Can Mood-Stabilizing Agents Increase the Activity of Complexes I and II of the Electron Transport Chain and the Level of Two Tricarboxylic Acid Cycle Enzymes in Euthymic Bipolar Patients during the Early and Late Stages of the Disease?

Luiz Arthur Rangel Cyrino (✉ 3657luizrangel@gmail.com)

UNIVILLE - Universidade da região de Joinville

Daniela Delwing-de Lima

UNIVILLE - Universidade da região de Joinville

Gislaine Tezza Rezin

Universidade do Sul de Santa Catarina

Gabriela Kozuchovski Ferreira

UNISOCIESC

Silvia Aparecida Ramos

UNIVILLE - Universidade da região de Joinville

Débora Delwing-Dal Magro

Universidade regional de Blumenau

Natalia Cavichioli

Universidade regional de Blumenau

Oliver Matheus Ullmann

UNIVILLE - Universidade da região de Joinville

Yasmin Mayer

UNIVILLE - Universidade da região de Joinville

Mariana Pacheco de Oliveira

Universidade do Sul de Santa Catarina

Larissa Espindola da Silva

Universidade do Sul de Santa Catarina

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Abstract

Bipolar disorder (BD) is associated with changes in biomarkers and mood episodes, leading to neurological damage, which may reflect alterations in cognitive functions and functionality. We analyzed the effect of four biomarkers related to metabolic energy in leukocytes. Two enzymes are related to the tricarboxylic acid (TCA) cycle (citrate synthase and succinate dehydrogenase), and two proteins are related to electron transport chain (ETC) (complexes I and II). We studied 50 euthymic patients divided into two subgroups with 25 patients (≤ 3 years and ≥ 10 years of diagnosis, from the first episode of mania) and 25 control patients. All the patients were using mood stabilizers. There were no changes in the levels of TCA cycle enzymes in the leukocytes in any subgroup. However, complex I activity showed a significant increase in patients ≤ 3 years (461.80 ± 162.76) and ≥ 10 years (561.21 ± 328.13) since disease onset compared to the control group (335.39 ± 167.64) ($p < 0.01$). Complex II activity was significantly increased in the ≥ 10 years (3.42 ± 1.38) group compared to the ≤ 3 years (2.66 ± 1.17) group and the control group (2.43 ± 0.95) ($p < 0.01$). These results suggest that stabilizing medications improve the activity of ETC complexes and stabilize TCA cycle enzymes.

1. Introduction

Psychiatry has made significant scientific advances in the last three decades. However, it is still necessary to clarify several aspects of the pathophysiological basis of bipolar disorder (BD). This disease is highly complex, multifactorial in character, determined by the interaction between genetic, environmental, and systemic factors, and has been associated with systemic toxicity, compromising not only the central nervous system but also other peripheral physiological systems. This toxicity occurs due to alterations in inflammatory biomarkers, oxidative stress mediators, neurotrophins, and energy metabolism. These biomarkers show differences between patients in the early and late stages of the disease^[1]. In addition, BD patients in the acute phases of the disease have been observed to have a significantly higher systemic toxicity rate than healthy controls but a lower systemic toxicity rate than sepsis patients^[2]. All of them seem to be related to mood episodes, leading to microglial activation and causing neural damage. These alterations in neuroplasticity are reflected in synapses and in particular, in the formation and expression of dendritic spines, as well as in various neurotransmission systems, which can therefore be reflected in cognitive functions and functionality, leading to disease progression^[3]. Although biomarkers related to mitochondrial energy function are still poorly studied, evidence suggests an important role of mitochondrial dysfunction in BD, producing altered energy metabolism, changes in electron transport chain (ETC) enzymes, altered levels of cytoplasmic calcium, and changes in the activation of mitochondrial genes. The influx of calcium from the cytosol controls the rate of mitochondrial ATP production. Intramitochondrial calcium activates four dehydrogenases coupled to the TCA cycle: citrate synthase, pyruvate dehydrogenase, isocitrate dehydrogenase, and α -ketoglutarate dehydrogenase. In addition, calcium uptake modulates the spatial and temporal profile of intracellular calcium signaling, regulating mitochondrial motility and morphology, which can lead to cell death^{[4][5]}. Changes in lactate concentrations were also significantly higher in BD patients than in controls,

indicating increased mitochondrial metabolism and anaerobic glucose utilization, which is consistent with altered mitochondrial metabolism in BD. Mitochondrial dysfunction involving ETC disruption is considered the main cause of chronic oxidative stress in BD, resulting in damage to lipids, proteins, and cell membrane DNA, which further aggravates oxidative stress, creating a cycle-perpetuating pathogen contributing to a chronic neuroinflammation process. Thus, studies have contributed to the understanding of these mechanisms, partially explaining the toxicity related to cumulative mood episodes in BD [6]. Therefore, this article aimed to study two enzymes of the TCA cycle (citrate synthase and succinate dehydrogenase) and the activity of mitochondrial complexes (I, II) of the ETC in leukocytes from euthymic bipolar patients with ≤ 3 years and ≥ 10 years of disease compared to healthy control patients and to relate the possible mechanisms responsible for energy metabolism in BD, focusing on neuroinflammation.

