# IMPROVING THE DIAGNOSTIC ACCURACY OF BIPOLAR DISORDER THROUGH METABOLOMIC PROFILING

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## LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURES

| | |
|---|---|
| NMDA | N-methyl-D-aspartate |
| USM | Universiti Sains Malaysia |
| HUSM | Hospital Universiti Sains Malaysia |
| BD | Bipolar Disorder |
| HPLC | High performance liquid chromatography |
| 5-HT | Serotonin |
| YMRS | Young Mania Rating Scale |
| BDI | Beck Depression Inventory |
| SSKM-20 | Saringan Status Kesihatan Mental-20 |
| 95% CI | 95% Confidence Interval |
| SD | Standard Deviation |
| IQR | Inter Quartile Range |

## ABSTRAK (BAHASA MELAYU)

**Pengenalan:** Kajian telah menunjukkan bahawa penglibatan disfungsi pada serotonin dan reseptor glutamat *N-methyl-D-aspartate (NMDA)* boleh menyebabkan beberapa gangguan neuropsikiatri. Akan tetapi, hubung kait sebenar disfungsi ini dengan penyakit bipolar masih tidak jelas. Oleh itu, tujuan kajian ini adalah untuk mengukur tahap kepekatan asid amino glutamat, glisina dan triptofan di dalam darah pesakit bipolar dan membandingkannya dengan kumpulan kawalan.

**Kaedah:** Satu kajian kes-kawalan telah dijalankan dari Oktober 2017 sehingga Jun 2018 di Hospital Universiti Sains Malaysia (HUSM). Kami telah mengukur tahap asid amino glutamat, glisina dan triptofan di dalam darah 83 pesakit bipolar dan data itu kemudiannya dibandingkan dengan 82 orang kumpulan kawalan menggunakan kaedah kromatografi cecair prestasi tinggi (HPLC).

**Keputusan:** Min (sisihan piawai) umur bagi pesakit bipolar adalah 40.87 (12.08) manakala bagi kumpulan kawalan adalah 35.60 (7.68) tahun. Peserta daripada kumpulan bipolar dan kumpulan kawalan berbeza dengan ketara dari segi umur ($p$=0.001), tetapi, tidak berbeza dari segi jantina. Median (julat antara kuartil) bagi tahap glutamat dan triptofan dalam pesakit bipolar masing-masing adalah 61.5 (122) dan 33.5 (24) µmol/L, manakala Min (sisihan piawai) bagi tahap glisina dalam pesakit bipolar adalah 273.6 (66.4) µmol/L. Tahap asid amino glutamat dan glisina di dalam darah pesakit bipolar adalah lebih tinggi terutamanya bagi bipolar I dalam episod manik berbanding dengan kumpulan kawalan. Akan tetapi, tiada perbezaan yang ketara bagi tahap kepekatan asid amino triptofan di dalam pesakit bipolar berbanding dengan kumpulan kawalan.

**Kesimpulan:** Keputusan ini memberi penambahan bukti mengenai penglibatan serotonin dan reseptor glutamat *N-methyl-D-aspartate (NMDA)* di dalam pathogenesis kecelaruan bipolar dan kebarangkalian untuk asid amino ini dijadikan sebagai biomarker bagi menyokong diagnosis kecelaruan bipolar.

**Kata kunci:** kecelaruan bipolar; triptofan; glutamat; glisina; reseptor NMDA

## ABSTRACT (ENGLISH)

**Background:** Studies have suggested the involvement of serotonergic and N-methyl-D-aspartate (NMDA) glutamate receptor complex dysfunction may involve in the pathophysiology of several neuropsychiatric disorders. However, the exact association with bipolar disorder remains unclear. Hence, the purpose of this study was to measure the plasma concentrations of glutamate, glycine and tryptophan in bipolar disorder patients and compared with the healthy controls.

**Methods:** A case-control study was conducted from October 2017 till June 2018 in Hospital Universiti Sains Malaysia (HUSM). We had measured the plasma levels of glutamate, glycine and tryptophan in 83 bipolar patients and data were compared to a group of 82 healthy controls using high performance liquid chromatography (HPLC) method.

