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Blood metabolomics analysis identifies abnormalities in the citric acid cycle, urea cycle, and amino acid metabolism in bipolar disorder

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ABSTRACT

Background: Bipolar disorder (BD) is a severe and debilitating psychiatric disorder. However, the precise biological basis remains unknown, hampering the search for novel biomarkers. We performed a metabolomics analysis to discover novel peripheral biomarkers for BD.
Methods: We quantified serum levels of 116 metabolites in mood-stabilized male BD patients (n = 54) and age-matched male healthy controls (n = 39).
Results: After multivariate logistic regression, serum levels of pyruvate, *N*-acetylglutamic acid, α-ketoglutarate, and arginine were significantly higher in BD patients than in healthy controls. Conversely, serum levels of β-alanine, and serine were significantly lower in BD patients than in healthy controls. Chronic (4-weeks) administration of lithium or valproic acid to adult male rats did not alter serum levels of pyruvate, *N*-acetylglutamic acid, β-alanine, serine, or arginine, but lithium administration significantly increased serum levels of α-ketoglutarate.
Conclusions: The metabolomics analysis demonstrated altered serum levels of pyruvate, *N*-acetylglutamic acid, β-alanine, serine, and arginine in BD patients.
General significance: The present findings suggest that abnormalities in the citric acid cycle, urea cycle, and amino acid metabolism play a role in the pathogenesis of BD.
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1. Introduction

Bipolar disorder (BD) is a chronic mental illness characterized by recurrent episodes of depression, mania, and hypomania. Worldwide, the combined lifetime prevalence of BD I and II is estimated between 1 and 3% [1]. People with BD are more prone to seek care when they are depressed than when experiencing mania or hypomania. The condition might therefore mistakenly be diagnosed as major depression and BD patients are frequently treated with antidepressants in primary care [2]. This is a problem because monotherapy with antidepressants increase the risk of switch to mania in BD [3] and might sometimes worsen symptoms [4,5]. Treatment guidelines therefore recommend antidepressants only as adjunct therapy to mood stabilizers [6,7]. Misdiagnosis will thus prevent BD patients from receiving the correct medication, and risk worsening the outcome. Biomarkers for aiding the diagnosis of BD are therefore warranted.

Although the precise pathogenesis of BD is not well understood, accumulating evidence suggests that inflammation [8–10], mitochondrial dysfunction, and oxidative stress [11–19] play a role in the pathogenesis of BD. In addition, brain-derived neurotrophic factor (BDNF) and its precursor proBDNF, have been suggested as peripheral biomarkers for BD [17,20–25]. Recently, we also reported that serum levels of glutamine, glycine, and D-serine were significantly higher in BD patients than in healthy controls, whereas serum levels of L-serine were significantly lower in BD patients than in healthy controls [26]. However, we did not find alterations in these amino acids in cerebrospinal fluid (CSF) from the same BD patients [26]. The discrepancy between blood and CSF highlights the importance to measure a potential biomarker in

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both blood and CSF. Peripheral biomarkers are desirable since it is easy to collect blood samples.

Metabolomics is the profiling of small molecule metabolites and provides the potential to characterize specific metabolic phenotypes associated with a disease. Metabolomics has the advantage over other "omics" techniques in that it directly samples the metabolic changes in an organism and integrates information from changes at the gene, transcript, and protein level, as well as posttranslational modifications [27–29]. Metabolomics analysis with capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) [30] has been used in the discovery of biomarkers for psychiatric disorders, including BD [31], schizophrenia [32], and autism spectrum disorder (ASD) [33]. We recently found that CSF levels of isocitrate were significantly higher in BD patients than in healthy controls, and that the expression of isocitrate dehydrogenase (IDH3A) mRNA and protein were significantly lower in postmortem brain tissue from BD patients than in control samples [31]. These findings suggested that abnormal mitochondrial metabolism of isocitrate by IDH3A plays a key role in the pathogenesis of BD [31]. However, there are no corresponding metabolomics analyses of serum from BD patients and healthy controls.