2. Materials And Methods

2.1. Ethics

This study was approved by the Research Ethics Committee of Universidade da Região de Joinville - UNIVILLE (protocol number 655.037) and followed the ethical rules of the Helsinki Declaration of 1975. All participants provided written informed consent before entering the study. Each patient underwent a clinical and psychiatric evaluation, where demographic, anthropometric, pharmacological data and clinical variables were collected.

2.2. Participants

The study evaluated 50 outpatients with BD types I/II in euthymia. The participants were divided into three distinct groups, each with 25 individuals: 25 euthymic BD patients in the early stage of disease (≤ 3 years since the diagnosis of BD from the first manic episode); 25 euthymic BD patients in the late stage of disease (≥ 10 years since the diagnosis of BD from the first manic episode); and 25 healthy controls. Bipolar patients were recruited from the Porto Seguro Psychiatric Hospital, located in the city of Curitiba, Brazil, and the healthy control group was selected among hospital staff. The psychiatric diagnosis of BD patients for types I/II was defined in the Manual Diagnosis and Statistics of Mental Disorders (DSM-V) and confirmed by a semi-Structured Clinical Interview, according to DSM-V (SCID-5-CV). Manic and depressive symptoms were assessed using the Young Mania Rating Scale (YMRS) **Young et al., (1978)**^[7], and the 17-item version of the Hamilton Depression Rating Scale (HAMD-17) **Hamilton, (1960)**^[8], respectively. With the HAMD-17 scale, depressive symptoms that had occurred within the last week were evaluated, and in the YMRS, manic symptoms that had presented themselves within the last 48 h were evaluated. The cutoff scores used in the study were YMRS > 7 as indicative of mania and HAMD-17 > 7 as indicative of depression.

2.3. Criteria

The inclusion criteria of bipolar patients in the euthymic stage were as follows: (a) the patients had been in the euthymic phase for at least six months; (b) active age (18 - 60 years); (c) no history of neurodegenerative diseases, cancer, morbid obesity or trauma; (d) none of the patients had a history of addiction or substance abuse in the last year; (e) not pregnant or breastfeeding; (f) nonsmokers; (g) patients had no significant comorbid medical conditions and did not receive medication in addition to those prescribed for their psychiatric condition; these patients should have been used for at least four weeks; and (h) patients were able to understand the procedures and protocol and provided written informed consent and did not present cognitive impairment with disability or dementia or physical disabilities, e.g., visual or hearing impairments.

2.4. *In vivo* studies

2.4.1. Leukocyte preparation

To analyze the energy metabolism enzymes citrate synthase, succinate dehydrogenase, complex I and complex II, 20 mL of peripheral blood was taken from each individual by venipuncture in a vacuum tube with heparin. The blood was centrifuged at 1000 x g for 30 minutes, and the leukocytes were separated using a Ficoll gradient (Sigma)^[9]. The leukocytes were then transferred to another tube, where they were washed twice with phosphate buffered saline (PBS) and centrifuged at 1300 x g for 10 minutes. After centrifugation, PBS and dimethyl sulfoxide (DMSO) <0.01% were added to the samples, which were progressively frozen at -10°C for 30 minutes, at -20°C for 30 minutes, and frozen at -80°C until the assay. All samples were assessed in duplicate.

2.4.2 Citrate synthase assay

The citrate synthase levels were determined using spectrophotometry according to the method described by **Alp et al. (1976)**^[10]. Homogenized leukocytes (10 µL) were added to a cuvette with 800 µL buffer (Tris-HCl 1 M, pH 8), 100 µL DTNB 1 mM, 40 µL acetyl-CoA 2.5 mM and 10 µL Triton 10%. The reaction was started by the addition of 50 µL of oxaloacetate (4 mM) and watching the changes in absorbance for 3 minutes at 412 nm.

2.4.3 Succinate Desidrogenase assay

The succinate desidrogenase levels were determined using spectrophotometry according to the method described by **Fischer et al. (1985)**^[11]. SDH and complex II (CII) activities of succinate: phenazine oxyreductase (soluble SDH) and complex II (succinate: DCIP oxyredutase) were measured in leukocyte homogenates by following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm with 700 nm as the reference wavelength ($\epsilon = 19.1 \text{ mM}^{-1} \text{ cm}^{-1}$) in the presence of phenazine methasulphate (PMS). The reaction mixture, consisting of 40.0 mM potassium phosphate, pH 7.4, 16.0 mM succinate and 8 µM DCIP, was preincubated with 40-80 µg homogenate protein at 30°C for 20 min. Subsequently, for complex II activity, 4.0 mM sodium azide and 7 µM rotenone were added, and the reaction was initiated by the addition of 40 µM DCIP and monitored for 5 min. The activity of SDH was assessed in the same incubation medium by the addition of 1.0 mM PMS and monitored for 5 min.

2.4.4 Complex I and II assay

Complex I was determined using spectrophotometry according to the method described by **Cassina and Radi (1996)**^[12]. Complex I activity was measured by determining the NADH-dependent ferrocyanide reduction rate, monitored in a spectrophotometer at 420 nm, and the specific activity is reported by nmol units of Complex I per min per milligram of protein and was measured by the **Lowry et al. (1951)**^[13] method, using serum bovine albumin as a standard.