**Results:** The mean (SD) age of bipolar patients were 40.87 (12.08) while the mean (SD) age for control groups was 35.60 (7.68) years. The participants from the bipolar group and control group differ significantly in age ($p$=0.001), however, there was no significant difference between patients and controls with respect to gender. The median (IQR) of glutamate and tryptophan level in bipolar patients was 61.5 (122) and 33.5 (24) respectively, while the mean (SD) of glycine level in bipolar patients was 273.6 (66.4). A significant higher glutamate and glycine levels were found in bipolar disorder patients more markedly in bipolar I in manic episode as compared to the healthy controls. However, there was no significant difference in the level of plasma tryptophan between bipolar patients and control group.

**Conclusions:** The findings provide additional evidence regarding the involvement of serotonergic and N-methyl-D-aspartate (NMDA) glutamate receptor complex in the pathogenesis of bipolar disorder and the possibility of these amino acids to serve as the peripheral biomarkers to support the diagnosis of bipolar disorder.

**Keywords:** bipolar disorder; tryptophan; glutamate; glycine; NMDA receptor

# CHAPTER 1: INTRODUCTION

## 1.1 Introduction

Bipolar disorder (BD) is a severe, burdensome and debilitating psychiatric illness with a lifetime prevalence of approximately 1.3% (1). The exact cause is still unknown, hampering the search for biomarkers to support laboratory testing for this disorder. Currently, there is no independent test to confirm the BD and mostly diagnosis is depending on judgement and clinical expertise (2).

Growing evidence showing that serotonergic and N-methyl-D-aspartate (NMDA) glutamate receptor complex dysfunction plays an important role in pathophysiology of BD. The NMDA receptor is a glutamate ion channel protein receptor that is activated when glutamate and glycine bind to it (3, 4). Previous studies involving NMDA receptor agents reported increased level of plasma glutamate, glycine and serine in schizophrenia patients concurrently with the improvement of the symptoms (5, 6). Another study also has shown that increased in the glycine level in relapsing mania which suggests a significant role of glycine as a coupled modulatory site for NMDA receptor with glutamate (6). Furthermore, previous in vivo radiological study also showed significantly higher glutamate and glycine in both the anterior and posterior cingulate cortex of patients with schizophrenia and bipolar disorder (7). From recent evidence, it could be postulated that the dysfunction of this receptor complex also plays an important role in the emergence of psychotic relapses.

Tryptophan is one of the essential amino acids and precursor of serotonin (5-HT) synthesis. Previous studies have reported that rapid depletion of tryptophan level may specifically reduce brain 5-HT function (8, 9) and reduction of plasma tryptophan

level up to 80% can lead to mild impairment in attentive performance and increased reports of negative mood, without clinical depression in healthy people (10, 11). In addition, lower levels of tryptophan also been demonstrated repeatedly in patients with schizophrenia (12, 13) and with affective disorders (14-16). The role of 5-HT in the pathogenesis of mood disorders has been widely studies. However, questions have been raised about the precise role of 5-HT in bipolar disorder patients. As mentioned in the previous studies, these amino acids were postulated to play an important role in the pathophysiology of mood disorder.

The dysregulation of glutamate and glycine levels were previously studied in the affective patients by postmortem, radiological and cerebrospinal fluid examination. However, to the best of our knowledge, there has been little discussion about the peripheral plasma level of glutamate, glycine and tryptophan in patient with bipolar disorder (6). On top of that, most of the studies were conducted in a Western country and very limited data available in the Asian population.

This study was carried out to find the potential biomarkers for BD. The importance of biomarkers is they can improve the diagnostic accuracy of BD and more importantly for early diagnosis in patients that susceptible to the disease. Hence, these findings could improve the accuracy of the diagnosis and possible treatment in future.

Therefore, the aim of this study is to investigate the plasma concentrations of amino acids related to the NMDA receptor which are glutamate and glycine and the role of the serotonergic system through assessing the central availability of the precursor amino acid of 5-HT by determining the plasma tryptophan level in bipolar disorder patients in Kelantan.