Here, we performed a metabolomics analysis using CE-TOFMS of serum samples from mood-stabilized BD patients (n = 54) and age-matched healthy controls (n = 39) [31]. We selected 116 major metabolic compounds from the following pathways for metabolomics analysis: the glycolytic system, the pentose phosphate pathway, the citric acid cycle, the urea cycle, the polyamine-creatine metabolism pathway, the purine metabolism pathway, the glutathione metabolism pathway, the nicotinamide metabolism pathway, the choline metabolism pathway and several amino acid metabolism pathways (Supplemental Table 1). To examine potential effects of medication on the studied metabolites, we also performed a metabolomics analysis of serum samples from rats chronically treated with lithium (Li) or valproic acid (VPA).

2. Methods and Materials

2.1. Participants

The BD patients were recruited from the St. Göran bipolar project, enrolling patients from the bipolar unit at the Northern Stockholm Psychiatric Clinic, Stockholm, Sweden. The work-up and diagnostic assessments have been described in detail previously [26,34,35]. The key clinical assessment instrument was the Affective Disorder Evaluation (ADE), developed for the Systematic Treatment Enhancement Program of Bipolar Disorder (STEP-BD) [36]. The full diagnostic assessment was based on all available sources of information including patient interview, case records, and if possible interviews with the next of kin. To reduce inter-rater bias, a best-estimate diagnostic decision based on all information available at admission was made at a diagnostic case-conference by a consensus panel of experienced board certified psychiatrists (n = 2–5) specialized in BD.

The general criteria for inclusion were: 1) age of at least 18 years and 2) meeting the Diagnostic and Statistical Manual (DSM)-IV criteria for bipolar spectrum disorder (i.e., type I, type II, or not otherwise specified). Information regarding age, sex, number of lifetime manic/hypomanic/depressive/total episodes, duration of illness (defined as years since the first hypomanic or manic episode), body mass index (BMI), and previous psychotic episodes was collected. The severity of BD was rated using the Clinical Global Impression (CGI) and Global Assessment of Functioning (GAF) rating scales. For ethical reasons, patients continued to take their prescribed medications at the time of serum sampling.

Population-based controls were randomly selected by Statistics Sweden (SCB) and contacted by mail. Given an expected response rate of 1:7, seven invitations were sent out per enrolled case. Fourteen percent of the invited controls responded to the invitation, and were subjected to a preliminary telephone screening by a research nurse to exclude severe mental health conditions, neurological diseases, and substance abuse. Eligible persons were scheduled for a one-day comprehensive assessment where they underwent further psychiatric interviewing by experienced clinicians using the Mini-International Neuropsychiatric Interview (M.I.N.I.) to exclude psychiatric disorders [37]. Substance abuse was screened for at the telephone interview by the nurse, in the psychiatric interview, by the Alcohol Use Disorders Identification Test (AUDIT), and by the Drug Use Disorders Identification Test (DUDIT), as well as by determining serum levels of carbohydrate-deficient transferrin (CDT) [38]. Overconsumption of alcohol as revealed by CDT or responses indicating large consumption (>8 standard drinks per time more than 2 times per week), and/or amnesia and/or loss of control more than once per month resulted in the exclusion of these individuals from the study. Other exclusion criteria were neurological conditions other than mild migraines, untreated endocrinological disorders, pregnancy, dementia, recurrent depressive disorder, and suspected severe personality disorders (based on interview and the Structured Clinical Interview for DSM (SCID-II) screen personality assessment), as well as a family history of schizophrenia or BD in first-degree relatives.

The study was approved by the Regional Ethics Committee in Stockholm and conducted in accordance with the latest Helsinki Protocol. All patients and controls consented orally and in writing to participate in the study. Informed consent was obtained during a euthymic period (i.e., during a time period when patients did not meet criteria for a depressive, hypomanic, or manic episode). All patients were capable of freely giving fully informed consent, as determined by the physicians who enrolled the patient. A total of 54 male BD patients and 39 male healthy controls were included (Table 1).

2.2. Serum sampling

Serum samples were collected from fasting subjects between 8:00 and 9:00 am. The samples were centrifuged on site and stored at –80 °C pending analysis at the Biobank at Karolinska Institutet, Stockholm, Sweden. An identical procedure was performed for the controls. The samples were stored at –80 °C until delivered by courier mail, frozen on dry ice, to Chiba University (Japan) for metabolomics analysis. This study was also approved by the Research Ethics Committee of the Graduate School of Medicine (Chiba University).

