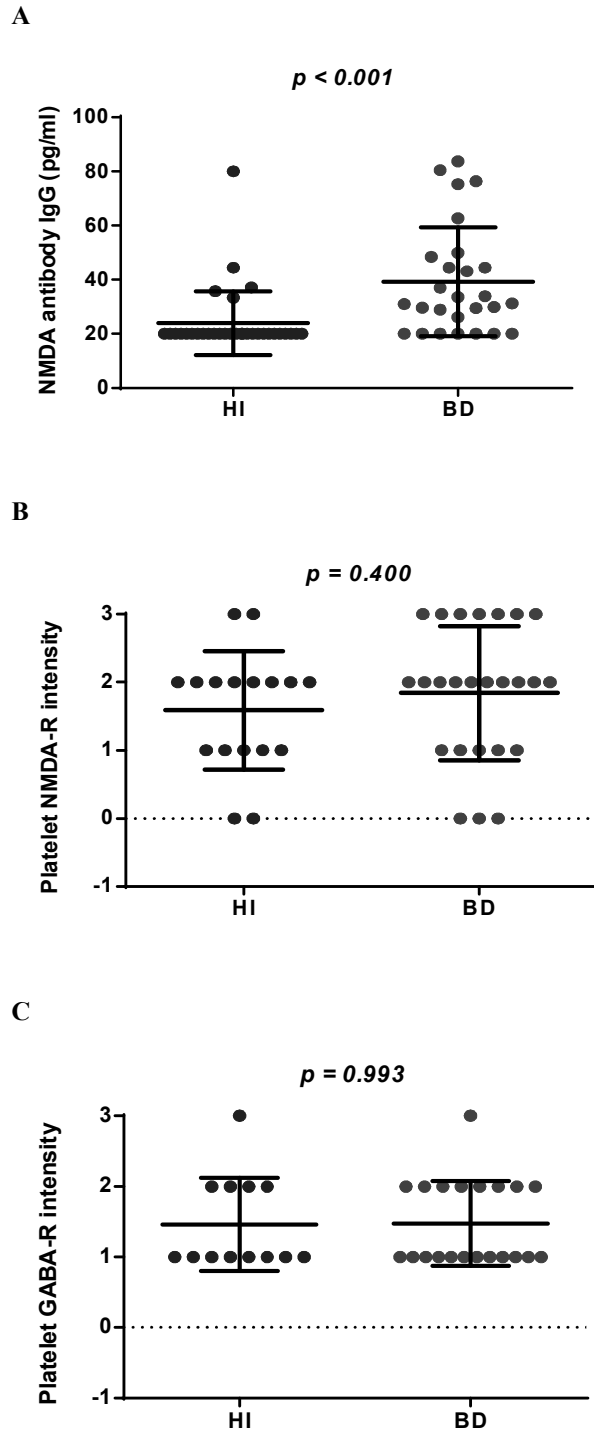
	<b>BD (n = 27)</b>	<b>HI (n = 33)</b>	<b>p-value</b>
<b>Gender (number of females, %)<sup>a</sup></b>	n = 16, 59.3 %	n = 17, 51.5 %	$\chi^2=0.360$ , df = 1, p = 0.549
<b>Age<sup>b</sup></b>	40.74 ± 8.24	34.97 ± 8.29	F= 0.008, df = 58, p = 0.009
<b>Smoking (number of smokers, %)<sup>b</sup></b>	n = 12, 44.4 %	n = 7, 24.1%	$\chi^2=2.572$ , df = 1, p = 0.109
<b>Body Mass Index<sup>b</sup></b>	28.37± 4.78	26.92 ± 3.81	F= 1.962, df = 45, p= 0.262
<b>Age of illness onset</b>	25.70±8.44		
<b>Duration of illness (years)</b>	14.74 ± 6.67		
<b>N of hospitalizations</b>	2.33±1.40		
<b>Duration of current euthymia (months)</b>	35.35 ± 36.17		
<b>N of previous episodes</b>	6.04 ± 4.36		
<b>N of manic episodes</b>	2.26 ± 1.46		
<b>N of hypomanic episodes</b>	1.70 ± 3.99		
<b>N of depressive episodes</b>	1.67 ± 1.75		
<b>N of mixed episodes</b>	0.52±0.82		
<b>Clinical Global Impressions score</b>	1.85 ± 0.46		
<b>Global Assessment of Functionality score</b>	81.67 ± 8.09		
<b>Hamilton Depression Scale score</b>	2.89 ± 1.89		
<b>Young Mani Rating Scale score</b>	0.89 ± 1.58		

a: Chi-Square; b: independent samples t test.