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# CHAPTER 2: OBJECTIVES OF THE STUDY

## 2.1 General Objectives

To determine the plasma amino acids profiles in bipolar disorder patients and compared with the healthy control.

## 2.2 Specific Objectives

1) To determine the plasma amino acid profiles (Glutamate, Glycine and Tryptophan) in patients with bipolar disorder.
2) To compare the plasma amino acid profiles (Glutamate, Glycine and Tryptophan) between patients with bipolar disorder and healthy control.

# CHAPTER 3: MANUSCRIPT

## 3.1 Title

**AMINO ACIDS PROFILING: ALTERATION OF GLUTAMATE AND GLYCINE CONCENTRATIONS IN PATIENTS WITH BIPOLAR DISORDER**

## 3.2 Abstract

**Background:** Previous studies have suggested that the involvement of serotonergic and N-methyl-D-aspartate (NMDA) glutamate receptor complex dysfunction in the pathophysiology of several neuropsychiatric disorders. However, the exact association with bipolar disorder remains unclear. Hence, the purpose of this study was to measure plasma concentrations of glutamate, glycine and tryptophan in bipolar disorder patients and compared with the healthy controls.

**Methods:** A case-control study was conducted from October 2017 till June 2018 in Hospital Universiti Sains Malaysia (HUSM). We had measured the plasma levels of glutamate, glycine and tryptophan in 83 bipolar patients and data were compared to a group of 82 healthy controls using high performance liquid chromatography (HPLC) method.

**Results:** The mean (SD) age of bipolar patients were 40.87 (12.08) while the mean (SD) age for control groups was 35.60 (7.68) years. The participants from the bipolar group and control group differ significantly in age (p=0.001), however, there was no significant difference between patients and controls with respect to gender. The median (IQR) of glutamate and tryptophan level in bipolar patients was 61.5 (122) and 33.5 (24) respectively, while the mean (SD) of glycine level in bipolar patients was 273.6

(66.4). A significant higher glutamate and glycine levels were found in bipolar disorder patients more markedly in bipolar I in manic episode as compared to the healthy controls. However, there was no significant difference in the level of plasma tryptophan between bipolar patients and control group.

**Conclusions:** The findings provide additional evidence regarding the involvement of serotonergic and N-methyl-D-aspartate (NMDA) glutamate receptor complex in the pathogenesis of bipolar disorder and the possibility of these amino acids to serve as the peripheral biomarkers to support the diagnosis of bipolar disorder.

**Keywords:** bipolar disorder; tryptophan; glutamate; glycine; NMDA receptor

## 3.3 Introduction

Bipolar disorder (BD) is one of the most common psychiatric disorders worldwide with a lifetime prevalence of approximately 1.3% (1). It is still not known what is the exact cause of bipolar, yet a variety of biochemical, environmental and genetic elements that could be involved in both triggering and causing bipolar episodes. Currently, there is no independent test to confirm the BD and mostly diagnosis is depending on judgement and clinical expertise (2).

There is growing evidence which suggests that N-methyl-D-aspartate (NMDA) glutamate receptor complex and serotonergic abnormalities play an important role in the pathophysiology of BD. Glutamate is the major excitatory synaptic neurotransmitter in the brain and has several important roles including mediating neurotransmission across excitatory synapses and regulating numerous physiological functions in the mammalian central nervous system (CNS), such as synaptic plasticity, learning, and memory (17,

18). Antagonism of the NMDA glutamate receptor complex induces behavioural and cognitive deficits in normal subjects with a broad range of CNS symptoms such as psychotic phenomena, agitation and disorientation (19) whereas hypofunction of this receptor complex showing association with negative symptoms and cognitive deficits in patients with schizophrenia (3).