2.3. Metabolomics analysis of human serum samples

Metabolomics analyses of serum samples from healthy controls and BD patients were performed using the CE-TOFMS at Human Metabolome Technologies (Yamagata, Japan). The sampled volume of 50 μL was added to 450 μL methanol containing internal standards, and mixed. Then, 450 μL chloroform and 200 μL Milli-Q water was added to the mixture. The mixture was centrifuged at 2,300 ×*g* and 4 °C for 5 min. Subsequently, 800 μL of upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cutoff filter at 9100 ×g and 4 °C for 120 min to remove proteins. The filtrate was centrifugally concentrated and re-suspended in 25 μL of Milli-Q water for analysis.

Cationic compounds were measured in the positive mode of CE-TOFMS (Agilent CE-TOFMS system Machine No. 3, Fused silica capillary, i.d. 50 μM × 80 cm), and anionic compounds were measured in the positive and negative modes of CE-MS/MS (Agilent CE system and Agilent 6400 TripleQuad LC/MS Machine No. QqQ01, Fused silica capillary, i.d. 50 μM × 80 cm), as reported previously [39, 40]. Peaks detected by CE-TOFMS and CE–MS/MS were extracted using automatic integration software (MasterHands, Keio University, Tsuruoka, Japan) [41] and MassHunter Quantitative Analysis B.04.00, Agilent Technologies, Santa Clara, CA, USA) in order to obtain peak information including m/z, migration time (MT), and peak area. The peaks were annotated with putative metabolites from the HMT metabolite database based on their MTs in CE and m/z values determined by TOFMS. The tolerance range for the peak annotation was configured at ±0.5 min for MT and ±10 ppm for

Table 1
Characteristics of the participants.

	Controls		BD Patients	
Number (male)	39[a]		54	
	Median	IQR	Median	IQR
Age (years)	36	29–47	41	32–52
BMI	24.1	22.8–25.8	25.7	24.1–28.1
Diagnosis			N	%
Bipolar disorder type I (BP I)			31	57.4
Bipolar disorder type II(BP II)			17	31.5
Not otherwise specified (NOS)			6	11.1
Clinical data			Median	IQR
Age first symptoms			20	14–25
Depressive episodes[c]			6	3–10
Hypomanic episodes[b]			2	0–5
Manic episodes[b]			1	0–2
Mixed episodes[b]			0	0–25
GAF[b]			70	60–80
MADRS[f]			4	0–11
YMRS[g]			1	0–2
No of episodes[b]			18	1–80
Audit total score[e]			8	2–11
Dudit total score[d]			0	0–0
			N	%
Psychosis episodes[b]			25	46.3
Family history of bipolar[a]			26	49.1
Family history of unipolar[a]			30	56.6
Alcohol dependence[c]			14	26.9
Alcohol abuse[c]			13	25.0
Substance abuse[c]			8	15.4
Medication			N	%
Mood stabilizer			44	81.5
Lithium (Li)			34	63.0
Anticonvulsants			19	35.2
Valproate (VPA)			7	13.0
Lamotrigine			12	22.2
Antidepressants			20	37.0
Anxiolytics			11	20.4
Antipsychotics			16	29.6

IQR: Interquartile Range.
GAF: Global Assessment of Functioning.
MADRS: Montgomery–Åsberg Depression Rating Scale.
YMRS: Young Mania Rating Scale.
[a] Missing data for 1 individual in the control group.
[b] Missing data for 1 individual in the patient group.
[c] Missing data for 2 individual in the patient group.
[d] Missing data for 6 individual in the patient group.
[e] Missing data for 9 individual in the patient group.
[f] Missing data for 10 individual in the patient group.
[g] Missing data for 11 individual in the patient group.

m/z. In addition, concentrations of metabolites were calculated by normalizing the peak area of each metabolite with respect to the area of the internal standard and by using standard curves, which were obtained by three-point calibrations. A total of 116 major metabolic substances on various pathways (glycolytic system, pentose phosphate pathway, citric acid cycle, urea cycle, polyamine–creatine metabolism pathway, purine metabolism pathway, glutathione metabolism pathway, nicotinamide metabolism pathway, choline metabolism pathway and diverse amino acid metabolism pathway) were selected for metabolomics analysis (Supplemental Table 1).