. Complex II was determined using spectrophotometry according to the method described by **Fischer et al. (1985)**^[11]. The Complex II activity was measured following the decrease in absorbance due to the reduction of 2,6-DCIP, monitored in a spectrophotometer at 600 nm, and specific activity is reported in nmol units of Complex II per min per milligram of protein was measured by **Lowry et al. (1951)**^[13] method, using serum bovine albumin as standard.

2.5. Statistical analysis

Demographic and clinical variables were analyzed using descriptive statistics, including (mean) and (standard deviation) for quantitative variables and absolute frequency (n) and relatives (%), for qualitative variables with a confidence interval of 95% in both cases. For the qualitative nominal and ordinal data, we used the chi-square test (χ^2) of Pearson, and for two or more groups, we used Fisher's exact test. Parametric and nonparametric tests were used for the analysis of qualitative variables. The assumption of normality and homoscedasticity of each variable was analyzed with the Kolmogorov-Smirnov normality test and Levene's test, respectively. For comparisons of parametric variables between two groups, Student's *t*-test was used, and for more than two groups, Tukey's test of analysis of variance (ANOVA) was used. To compare nonparametric variables between two and three independent samples, Mann-Whitney tests and Kruskal-Wallis tests were used. Dunn's post hoc test was performed for peer comparisons if the main effects were significant. The SPSS software program (SPSS Inc., Chicago, USA) was used. To calculate the statistical power analyses, we used the program G*Power 3.1. Statistical significance was set at $p < 0.05$ for all tests, or a level of significance of 5% was adopted to reject the null hypotheses.

3. Results

3.1 Demographic, Clinical and Pharmacological Characteristics

We evaluated the demographic and clinical characteristics of the different groups studied. Fifty patients with BD were divided into two groups of euthymic patients (≤ 3 and ≥ 10 years of the disease) with 25 patients each. Thirty-six euthymic patients (72%) were female. There was a significant difference in the mean age between the euthymic patient groups (≤ 3 years and ≥ 10 years of the disease) ($p < 0.01$) and a significant difference in the mean years of education between the healthy control group and both

euthymic patient groups (≤ 3 years and ≥ 10 years of the disease) ($p < 0.01$). There was a nonsignificant difference between groups in gender, occupational status, or marital status ($p > 0.05$), as shown in Table 1.

The bipolar patients had a mean disease duration of ≤ 3 years (2.52 ± 0.65) and ≥ 10 years (15.64 ± 6.81), and the mean age at onset of the disease was ≤ 3 years (22.1 ± 7.01) and ≥ 10 years (25.1 ± 6.17). There was a significant difference in the mean illness duration between the euthymic patient groups (≤ 3 years and ≥ 10 years of the disease) ($p < 0.001$). There was a nonsignificant difference between groups in age onset, hospitalizations, and duration of hospitalizations ($p > 0.05$). Regarding pharmacologic treatment, our results showed that 10 (20%) of the patients were on monotherapy. Among the patients on polypharmacy, 18 (36%), 16 (32%), and 6 (12%) of the patients received 2, 3, and 4 psychotropic medications, respectively, as shown in Table 2.

Table 1
Sociodemographic Characteristics of the Sample

	Healthy Controls n = 25	Bipolar Patients ≤ 3 years of the disease n = 25	Bipolar Patients ≥ 10 years of the disease n = 25	p - Value
Age, years ^b	35.0 (±9.96)	34.9 (±10.04)	47.4 (±8.21)	p < 0 .01 c
Gender, n				p = 0.77 a
Male	9	7	7	
Female	16	18	18	
Marital status n (%)				p = 0.08 d
Married	12 (48)	9 (36)	15 (64)	
Divorced	1 (4)	2 (8)	5 (20)	
Widowed	1 (4)	0 (0)	1 (4)	
Single	11 (44)	14 (56)	4 (12)	
Years of education ^b	14.7 (±2.18)	13.8 (±2.70)	12.4 (±2.77)	p < 0.01 e
Education n (%)				p = 0 .13 d
Illiterate	-	-	-	
Up to primary school	0 (0)	3 (12)	4 (16)	
Up to high school	10 (40)	10 (40)	12 (48)	
Graduate	12 (48)	12 (48)	9 (36)	
Postgraduate	3 (12)	0 (0)	0 (0)	
Work situation n (%)				p = 0.17 d
Employed	23 (92)	18 (72)	13 (52)	
Unemployed	2 (8)	6 (24)	10 (40)	
Medical benefits	0 (0)	1 (4)	0 (0)	

^a χ^2 ^b Mean (±SD) ^c One-way ANOVA followed by Dunn's post hoc ^d Fisher's exact test ^e Kruskal Wallis

	Healthy Controls n = 25	Bipolar Patients ≤ 3 years of the disease n = 25	Bipolar Patients ≥ 10 years of the disease n = 25	p-Value
Invalidity	0 (0)	0 (0)	2 (8)	
^a χ^2 ^b Mean ($\pm$ SD) ^c One-way ANOVA followed by Dunn's post hoc ^d Fisher's exact test ^e Kruskal Wallis				