In addition to the binding site for the agonist glutamate, a glycine-coupled modulatory site must be occupied by glycine in able to open the calcium channel of NMDA receptors (3, 4). Few studies also have shown that increased in the glycine level not only in schizophrenia but also in relapsing mania which suggests a significant role of glycine as coupled modulatory site for NMDA receptor with glutamate (6). Besides, in vivo radiological study showed significantly higher glutamate and glycine in both the anterior and posterior cingulate cortex of patients with schizophrenia and bipolar disorder (7). This could be postulated that the dysfunction of this receptor complex also plays an important role in the emergence of psychotic relapses.

Tryptophan is one of the essential amino acids and precursor of serotonin (5-HT) synthesis. Previous studies have reported that rapid depletion of tryptophan level may specifically reduce brain 5-HT function (8, 9) and reduction of plasma tryptophan level up to 80% can lead to mild impairment in attentive performance and negative mood, without clinical depression in healthy people (10, 11). In addition, lower levels of tryptophan also been demonstrated repeatedly in patients with schizophrenia (12, 13) and with affective disorders (14-16). The role of tryptophan in the pathogenesis of mood disorders has been widely studied. However, questions have been raised about the precise role of tryptophan in bipolar disorder patients.

A number of researchers have reported the relationship between glutamate receptors and the serotonergic system and postulated that glutamate and 5-HT2A receptors are anatomically and functionally coupled. Recent evidence showed that glutamate agonists activate 5-HT2A receptors whereas serotonin activation results in inhibition of the NMDA response (20).

Recently, metabolomics, which quantifies and identifies metabolites in various bio fluids such as serum/plasma and urine, has been used in diagnosing neuropsychiatric disorders especially in schizophrenia and autism (21, 22). However, with regards to BD, there are no biomarkers offered to support laboratory testing for this disorder (23). Previous studies also determined the dysregulation of glutamate and glycine level in the affective patients by postmortem, radiological and cerebrospinal fluid examination. However, to the best of our knowledge, there has been little discussion about peripheral plasma level of glutamate and glycine in patients with bipolar disorder (6).

The aim of this study is to investigate the plasma concentrations of amino acids related to the NMDA receptor which are glutamate and glycine and the role of the serotonergic system through assessing the central availability of the precursor amino acid of 5-HT by determining the plasma tryptophan level in the patients with bipolar disorder.

## 3.4 Methodology

### Participants

A case control study was conducted from October 2017 till June 2018 in Hospital Universiti Sains Malaysia (HUSM). Bipolar patients aged 18 to 65 years old who fulfilled the DSM-5 criteria of Bipolar I and Bipolar II were recruited from the

psychiatric outpatient clinic. They were informed prior to clinic visit to fast for at least 10 hours to make sure the amino acids level were not being influenced by the diet intake. Those with comorbid psychiatric illnesses, chronic medical illnesses, pregnant lady and either had acute surgery or infarctions to the brain which may interfere with the amino acids level also were excluded from the study. These illnesses were ruled out by clinical interview by the researcher and team.

Healthy control aged from 18 to 65 also being recruited from HUSM employee who volunteered to take part in the study. They were assessed to ensure that they were in good health and had no underlying medical or psychiatric illnesses. All protocols were approved and conducted in compliance with the Human Research Ethics Committee of USM (USM/JEPeM/17020091) and written informed consent was obtained from participants before the studies.

**Sample size calculation and sampling method**

In the first objective, we want to evaluate the plasma level of glutamate, glycine and tryptophan in BD patients. Hence, the sample size were calculated by using the formula for estimation of single mean with type 1 error ($\alpha$) of 5%, standard deviation of the parameters of interest from previous study ($\sigma$), precision of estimation ($\Delta$) and anticipated dropout rates of 10% which gives to a total number of 64 BD patients that need to be recruited.

For the second objective, we want to compare the plasma level of glutamate, glycine and tryptophan in BD and healthy control. Hence, the sample size was calculated by using the formula for comparison of two independent means with type 1 error ($\alpha$) of 5%, 1- type 2 error (power) of 80%, standard deviation of the parameters of

interest among control group from previous study (σ), detectable difference (Δ), ratio between control to cases (m) is equal to 1, and anticipated dropout rates of 10% which gives to the largest sample size, 75 bipolar patients. As for this study, 83 patients with bipolar disorder and 82 healthy controls were recruited with total numbers of study participants were 165.