2.4. Statistical analyses

Data from human samples are presented as mean ± standard deviation (SD). Statistical analyses were performed using SAS software version 9.3 (SAS Institute, Cary, NC). Analyses of metabolites between control and BD groups were performed using unpaired *t*-tests (Table 1) and Wilcoxon rank sum tests (data not shown). In addition we also performed multiple logistic and linear regression models with a stepwise selection method [42,43]. Internal validation of the logistic regression models was performed using the Hosmer–Lemeshow goodness-of-fit test, in which a p-value greater than 0.1 indicates a good fit [44], and the performance was evaluated by the jack-knife method (a performance evaluation method in which a measured value predicted from the n − 1 observations, removing the own predicted observation) [45]. False discovery rate was used to control for multiple comparisons as indicated in the results [46] and $P < 0.05$ was considered statistically significant. Data from rat experiments are presented as mean ± SD. To determine the effects of drug treatment, a one-way ANOVA, followed by the *post hoc* Dunnett's test was used. P-values of less than 0.05 for two-tailed tests were considered statistically significant.

3. Results

3.1. Metabolomics of serum samples from BD patients and controls

As we previously reported [31], there were now differences between the 39 healthy controls and the 54 BD patients in regard to age and BMI. Out of the 116 major metabolic substances measured, 82 were detected in serum, while the remaining 34 were under the detection limit (Table 2). To select metabolites showing significant differences between BD patients and healthy controls, we first performed both unpaired *t*-tests and Wilcoxon rank sum tests between BD patients and healthy controls. Eighteen compounds, including uric acid, CoA, glycerol 3-phosphate, pyruvic acid (pyruvate), *N*-acetylglutamic acid (*N*-acetylglutamate), 2-hydroxyglutaric acid, 2-oxoglutaric acid (α-ketoglutarate), citric acid (citrate), cis-aconitic acid (cis-aconitate), isocitric acid (isocitrate), urea, β-alanine, serine, valine, threonine, ornithine, glutamine, and arginine were altered (Table 2). These metabolites are molecules in the citric acid cycle, the urea cycle, and the amino acid metabolism (Fig. 1).

A multiple logistic regression analysis was performed to evaluate the association between these 18 metabolites and BD. A stepwise selection-elimination method was used, and the significance level was set at 5%. Six parameters – pyruvate, *N*-acetylglutamate, α-ketoglutarate, β-alanine, serine, and arginine – were independently associated with BD (Supplemental Table 2, and Table 3; see footnote for the resulting equation). In this model, after cross-validation testing using the jack-knife procedure, the model's sensitivity was 85.2% and the specificity was 76.9%. The Hosmer–Lemeshow goodness-of-fit statistic (the internal validation of the logistic regression model) was 3.61 with 8 DF ($P = 0.89$), indicating a good fit of the model (Fig. 2).

To investigate whether these six substances were associated with clinical features or medication in BD patients, we performed additional multiple linear regression analyses. The independent variables were: age at first symptoms, GAF score, total number of mood episodes (depressive, hypomanic, manic and mixed), MADRS score, YMRS score, AUDIT total score, DUDIT total score, psychotic episodes, family history (e.g., bipolar and unipolar), alcohol dependence, alcohol use, substance abuse, and medication (mood stabilizer, Li, anticonvulsant, VPA, lamotrigine, antidepressant, anxiolytic, and antipsychotic). Results demonstrated that a history of a manic episode ($P = 0.009$) and YMRS ($P = 0.013$) were positively correlated with pyruvic acid. The number of depressive episodes ($P = 0.012$) and Li medication ($P = 0.002$) were positively related with *N*-acetylglutamic acid, whereas alcohol dependence ($P = 0.002$) was negatively correlated with β-alanine. Li medication ($P = 0.004$) was positively correlated with serine, while anxiolytic medication ($P = 0.001$) and antipsychotic medication ($P = 0.011$) were correlated with arginine. No variables were related with α-ketoglutarate (Supplemental Table S3 and S4).