Table 2
Clinical and Pharmacological Characteristics of the Sample

	Healthy Controls n = 25	Bipolar patients ≤ 3 years of the disease n = 25	Bipolar patients ≥ 10 years of the disease n = 25	p-Value
Illness duration (years) ^a	N/A	2.52 ($\pm$ 0.65)	15.64 ($\pm$ 6.81)	$p < 0.001$ ^d
Age of onset (years) ^a	N/A	22.1 ($\pm$ 7.01)	25.1 ($\pm$ 6.17)	$p = 0.62$ ^d
HAMD-17 total score ^a	4.32 ($\pm$ 2.49)	4.10 ($\pm$ 2.02)	3.71 ($\pm$ 1.46)	$p = 0.53$ ^b
YMRS total score ^a	0.56 ($\pm$ 0.86)	0.88 ($\pm$ 1.01)	1.28 ($\pm$ 1.13)	$p = 0.08$ ^c
Hospitalizations n (%)	N/A	12 (48)	8 (32)	
Duration hospitalizations (day) ^a	N/A	13.4 ($\pm$ 24.3)	13.0 ($\pm$ 36.6)	$p = 0.90$ ^d
Suicide attempts n	N/A	18	40	
Family history of affective disorders n (%)	N/A	10 (40)	13 (52)	
Treatment n (%)				
Lithium	N/A	13 (52)	15 (60)	
Other mood stabilizers	N/A	11 (44)	13 (52)	
Atypical antipsychotics	N/A	8 (32)	12 (48)	
Typical antipsychotics	N/A	2 (8)	0 (0)	
Antidepressants	N/A	7 (28)	7 (28)	
Benzodiazepines	N/A	2 (8)	7 (28)	

HAMD-17 = Hamilton Depression Rating Scale; YMRS = Young Mania Rating Scale; N/A = not available; ^a Mean ($\pm$ SD)

^b t test ^c Mann-Whitney ^d Kruskal Wallis

3.2. Evaluation of the activity of tricarboxylic acid cycle enzymes in leukocytes

Regarding the TCA cycle, no changes in the activity of the citrate synthase and succinate dehydrogenase enzymes in leukocytes in any of the groups were observed, $p > 0.05$, as shown in **Table 3**.

Table 3: Comparison of activity of the citrate synthase and succinate dehydrogenase enzymes in leucocytes between the healthy control patients and the Euthymic bipolar patients with ≤ 3 and ≥ 10 years of the disease.

	Control Patients n = 25	Euthymic Patients BD ≤ 3 years n= 25	Euthymic Patients BD ≥ 10 years n= 25	p – Value
Tricarboxylic Acid Cycle enzymes				
Citrate synthase (nmol/min x mg protein) Means ($\pm$ SD)	1.50 ($\pm$ 0.39)	1.76 ($\pm$ 0.53)	1.68 ($\pm$ 0.38)	0.15 ^a
Succinate dehydrogenase (nmol/min x mg protein) Means ($\pm$ SD)	3.29 ($\pm$ 1.67)	2.83 ($\pm$ 2.05)	3.33 ($\pm$ 1.49)	0.06 ^b

Means $\pm$ standard deviation (SD). The normality of each variable was analyzed using the Kolmogorov-Smirnov normality test. ^a To compare parametric variables between the three independent groups, two-way analysis of variance (ANOVA) followed by Tukey's test was used. ^b To compare nonparametric variables between the three independent samples, the Kruskal-Wallis test was used. Dunn's post hoc test was performed for pairwise comparisons if the main effects were significant. Statistical significance was set at $p < 0.05$.

3.3. Evaluation of the energy metabolism activity of complexes I and II enzymes in the electron transport chain in leukocytes

Complex I activity had a significant increase in both patients; ≤ 3 years (461.80 ± 162.76) and ≥ 10 years (561.21 ± 328.13) since disease onset compared to the control group (335.39 ± 167.64) [$F(2,72) = 5.921$; $df = 72$; $p < 0.01$]. Complex II activity had a significant increase in the group of patients ≥ 10 years (3.42 ± 1.38) compared to patients ≤ 3 years (2.66 ± 1.17) at disease onset and compared to the control group

(2.43 ± 0.95) [$F(2,72) = 4.828$; $df = 74$; $p < 0.01$]. Thus, euthymic patients showed an increase in complex I and II activity levels, especially in the ≥ 10 years group, as shown in **Fig. 1 Fig. 2**.