For sampling methods of the cases, all eligible BD patients that came for follow up in psychiatric outpatient clinic HUSM within the study period was recruited while for the controls, we have obtained the healthy subjects who voluntarily to participate in the study after the advertisement placed in the HUSM clinic.

**Instruments**

The self-administered questionnaires included questions on sociodemographic characteristics, Beck Depression Inventory (BDI) and Saringan Status Kesihatan Mental – 20 (SSKM-20). The clinician-rated questionnaire used in this study was the Young Mania Rating Scale (YMRS).

*Young Mania Rating Scale (YMRS)*

The severity of manic symptoms was assessed according to 11-items YMRS that ranges from 0 to 60 (24) with higher scores indicate greater severity of symptoms. This question tool is a validated, clinician rated in English language and was assessed by a trained psychiatry medical officer throughout the study duration.

*Beck Depression Inventory (BDI)*

BDI was used to assess the status of depression for the patient with bipolar disorder in the depressive episode. This questionnaire is a validated tool, translated to Malay

language and had 21 questions with multiple choice self-report inventory. It assessed how the subjects had been feeling in the last week. The standard scores were divided into 4 categories from 0 to 9 indicates minimal depression, 10 to 18 mild depression, 19 to 29 moderate depression and 30 to 63 indicates severe depression. The higher the total scores indicate more severe depressive symptoms.

*Saringan Status Kesihatan Mental – 20 (SSKM-20)*

This is a validated screening tool to screen for any psychiatry issues in Malaysia. It contains 20 questions with *likert scale* answer which assess the participants' feeling within 1 month duration. The cut-off point used was 14, and participants with scores more than 14 will be referred for assessment.

*Sociodemographic porforma*

Demographic characteristics of the participants in this study included basic characteristics such as age, race, gender, BMI, smoking status, diagnosis, duration of illness and family history of mental illness.

**Amino acids analysis**

Samples were collected from 10 hours of fasting subjects between 8:00 am and 9:00 am. In this study, 5mls of venous blood was withdrawn by a qualified officer for plasma amino acids analysis. The blood taking was done in the treatment room of psychiatric clinic and collected in lithium heparin bottle. They were kept in ice for transportation and were analyzed using high-performance liquid chromatography (HPLC) method. Patient's glutamate and glycine level were compared with the reference value of 1 to 57µmol/L and 123 to 319µmol/L, while tryptophan level was compared to the reference value of 29 to 77µmol/L.

**Statistical analysis**

Characteristics of participants were compared using an independent sample t-test (for age) and chi-square test (for gender). Analyses of amino acids level between control and BD group were performed using an independent t-test and Mann Whitney test (25). In addition, we also carried out One-way ANOVA and Kruskal Wallis test to determine the difference within bipolar episodes. Significant level was set at $p<0.05$.

## 3.5 Results

*Demographic data*

A total of 83 bipolar patients were enrolled from HUSM psychiatric outpatient clinic and 82 healthy control employees volunteered to participate in this study. The sociodemographic characteristics of bipolar patients and the control groups are summarized in Table 1. The mean (SD) age of bipolar patients were 40.87 (12.08) while the mean (SD) age for control groups was 35.60 (7.68) years. The participants from the bipolar group and control group differ significantly in age ($p=0.001$), however, there was no significant difference between patients and controls with respect to gender. Among patients with bipolar disorder, 10 were identified to be in manic, 67 were in euthymic, 4 in hypomanic and 2 in depressive episodes at the time of assessment.