3.2. Effects of Li and VPA on rat serum levels

To examine the effects of medication on the metabolites, we performed metabolomics analyses of serum samples from rats treated with chronic (4-weeks) Li or VPA. We measured the serum

Table 2
Metabolomics data of human serum samples.

Compound name	Concentration (μM)						Comparative analysis
	Controls			BD patients			P value
	Mean	S.D.	N	Mean	S.D.	N	
Nicotinamide adenine dinucleotide (NAD^+)	1.0	0.06	38	1.0	0.04	51	0.703
Cyclic AMP (cAMP)	0.008	0.007	38	0.007	0.004	52	0.871
Cyclic GMP (cGMP)	0.013	0.014	8	0.007	0.004	16	0.254
Xanthine	3.5	1.1	39	3.8	1.2	54	0.271
Mevalonic acid	0.03	0.02	8	0.05	0.05	18	0.063
UDP-glucose	0.4	0.03	24	0.4	0.014	21	0.445
Uric acid	339	46	39	368	66	54	0.014*
IMP	0.09	0.010	21	0.08	0.005	26	0.401
Oxidised nicotinamide adenine dinucleotide phosphate ($NADP^+$)	0.10	0.015	11	0.10	0.004	16	0.396
Glucose 6-phosphate	0.2	0.05	39	0.2	0.06	53	0.321
Fructose 6-phosphate	0.05	0.02	37	0.06	0.02	45	0.421
Ribose 5-phosphate	0.03	N.A.	1	N.D.	N.A.	0	N.A.
Acetoacetyl CoA	0.04	9.7E−04	5	0.04	1.2E−04	2	0.353
Acetyl CoA	0.02	0.03	3	0.002	0.003	3	0.450
Folic acid	N.D.	N.A.	0	0.03	6.0E−04	2	N.A.
CoA	0.3	0.002	2	0.3	0.004	5	0.049*
Ribose 1-phosphate	0.3	0.15	36	0.3	0.14	51	0.899
Ribulose 5-phosphate	0.10	0.02	14	0.11	0.013	16	0.302
Erythrose 4-phosphate	0.011	N.A.	1	0.011	N.A.	1	N.A.
Glyceraldehyde 3-phosphate	N.D.	N.A.	0	0.09	0.07	3	N.A.
Reduced nicotinamide adenine dinucleotide phosphate (NADPH)	0.7	0.008	2	0.7	0.02	7	0.686
Phosphocreatine	0.06	N.A.	1	N.D.	N.A.	0	N.A.
Adenylosuccinic acid	0.05	0.005	8	0.05	0.002	13	0.554
Fructose 1,6-diphosphate	0.2	0.05	2	0.13	0.02	3	0.598
6-Phosphogluconic acid	0.3	N.A.	1	0.2	N.A.	1	N.A.
N-carbamoylaspartic acid	0.08	0.04	39	0.10	0.04	54	0.170
PRPP	N.D.	N.A.	0	0.03	1.3E−03	2	N.A.
2-Phosphoglyceric acid	0.03	0.03	6	0.02	0.02	9	0.958
3-Phosphoglyceric acid	0.2	0.08	39	0.2	0.11	53	0.471
2-Oxoisovaleric acid	14	2.5	39	14	2.8	54	0.507
GDP	0.3	0.03	11	0.3	0.010	18	0.860
Lactic acid	2,173	592	39	2,061	489	54	0.321
ADP	0.6	0.05	15	0.6	0.02	18	0.348
GTP	2.3	0.005	2	2.3	0.08	6	0.917
ATP	5.0	0.15	5	4.9	0.2	8	0.513
Glycerol 3-phosphate	1.8	0.5	39	1.6	0.5	54	0.074
Glycolic acid	8.2	1.6	39	8.4	1.8	51	0.554
Pyruvic acid	35	23	39	48	24	54	0.009**
N-acetylglutamic acid	0.2	0.03	37	0.3	0.05	54	0.002**
2-Hydroxyglutaric acid	0.6	0.2	39	0.7	0.3	54	0.046*
Succinic acid	6.2	1.0	39	6.1	1.1	54	0.954
Malic acid	N.D.	N.A.	0	1.9	2.1	2	N.A.
2-Oxoglutaric acid	4.0	2.2	39	8.7	5.5	54	<.0001***
Citric acid	92	21	39	73	20	54	<.0001***
cis-Aconitic acid	1.2	0.3	39	1.0	0.3	54	0.012*
Isocitric acid	4.2	1.1	39	3.8	1.2	54	0.057
Urea	5,623	1,145	39	4,955	1,039	54	0.004**
Glycine (Gly)	360	50	39	359	54	54	0.947
Alanine (Ala)	449	79	39	475	76	54	0.121
Sarcosine	1.5	1.0	38	1.4	1.0	51	0.990
β-alanine (β-ala)	3.7	1.2	39	2.7	0.8	54	<.0001***
γ-Aminobutyric acid	N.D.	N.A.	0	0.2	0.07	6	N.A.
N,*N*-Dimethylglycine	3.5	1.7	39	3.5	2.0	54	0.998
Choline	13	4.2	39	12	3.8	54	0.366
Serine (Ser)	230	28	39	209	32	54	0.002**
Creatinine	84	9.7	39	85	13	54	0.884
Proline (Pro)	268	89	39	278	121	54	0.653
Valine (Val)	340	40	39	324	45	54	0.084
Betaine	50	13	39	48	16	54	0.526
Threonine (Thr)	163	24	39	150	27	54	0.020*
Hydroxyproline	16	7.3	39	17	8.9	54	0.562
Creatine	31	16	39	34	13	54	0.265
Isoleucine (Ile)	82	12	39	83	17	54	0.760
Leucine (Leu)	187	20	39	184	25	54	0.424
Asparagine (Asn)	55	5.7	39	54	7.8	54	0.252
Ornithine	79	19	39	71	16	54	0.019*
Asparagic acid (Asp)	37	7.5	39	36	7.9	54	0.690
Hypoxanthine	10	3.0	39	10	2.8	54	0.966
Glutamine (Gln)	723	63	39	701	98	54	0.194
Lysine (Lys)	225	33	39	223	31	54	0.729
Glutamic acid (Glu)	103	27	39	122	40	54	0.006**
Methionine (Met)	24	3.0	39	23	3.8	54	0.593