**Supplementary table.** Pearson's Correlations between markers and clinical variables

		<b>NMDA-R Antibody levels</b>	<b>Plattelet NMDA-R intensity</b>	<b>Plattelet GABA-R intensity</b>
<b>Age</b>	<b>r</b>	<b>0.270*</b>	0.192	-0.177
	<b>p</b>	<b>0.037</b>	0.223	0.263
	<b>n</b>	<b>60</b>	42	42
<b>Body mass index (kg/m<sup>2</sup>)</b>	<b>r</b>	0.029	-0.039	0.210
	<b>p</b>	0.845	0.819	0.213
	<b>n</b>	47	37	37
<b>Number of manic episodes</b>	<b>r</b>	0.148	<b>-0.610**</b>	-0.347
	<b>p</b>	0.463	<b>0.001</b>	0.089
	<b>n</b>	27	<b>25</b>	25
<b>Number of depressive episodes</b>	<b>r</b>	-0.081	-0.168	0.079
	<b>p</b>	0.690	0.423	0.706
	<b>n</b>	27	25	25
<b>Duration of illness (years)</b>	<b>r</b>	-0.190	0.074	-0.221
	<b>p</b>	0.343	0.724	0.289
	<b>n</b>	27	25	25
<b>Duration of euthymia (months)</b>	<b>r</b>	0.032	0.162	0.090
	<b>p</b>	0.876	0.451	0.677
	<b>n</b>	26	24	24
<b>Global Assessment of Functioning score</b>	<b>r</b>	0.247	.412*	0.247
	<b>p</b>	0.215	0.041	0.234
	<b>n</b>	27	25	25
<b>Hamilton Depression Rating Scale score</b>	<b>r</b>	-0.306	-0.233	-0.277
	<b>p</b>	0.120	0.263	0.181
	<b>n</b>	27	25	25
<b>Young Mania Rating Scale score</b>	<b>r</b>	-0.112	<b>-0.422*</b>	0.144
	<b>p</b>	0.577	<b>0.036</b>	0.493
	<b>n</b>	27	<b>25</b>	25
<b>Serum Lithium level (mmol/l)</b>	<b>r</b>	<b>-0.593*</b>	-0.049	-0.257
	<b>p</b>	<b>0.020</b>	0.875	0.396
	<b>n</b>	<b>15</b>	13	13
<b>Serum Valproate level (ug/ml)</b>	<b>r</b>	-0.025	0.551	0.124
	<b>p</b>	0.936	0.063	0.701
	<b>n</b>	13	12	12

**Fig. 1.** The levels of serum NMDA -R antibodies and platelet NMDA and GABA receptor intensities



**Figure 1:** A. Patients with BD had significantly higher serum NMDA-R antibody levels in comparison to healthy individuals ( $t = 4.363$ ,  $df = 58$ ,  $p < 0.001$ ). B. No significant difference between patients with BD and healthy individuals with respect to density of NMDA receptors on platelets. C. No significant difference between patients with BD and healthy individuals with respect to density of GABA receptors platelets ( $p = 0.400$ ;  $p = 0.993$ ).

there was no significant difference between healthy females and males with respect to NMDA-R antibodies ( $p=0.388$ ). Additionally, while female patients with BD had significantly higher levels of NMDA-R antibodies ( $46.06 \pm 22.01$  pg/mL) compared to healthy females ( $22.22 \pm 6.57$  pg/mL) ( $t=5.145$ ,  $df=21,365$ ,  $p<0.001$ ), there was no significant difference between male patients with BD ( $29.33 \pm 11.94$ ) and healthy males ( $25.81 \pm 11.73$  pg/mL) with respect to serum NMDA antibody levels ( $p=0.532$ ) (Fig. 2).