**Table 3.1:** Characteristics of Study Participants

| Variables | Controls | Bipolar patients | *p* value |
|---|---|---|---|
| Number (n) | 82 | 83 | |
| | **Mean(SD)** | **Mean (SD)** | |
| Age (years) | 35.60 (7.68) | 40.87 (12.08) | **0.001** [a] |
| BMI | 25.55 (5.17) | 27.65 (5.33) | **0.011** [a] |
| | **n (%)** | **n (%)** | |
| Gender | | | 0.388 [b] |
| Male | 34 (42.0) | 29 (34.9) | |
| Female | 48 (58.0) | 54 (65.1) | |
| Race | | | 0.082 [b] |
| Malay | 82 (100) | 80 (96.4) | |
| Chinese | | 3 (3.6) | |
| Indian | | | |
| Others | | | |
| Smoking | | | 0.274 [b] |
| Yes | 5 (6.10) | 9 (10.8) | |
| No | 77 (93.9) | 74 (89.2) | |
| Family history of psychiatric illness | | | **<0.001** [b] |
| Yes | 1 (1.21) | 64 (77.1) | |
| No | 81 (98.8) | 19 (22.9) | |
| Diagnosis | | | |
| Bipolar 1 | | 76 (91.6) | |
| Bipolar 2 | | 7 (8.4) | |
| Episodes | | | |
| Euthymic | | 67 (80.7) | |
| Manic | | 10 (12.0) | |
| Hypomanic | | 4 (4.8) | |
| Depressive | | 2 (2.4) | |
| | | **Median (IQR)** | |
| Duration of illness (years) | | 10.0 (17.0) | |
| | **Mean (SD)** | **Mean (SD)** | |
| YMRS | | 17.43 (9.87) | |
| BDI | | 23.0 (8.49) | |
| SSKM | 8.27 (6.16) | | |

[a] Independent sample t-test
[b] $\chi^2$ test

As can be inferred from Table 2 and Table 3, the mean glutamate and glycine concentrations were significantly higher in bipolar patients as compared to the group of healthy controls with p=0.005 and 0.021 respectively. However, there was no significant difference in concentration of tryptophan between BD patients and healthy controls. In addition, a significant higher concentration of glutamate was found in Bipolar I than in Bipolar II and healthy control groups (Table 4).

**Table 3.2:** Comparison of Glutamate and Tryptophan Level of Bipolar Disorder Patients and Control Group

| **Variable** | **Median (IQR) level Concentration (µmol/L)** | | **Z statistics** | ***p* value** |
|---|---|---|---|---|
| | **Control (n=82)** | **Bipolar (n=83)** | | |
| Glutamate | 61.5 (122) | 111 (111) | -2.80 | **0.005*** |
| Tryptophan | 33.5 (24) | 30 (36) | -0.24 | 0.808 |

*Mann Whitney test

**Table 3.3:** Comparison of Mean Glycine Level between Bipolar Patients and Control Group

| **Variable** | **Mean (SD) level Concentration (µmol/L)** | | ***t* stats (df)** | ***p* value** |
|---|---|---|---|---|
| | **Control (n=82)** | **Bipolar (n=83)** | | |
| Glycine | 273.6 (66.4) | 304.0 (98.1) | -2.328 (163) | **0.021*** |

*Independent sample t-test

**Table 3.4:** Comparison of Glutamate and Tryptophan Level Among Bipolar I, Bipolar II and Control.

| **Variables level Concentration (µmol/L)** | **Median (IQR)** | | | | |
|---|---|---|---|---|---|
| | **Control (n=82)** | **Bipolar I (n=76)** | **Bipolar II (n=7)** | **Z stats (df)** | ***p* value** |
| Glutamate | 61.5 (122) | 111 (114) | 67 (115) | 8.606 (2) | **0.014*** |
| Tryptophan | 33.5 (24) | 31.5 (38) | 27 (38) | 0.579 (2) | 0.749 |

*Kruskal Wallis test

Multiple pairwise comparisons with Bonferroni correction revealed a significant difference in glutamate level between control and Bipolar I ($p$=0.011).

To compare the concentration levels in between bipolar episodes, BD patients who were in hypomanic and depressive episodes were excluded due to the small number of patients for group comparison. Therefore, manic patients (n=10), euthymic patients (n=67) and control group (n=83) were analyzed and showing a significantly higher concentration of glutamate and glycine during the manic episode (Table 5-6). However, no significant difference of tryptophan level was found in between bipolar episodes.