4. Discussion

Studies with bipolar patients show several changes in peripheral biomarkers, which have shown an association of BD with significant systemic toxicity (neuroinflammation) ^[14]. Thus, this toxicity may be related to changes in these peripheral physiological systems and influence changes in the central nervous system, contributing to a decrease in cellular resilience, producing cognitive and functional changes, and leading to a greater vulnerability of patients to stress and clinical worsening in their health, (neuroprogression). In this context, a better understanding of the mechanisms responsible for such biochemical, cognitive, and functional changes may help in the development of new therapies that are more effective in the treatment of BD. However, psychiatry still needs reliable and accessible biomarkers that facilitate early diagnosis. Regarding energy metabolism biomarkers, there are only a few studies, mainly relating to biomarkers of the TCA cycle and ETC complexes. When we refer to the mediators of energy metabolism, we must remember that mitochondria are the main cellular structure involved. Studies show that inflammatory activations can affect mitochondrial functions, altering energy production from dysfunctions mainly in the TCA cycle and ETC complexes, and alterations were found in several psychiatric pathologies, such as BD. As an example, we can observe that higher levels of inflammatory cytokines produce decreased ETC complex I activity in the brain and peripheral cells of bipolar patients, leading to increased reactive oxygen species (ROS) production by mitochondria ^[15]. Another example was studied by Kim et al. (2016) ^[16], showing that the activation of the inflammatory system, by increased levels of caspase-1, interleukins (IL) IL-1, IL-6, IL-10, and tumor necrosis factor- α , induces the production of ROS, causing mitochondrial changes in the ETC and in the TCA cycle, and the same ROS produced through oxidative stress worsens mitochondrial functions both in the TCA cycle and in the ETC complexes, stimulating the mitochondria to produce more ROS, which will generate a chronic cycle that feeds back. As previously commented, increased neuronal oxidative stress produces deleterious effects on signal transduction, plasticity, and cellular resilience that can induce mitochondrial dysfunction by reducing ETC and TCA cycle activity ^[17]. These mitochondrial dysfunctions can be measured in the peripheral blood and *postmortem* brains of BD patients ^{[18][19]}.

In a review study, Zuccoli et al. (2017) ^[20] compiled 95 expressed proteins that were found to be differentially related to energy metabolism in BD in *postmortem* brain tissue. Forty-eight of these proteins were primarily related to the TCA cycle and ETC. TCA cycle proteins are composed of a set of eight enzymes present in the mitochondrial matrix, with most enzymes encoded in the nucleus in higher eukaryotes. These proteins are essential for mitochondrial energy production and produce substrates used in oxidative phosphorylation by the ETC. Thus, inactivating any of them can reduce mitochondrial energy generation ^[21]. In this manner, the TCA cycle is critical for mitochondrial activity, as it produces substrates that feed oxidative phosphorylation in the mitochondrial ETC, which, when present in mitochondrial dysfunctions, can play an essential role in the pathophysiology of neuropsychiatric

diseases. This notion is consistent with parallel studies of transcriptomics, proteomics, and metabolomics showing differential expression of several genes related to mitochondrial functions between patients with mental disorders and healthy controls ^[22]. Despite its importance, few studies have evaluated the activity of TCA cycle enzymes in patients with mental illnesses, particularly in bipolar patients. One of these studies was carried out with 18 patients with BD during the depressive phase at the beginning of the disease and was treatment-naïve ^[23]. The research evaluated the activity of the main enzymes of the TCA cycle, citrate synthase, malate dehydrogenase, and succinate dehydrogenase in leukocytes from BD patients. There was no change in enzymatic activity in the TCA cycle compared to controls. There was also no correlation between any enzyme activity of the TCA cycle and the severity of depressive symptoms in recent-onset BD. Thus, **De Sousa et al. (2014a)** ^[24] suggested that even if mitochondrial changes occur early in the disease, the organism might have compensatory mechanisms that prevent changes in the TCA cycle at the beginning of the disease. In our study, we found similar results, in which we did not observe significant changes in the levels of citrate synthase and succinate dehydrogenase enzymes in leukocytes from euthymic patients, both in the group of patients who started the disease with ≤ 3 years and in the group with ≥ 10 years of the disease. Analyzing our results, we were able to observe that even patients with a longer duration of the disease remain in the euthymic phase and present normal values of these enzymes. It is also important to point out that we did not find any studies focusing on the later stages of the disease, which makes this work relevant.

In a study carried out in rats, **Feier et al. (2013)** ^[25], using methamphetamine as a model for inducing mania, observed a decrease in the enzymes of the TCA cycle (citrate synthase and succinate dehydrogenase) and consequently of complexes I, II, III, IV of the ETC, where after the use of mood stabilizers (lithium and valproic acid), the values returned to normal. This reinforces the idea that treated patients show reversal of energy metabolism dysfunction in TCA and ETC cycle enzymes, developing the hypothesis that TCA cycle enzymes may be involved in BD. In another similar work, **Valvassori et al. (2014)** ^[26] found reduced levels of citrate synthase, succinate dehydrogenase, and malate dehydrogenase enzymes in the prefrontal cortex, hippocampus, and striatum in rats treated with amphetamine. These reduced levels were associated with behavioral hyperactivity, which later, when treated with tamoxifen, showed significant improvement in the levels of these enzymes and in behavior. These findings suggest that inhibition of TCA cycle enzymes may be an important link to the mitochondrial dysfunctions seen in BD. However, we should note that **Bubber et al. (2004)** ^[27] demonstrated that although the enzymatic activities of the TCA cycle in rat brains have a similar pattern to humans, most brain enzymatic activities of these animals are 2 to 3 times higher than those in human brains. On the other hand, the same researcher showed that the activities of TCA cycle enzymes varied considerably in the human brain in schizophrenia, where they had reduced activity in some of them, suggesting that similar changes also occurred in other psychiatric pathologies ^[28]. In contrast, **Yoshimi et al. (2016)** ^[29] showed that serum levels of pyruvate and α -ketoglutarate in BD patients were significantly higher than those of healthy controls and that the latter likely plays a role in the pathogenesis of BD, although the underlying reasons are still unknown. The serum levels of acetyl-CoA and oxaloacetate were not altered. In addition, no studies were conducted during the later stages of the disease, and α -ketoglutarate is a key metabolite in

the TCA cycle and is involved in DNA and histone methylation, producing an epigenetic impact. It has been suggested that epigenetic modifications play an important role in the pathogenesis of many psychiatric disorders, including BD [30].