Serum NMDA-R antibody levels showed a weak correlation with age ( $r=0.270$ ,  $p=0.037$ ), and a moderately negative correlation with serum lithium levels of patients receiving lithium ( $r=-0.593$ ,  $n=15$ ,  $p=0.020$ ). A significant linear regression analyses that included age, sex, body mass index, smoking status and serum Lithium levels as independent factors, revealed the significant effects of serum lithium levels on serum NMDA-R antibody levels ( $F=-56.26$ ,  $t=-2.53$ ,  $p=0.013$ ,  $CI:-101.12/-11.40$ ). A three-way univariate analysis of covari-

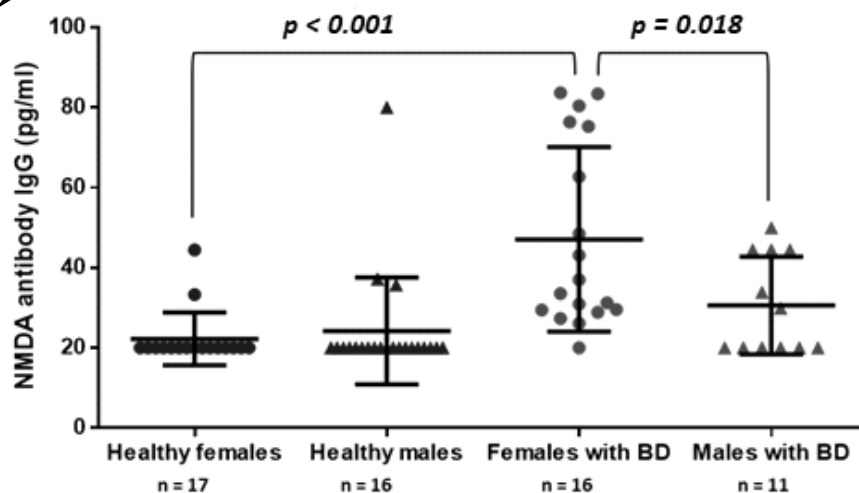
ance revealed a significant impact of diagnosis and sex on NMDA-R antibody levels even after adjusting for age and smoking status ( $F=5.813$ ,  $df=1$ ,  $p=0.020$ ).

#### Density of NMDA and GABA receptors on the surface of platelets

There were no significant differences between patients with BD and healthy individuals with respect to NMDA ( $p=0.400$ ) and GABA receptor on platelets ( $p=0.993$ ) (Fig. 1). There were no significant differences between female patients with BD and healthy females with respect to NMDA and GABA receptor profiles on platelets ( $p=0.434$ ,  $p=0.466$ , respectively), nor between male patients with BD and healthy males with respect to NMDA and GABA receptor profiles on platelets ( $p=0.401$ ;  $p=0.881$ , respectively).

The density of NMDA receptors on platelets were positively correlated with density of GABA receptors on platelets ( $r=0.384$ ,  $p=0.012$ ). The density

Fig. 2. The levels of serum NMDA -R antibodies with respect to sex and BD diagnosis



**Figure 2:** Females with BD ( $46.06 \pm 22.01$  pg/mL) had significantly higher serum levels of NMDA antibody than males with BD ( $29.33 \pm 11.94$  pg/mL) ( $t=2.478$ ,  $df=25$ ,  $p=0.020$ ), whereas there was no significant difference between healthy females and males with respect to NMDA-R antibodies ( $p=0.388$ ). Additionally, while female patients with BD had significantly higher levels of NMDA-R antibodies ( $46.06 \pm 22.01$  pg/mL) compared to healthy females ( $22.22 \pm 6.57$  pg/mL) ( $t=5.145$ ,  $df=21,365$ ,  $p<0.001$ ), there was no significant difference between male patients with BD ( $29.33 \pm 11.94$ ) and healthy males ( $25.81 \pm 11.73$  pg/mL) with respect to serum NMDA antibody levels ( $p=0.532$ ).

of NMDA receptors on platelets was negatively correlated with total number of manic episodes ( $r=-0.610$ ,  $p=0.001$ ) and YMRS score ( $-0.422$ ,  $p=0.036$ ). A significant linear regression analyses that included age, sex, smoking status, total number of manic episodes and YMRS scores as independent factors, confirmed the significant effects of total number of manic episodes ( $F=-0.435$ ,  $t=-4.87$ ,  $p<0.001$ , CI:  $-0.614/-0.256$ ) and YMRS scores ( $F=-0.238$ ,  $t=-2.745$ ,  $p<0.008$ , CI:  $-0.412/-0.064$ ) on the densities of NMDA receptors on platelets.

## DISCUSSION

To the best of our knowledge, this is the first study addressing NMDA-R antibodies and density of GABA receptors on the surface of platelets in euthymic patients with BD in comparison to healthy individuals. Our findings show increased levels of NMDA-R IgG antibodies in euthymic patients with BD compared to healthy individuals. The increase is mainly driven by female participants. However, we detected no significant change in the platelet NMDA or GABA receptor density in BD patients compared to controls.