As described above, evidence suggests that mitochondrial dysfunction and oxidative stress are correlated in the pathogenesis of BD, more specifically with the impairment of ETC complexes. In our work, we evaluated the activity of complexes I and II, which showed altered levels. The values showed a significant increase in activity between the groups, in which higher complex I activity was observed in euthymic bipolar patients, both in the group with ≤ 3 years and more than ≥ 10 years of illness, when compared to the control group ($p < 0.01$), as shown in **Fig. 1**. Additionally, regarding complex II, we found a significant increase only in the group with ≥ 10 years of the disease and not in any other group ($p < 0.01$). However, it should be noted that in the group of ≤ 3 years of the disease in complex II, there was an increase in activity compared to the control group, but it was not statistically significant, as shown in **Fig. 2**. The question to be answered regarding the increase in the activity of these two complexes would be whether this increase is due to an adaptive response of the mitochondria with an increased expression of these mitochondrial ETC protein complexes, a primary deficit in BD, or the use of specific drugs for the treatment of BD.

To answer this question, further research will be needed ahead, including another group of bipolar patients, this time untreated, and comparing them with the current groups. However, to discuss our results, we sought some answers in the literature. The articles studied presented very conflicting results, and this was due to the different materials and methods used, which we will discuss next. These differences in methodology are observed relating to the tissue used (*postmortem* neurons; leukocytes, platelets, or in cell culture); the stage of the disease (mania, depression, and euthymia); the brain area evaluated (prefrontal, cerebellum, and parietal); use or not of stabilizing medication, methodology for measurement (through gene expression or measurements of ETC proteins, or their subunits). All these variables can become factors that enable different results.

All ETC complexes have several subunits, as shown in Fig. 3. Complex I is the most studied. This complex is composed of 26 polypeptide chains (subunits) and is the largest of the ETC complexes. Thus, many studies have shown that bipolar patients in the manic or depressive phase have lower levels of complex I activity. **Ben-Shachar and Karry (2008)** [31] evaluated complex I subunits in *the postmortem* cerebellum of bipolar patients and compared them with control patients. Decreased levels of the following subunits were observed: NDUFV1 (NADH dehydrogenase [ubiquinone] flavoprotein 1); NDUFV2 (NADH dehydrogenase [ubiquinone] flavoprotein 2) and NDUFS1 (NADH dehydrogenase [ubiquinone] iron-sulfur protein 1). However, in this same work, when the levels of NDUFV1 and NDUFV2 in the parietal-occipital cortex were evaluated, there was an increase in the activity. These results suggest that there may be regional changes in the brain related to mitochondrial complex dysfunction in BD patients. In other studies, **Sun et al. (2006)** [32] and **Kim et al. (2016)** [16] evaluated the complex I subunit NDUFS7 (NADH dehydrogenase [ubiquinone] iron-sulfur protein 7) in the prefrontal cortex of *postmortem* bipolar patients. In all of them, reduced levels of this protein were found. Another study carried out by **Andreazza et al.**

(2013) ^[33] also observed a decrease in complex I but no change in complex III in the *postmortem* prefrontal cortex of bipolar patients in mania.

We must remember that complexes I and III are primarily responsible for the increased production of ROS, especially superoxide anion ($O_2^{\bullet-}$), with complexes I and III being the main producers of ROS under normal metabolic conditions ^[34]. This increase in $O_2^{\bullet-}$ by ETC can secondarily cause more mitochondrial dysfunction, feedback the process of compromising respiratory chain proteins, and lead to more mitochondrial dysfunction and, consequently, decreased ATP synthesis ^[35]. The same results were found in rats submitted to an experimental model of depression, which showed impaired mitochondrial function in complexes I and IV of the cerebral cortex and cerebellum ^[36]. On the other hand, **De Sousa et al. (2014b)** ^[18] found no changes in the copy number of mitochondrial DNA, which encodes proteins related to ETC activity in leukocytes from untreated patients with bipolar depression, compared with healthy patients. Even after six weeks of lithium treatment, the mitochondrial DNA copy number remained unchanged.

In another study, **Gubert et al. (2013)** ^[37] evaluated 12 euthymic bipolar patients all on treatment and compared them with 30 healthy volunteers. A significant decrease in complex I activity was observed in peripheral blood mononuclear cells. These results differ from ours, where increases in complexes I and II were found in euthymic patients treated with mood stabilizers. However, the results of **De Sousa et al. (2015a)** ^[23], after evaluating complex I in peripheral blood using leukocytes from patients with BD in the depressive phase, reported a decrease in complex I activity, but after treatment with lithium, they showed an increase in ETC complex I activity, without any changes in other complexes, and these results are in line with ours. Similar data to ours were observed by **Sun et al. (2006)** ^[32], where the expression of the complex I subunit gene (NDUFS7) was increased in the prefrontal cortex of patients treated with lithium compared to patients without lithium treatment. When correlating the plasma lithium level, they observed a positive correlation with the levels of mitochondrial complex I activity after treatment. Finally, in a study similar to ours, **Valvassori et al. (2018)** ^[19] found results that reflect much of those found by our group. By analyzing the activity levels of the mitochondrial complex (I, II, II-III and IV) in peripheral blood using lymphocytes from 51 patients with BD, 34 of whom were euthymic and 37 in the depressive phase, the researchers observed that during the depressive phase, there is an increase in mitochondrial $O_2^{\bullet-}$, carbonyl, thiobarbituric acid reactive substances (TBA-RS) and superoxide dismutase (SOD) levels, which may reduce mitochondrial complex II activity levels, producing a negative correlation between the HAM-D 17 scale and complex II activity in lymphocytes, a positive correlation between the HAM-D 17 and TBA-RS scale, and a negative correlation between the activity of complex II and the parameters of oxidative stress, i.e. represented by SOD. A decrease in complex II has also been reported in lymphocytes from bipolar patients in the depressive phase compared to euthymic patients. This reinforces the idea that euthymic patients present an improvement in the functioning of ETC proteins, at least regarding complexes I and II, as observed by our group. This is possibly due to the effect of mood stabilizers on ETC or the decrease in oxidative stress, as we observed in a previous study carried out by our research group. Regarding new studies, **Zvěřová et al. (2019)** ^[38] and **Hroudová et al. (2019)** ^[39] evaluated ETC

complexes and platelets in the peripheral blood of bipolar patients. In the first study, they used 24 patients in the depressive phase and compared them with healthy controls. In the second study, they used bipolar patients in a manic episode and patients in remission and compared them with normal control patients. Almost all patients were using medication. In the first study, a significant increase in complex I was observed, but a decrease in citrate synthase, complex II and complex IV was observed in bipolar patients compared to healthy controls. In the second study, the activities of citrate synthase and ETC complexes I, II and IV were examined. The activities of citrate synthase, complex II and complex IV were decreased in patients with BD, the activity of complex I was increased, and the relationship between complex I and citrate synthase was significantly increased, corroborating findings by [38]. Thus, curiously, the findings were similar in patients in the depressed and manic phases of BD. Both studies suggest that increased complex I activity may be a compensatory mechanism in response to disturbances in the oxidative phosphorylation system, for example, decreased complex II and complex IV activities. As previously described, our research observed a similar result with an increase in complex I both in the groups (≤ 3 years and ≥ 10 years of the disease); however, relating to complex II patients, we only observed an increase in the group of patients with ≥ 10 years of the disease. These studies consistently demonstrate that the use of medication seems to produce an improvement in the functioning of ETC proteins, reinforcing the hypothesis of several authors. **Iwamoto et al. (2005)** [40] found that each of the drug groups used to treat BD (lithium, valproic acid, and antipsychotics) appeared to significantly affect the overall repressive process in the expression of mitochondrial-related genes. Studies in cell culture and animal models have also shown that chronic treatment with lithium or valproic acid can increase mitochondrial function and protect against mitochondria-mediated toxicity [41]. Lithium has also been shown to increase complex I - III activity in *postmortem* human brain tissue extracts at therapeutically relevant concentrations [42]. In this way, we can observe that there are very different results between the different articles, where previous studies, based on *postmortem* brain samples, differ from the findings based on blood samples isolated from living individuals with BD. There may also be significant differences regarding studies using gene expression, where ETC genes are unregulated in the blood but downregulated in some regions of the central nervous system of patients with BD. For the dosages of ETC proteins in leukocytes, these seem to present more consistent results and less variation, demonstrating that generally the proteins of the complexes or their subunits, especially those of complexes I and II, seem to have their activities reduced during the phases of manic and depressive symptoms of the disease, but they improve after returning to euthymia due to the use of stabilizers.

5. Conclusion

The conclusions of this study reinforce the hypothesis of neuroprogression and neuroinflammation developed by several researchers. This hypothesis states that during acute mood episodes, there are changes associated with the systemic toxicity of the disease and the damage resulting from multiple episodes. The recurrence of episodes in BD produces worse outcomes, producing functional and cognitive impairments and decreased responsiveness to treatment. Our results suggest that peripheral biomarkers related to energy metabolism pathways show greater stabilization and normalization of their

levels during the euthymia phase. This stabilization occurs in part due to the use of mood stabilizers, as observed in our study, with increased activity of complexes I and II during euthymia, suggesting that there was an improvement in the activity of these complexes due to the use of stabilizing medications, as shown in **Fig. 1** and **Fig. 2**. In light of this knowledge, we can propose that the use of mood stabilizers, in addition to producing better management of acute episodes, prevents their recurrence with an improvement of mitochondrial functions.