### NMDA-R antibodies

Possible associations between autoimmune mechanisms and the underlying neurobiology of BD are suggested by three types of studies: Epidemiologic studies reporting that autoimmune diseases are more prevalent in BD (4,61,67); studies reporting that patients with autoimmune diseases are more prone to develop BD (4,68); and several case-control studies reporting higher prevalence of several antibodies in BD (69-71). More recently, increased NMDA-R antibodies have been shown in BD (35,36,40) suggesting that the well-documented disturbances in NMDA functioning in BD may be caused by increased levels of circulating NMDA-R antibodies. In accordance with the previous evidence, we found higher levels of NMDA-R antibodies in euthymic patients with BD compared to healthy individuals (36,38-40). As our BD patient group was significantly older than the control group, we adjusted the results for age following previous literature showing an effect of age on antibody levels (72,73). However, even after adjust-

ments with age, sex and smoking status, the group of patients with BD had higher levels of NMDA-R IgG antibodies than the group of healthy individuals.

The previous literature focusing on the serum NMDA-R antibodies in psychiatric disorders is predominantly based on qualitative assessment of NMDA-R antibody sero-positivity ratios. These studies reported increased prevalence of serum NMDA-R antibody positivity in 3-10 % of psychotic patients (38,74). However, these studies have substantial discrepancies, including study population selection and types of immunoglobulins or NMDA receptor variants or assays (i.e. cell based assay or ELISA) (40). Therefore, caution should be taken while comparing our results with those of previous studies. Our study population focused exclusively on patients with BD, whereas previous studies used mixed groups of BD and psychosis patients (36,39).

Of note, we detected serum levels (i.e. titers) of NMDA-R antibodies quantitatively using a commercial ELISA assay. Majority of the previous studies evaluating anti-NMDA-R antibodies did not use ELISA. Instead, they used other methods due to concerns regarding the tridimensional structure of the related antigen (34). Only one previous study provided quantitative data on serum titers of NMDA-R IgG antibodies, and showed significantly increased serum levels of NMDA-NR2 antibodies in manic patients (36). We focused exclusively on IgG subtype of NMDA-R antibodies in line with the previous data, which showed increased levels of IgG antibodies alone in patients with psychosis compared to controls, in the absence of any change in the levels of any subtype (IgG, IgM, IgA) of NMDAR antibodies (33).

It is important to note that our study population consisted of euthymic patients with BD who had experienced no mood episodes for at least 6 months. Dickerson et al. (2012) showed significantly increased serum levels of NMDA-NR2 antibodies only in manic patients with BD or schizoaffective disorder, in comparison to healthy individuals (36). Furthermore, manic patients' serum NMDA-NR2 antibody levels decreased at the six-month



follow-up; which implies an association between antibody levels and the manic state. Previous data from case reports (75-78) and case-control studies (36,38,79) suggest a possible relationship with NMDA-R antibody seropositivity and manic or depressive mood episodes. Despite focusing exclusively on euthymic patients, our results revealed significantly higher NMDA-R antibodies in patients with BD. This finding suggests that NMDA autoimmunity persists in euthymic phases of BD. Further data are needed to highlight the effects of different states of the illness (i.e., euthymia vs. being in episode) on NMDA-R antibody levels. On the other hand, our results revealed that NMDA receptor density on platelets was negatively correlated with both total number of previous manic episodes and also current manic symptom severity. These are in line with previous reports, implying a significant effect of mania on glutamatergic system.

Our further analysis (i.e. three-way univariate analysis of covariance) revealed that the increase in the levels of NMDA-R antibodies was driven by female patients with BD. The female vulnerability to autoimmunity is well documented (37-39). Significantly higher NMDA-R antibody levels in females with BD compared to healthy females, in the absence of any corresponding difference for males may suggest a female specific vulnerability to NMDA-R autoimmunity in BD. Despite similar ratios of sexes in BD type I, emerging evidence suggest sex differences in the presentation and clinical course of BD (45). Considering increased comorbidity with certain diseases in females (49-55), and previous data showing significant sex differences in BD, with respect to oxidative stress (56), cognitive decline (57,58) and brain structures (59,60); our finding of sex specific increase of serum NMDA-R antibodies may indicate the importance of a sex specific approach when investigating NMDA-R autoimmunity in BD.

Our data showed significant negative correlations between NMDA-R antibodies and serum lithium levels. Furthermore, a linear regression analysis confirmed the significant linear association between NMDA-R antibodies and serum lithium levels, even after adjustment for possible confounders including age, sex, body mass index and

smoking. Although lithium, the gold standard medication of BD, is considered to have immune modulatory effects, its mode of action is not fully understood (80). Several lines of clinical evidence suggest that lithium has neuroprotective effects via neurotrophic, antioxidant, anti-inflammatory mechanisms (81, 82). Evidence shows that lithium protects neurons from glutamatergic excitotoxicity (83, 84) or modulates glutamate receptors (85). Conforming the growing range of evidence showing neuroprotective effects of lithium against immune and glutamatergic insults, our preliminary data deserve further investigation.

#### NMDA and GABA receptors on surface of platelets

This is, as far as we know, the first study investigating NMDA and GABA receptors on surface of platelets in patients with BD. We measured density of NMDA and GABA receptors on platelets in plasma enriched plasma samples using immunocytochemical methods. As platelets express glutamatergic and GABAergic receptors that are analogous to those in the brain, platelets may be considered as a significant and easily accessible alternative to neuronal cells in the study of the GABA/Glutamate system (86-90). Only a limited number of studies demonstrated changes in glutamate receptor functions in platelets of patients with psychiatric disorders including schizophrenia (91, 92) and major depression (93). While one study reported alterations in glutamate uptake in platelets of patients with BD (94), and another showed GABA receptors on platelets (95), no study specifically investigated GABA receptors on platelets in psychiatric disorders.

Our results show no change in density of NMDA and GABA receptors on platelets in patients with BD in comparison to healthy individuals, and no correlations with density of NMDA on surface of platelets and patients' antibody titers. However, a possible relationship between surface NMDA receptor expressions and manic symptoms is implicated by negative linear associations between density of NMDA receptors, Young Mania Rating Scale scores, which may implicate subclinical or residual manic symptoms in the euthymia group, and number of previous manic episodes.

Supporting this point of view are previous animal studies showing significant decreases in surface expression of NMDA receptor subunits in hippocampus (96-98). Future longitudinal studies may highlight impacts of manic episodes on platelet NMDA receptor expressions.

### Strengths and limitations

The two main strengths of this study were, first, the combined investigation of both NMDA-R antibodies and density of NMDA and GABA receptors on platelet surface; and second, the study was carried out in a homogenous patient population based on stringent inclusion criteria. Nevertheless, certain limitations of the study should be noted. The small sample size and the inclusion of only medicated patients should be considered while interpreting the results. Small differences of NMDA and GABA receptor density might have become invisible not only due to the small sample size, but also possible protective effects of medications. Many drugs used in the treatment of BD including mood-stabilizers, antipsychotics and antidepressants were shown to have modulating effects on NMDA receptors (99-103) as well as GABA receptors (104-106). Therefore, further studies including medicated patients are needed to clarify the effect of illness on the glutamate/GABA systems. Older age in patient sample might be another limitation of the study. However, all results were controlled for age.

Using serum samples and platelets, rather than cerebrospinal fluid and brain tissue, might also have affected our results. Another limitation includes the laboratory techniques we used. Despite being coherent with previous results, our results of the ELISA assessments need further replication using cell-based assays. Moreover, further studies targeting subunits of NMDA receptors such as NR-1, NR-2 are needed. The immunocytochemical technique was used to identify receptor densities on platelet surface, and further studies using quantitative techniques (e.g. flow cytometry) would improve the understanding of platelet receptor functioning in BD.

Finally, as a major limitation of the study, the cross-

sectional design does not allow to control our results for the possible confounding effects of individual factors that predispose to autoimmunity including exposure to infectious agents, dietary components, chemicals, toxins or stress. Future follow up studies will raise the understanding in the field.

### CONCLUSION

Our finding of increased levels of NMDA IgG antibodies in euthymic patients with BD support the notion that the autoantibodies targeting NMDA receptors may represent a possible key area for understanding the neurobiology of BD, particularly for females. The negative correlation between the platelet surface density of NMDA receptors and number of the previous manic episodes, as well as current manic symptoms, may reflect the impact of mania on the glutamatergic system. Another implication of our results is that lithium may have a potential protective action against glutamatergic insults. However, further follow up studies with large sample size are needed to verify these results.

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