Studies have shown that mood stabilizers produce protective antioxidant effects, producing an increase in mitochondrial function and reflecting symptomatic improvement in bipolar patients. Both lithium and valproic acid directly increase the activity of complex I of the mitochondrial ETC, and both substances appear to induce complex II, III, and V phosphorylation, increasing energy production ^{[32][43]}. Thus, the early use of medications for treatment, despite their side effects, would be beneficial in the clinical evolution of the patient. In our sample, we observed that all patients used drugs from the most diverse categories – lithium carbonate, antidepressants, antipsychotics, and anticonvulsants – used alone or in combination. We know that, in general, they are effective in 80% of cases of BD and depression and have been proven to produce a neuroprotective effect ^[44]. However, in a study with bipolar patients, it was found that 40% of these patients, especially those with more severe conditions, could only keep the disease under control by taking three or more medications and that, in general, these diseases reach a very advanced stage and are more difficult to control after the tenth crisis, where the outbreaks gain autonomy and can become independent of the stressful conditions that previously triggered them (a process called kindling), which usually occurs approximately 10 years after the first manifestations of the disease. Usually, patients search for treatment many times after the onset of the disease. Sometimes the psychiatrist spends much time diagnosing and prescribing the correct medications. In BD, the time since the onset of the disease and the beginning of the treatment varies between 5 and 10 years. During this time, they face many challenges at work and social and family relationships ^{[45][46]}.

Finally, the mitochondrial alterations and differences in the expression of proteins of ETC complexes and the TCA cycle also present alterations in the morphological structures of mitochondria, which affect energy metabolism due to the inhibition of ETC and TCA cycles with ATP depletion. These alterations seem to support the idea that mitochondrial disorders are early in the physiopathology of BD ^[47]. Thus, more studies will be necessary to clarify the relationship between the diagnoses, moods, treatment, and expression of proteins in the ECT complex, TCA enzymes, and mitochondrial morphology in BD.

Declarations

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Research Ethics Committee This study was approved by the Research Ethics Committee of Universidade da Região de Joinville - UNIVILLE (protocol number 655.037) and followed the ethical rules of the Helsinki Declaration of 1975. All participants provided written informed consent before entering the study.

Datasets - The datasets generated during and/or analyzed during the current study are not publicly available because individual privacy could be compromised but are available from the corresponding author upon reasonable request.

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Figures

Figure 1

Box Plot - Comparison of activity of complexe I activity (nmol/min x mg protein) between the healthy control patients and the Euthymic bipolar patients with ≤ 3 and ≥ 10 years of the disease.

Figure 2

Box Plot - Comparison of activity of complex II activity (nmol/min x mg protein) between the healthy control patients and the Euthymic bipolar patients with ≤ 3 and ≥ 10 years of the disease.

OXIDATIVE PHOSPHORYLATION

NADH dehydrogenase

E	ND1	ND2	ND3	ND4	ND4L	ND5	ND6										
E	Ndufs1	Ndufs2	Ndufs3	Ndufs4	Ndufs5	Ndufs6	Ndufs7	Ndufs8	Ndufv1	Ndufv2	Ndufv3						
B/A	NuoA	NuoB	NuoC	NuoD	NuoE	NuoF	NuoG	NuoH	NuoI	NuoJ	NuoK	NuoL	NuoM	NuoN			
B/A	NdhC	NdhK	NdhJ	NdhH	NdhA	NdhI	NdhG	NdhE	NdhF	NdhD	NdhB	NdhL	NdhM	NdhN	HoxE	HoxF	HoxU
E	Ndufa1	Ndufa2	Ndufa3	Ndufa4	Ndufa5	Ndufa6	Ndufa7	Ndufa8	Ndufa9	Ndufa10	Ndufa11	Ndufa12	Ndufa13				
E	Ndufb1	Ndufb2	Ndufb3	Ndufb4	Ndufb5	Ndufb6	Ndufb7	Ndufb8	Ndufb9	Ndufb10	Ndufb11	Ndufb12	Ndufb13				

Succinate dehydrogenase / Fumarate reductase

E	SDHC	SDHD	SDHA	SDHB
B/A	SdhC	SdhD	SdhA	SdhB
	FrdA	FrdB	FrdC	FrdD

Cytochrome c reductase

E/B/A	ISP	Cyt b	Cyt 1							
E				COR1	QCR2	QCR6	QCR7	QCR8	QCR9	QCR10

Cytochrome c oxidase

E	COX10	COX3	COX1	COX2	COX4	COX5A	COX5B	COX6A	COX6B	COX6C	COX7A	COX7B	COX7C	COX8	E/B/A	COX11	COX15	COX17
B/A	CyoE	CyoD	CyoC	CyoB	CyoA	CoxD	CoxC	CoxA	CoxB	QoxD	QoxC	QoxA	QoxB	QoxA	SoxD	SoxC	SoxB	SoxA

Cytochrome c oxidase, cbb3-type

B	I	II	IV	III
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Cytochrome bd complex

B/A	CydA	CydB	CydX
-----	------	------	------

Cytochrome c

CYC

F-type ATPase (Bacteria)

alpha	beta	gamma	delta	epsilon
a	b	c		

F-type ATPase (Eukaryotes)

alpha	beta	gamma	delta	epsilon	
OSCP	a	b	c	d	e
f	g	f6/h	j	k	3

V/A-type ATPase (Bacteria, Archaea)

A	B	C	D	E	F	G/H
I	K					

V-type ATPase (Eukaryotes)

A	B	C	D	E	F	G	H
a	c	d	e	S1			

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Figure 3

Image origin: Kanehisa, M., & Goto, S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic acids research*, 28(1),27-30. This image was obtained with permission from KEGG.