Table 2 (*continued*)

Compound name	Concentration (μM)						Comparative analysis
	Controls			BD patients			P value
	Mean	S.D.	N	Mean	S.D.	N	
Histidine (His)	97	10	39	95	11	54	0.301
Carnitine	59	12	39	62	10	54	0.237
Phenylalanine (Phe)	87	10	39	87	13	54	0.990
Arginine (Arg)	122	19	39	131	23	54	0.039*
Citrulline	37	6.4	39	38	7.1	54	0.738
Thyosine (Tyr)	65	12	39	68	17	54	0.239
Triptophane (Trp)	72	9.3	39	71	14	54	0.737
Cystathionine	1.1	0.2	3	0.8	0.13	2	0.216
Inosine	3.2	1.2	11	4.5	2.2	13	0.087
Guanosine	N.D.	N.A.	0	1.4	0.3	5	N.A.

N.D.: Not Detected.
N.A.: Not Available.
Unpaired t-test or Wilcoxon rank sum t-test (*P < 0.05, **P < 0.01, ***P < 0.001)
P < 0.1

concentrations of the same 116 major metabolites of which 73 metabolites were detected in the rat serum. Results showed that treatment with Li significantly increased serum levels of 2-hydroxyglutaric acid, malic acid (malate), α-ketoglutarate, fumaric acid (fumarate), citrate, and betaine. Treatment with VPA significantly altered serum levels of phosphocreatine, glycine, *N*,*N*-dimethylglycine, creatine, threonine, glutamine, and tryptophan (Supplemental Table 5). Thus, serum levels of pyruvate, *N*-acetylglutamic acid, α-ketoglutarate, β-alanine, serine, and arginine in rats were not altered by chronic treatment with Li or VPA (Supplemental Table 5).

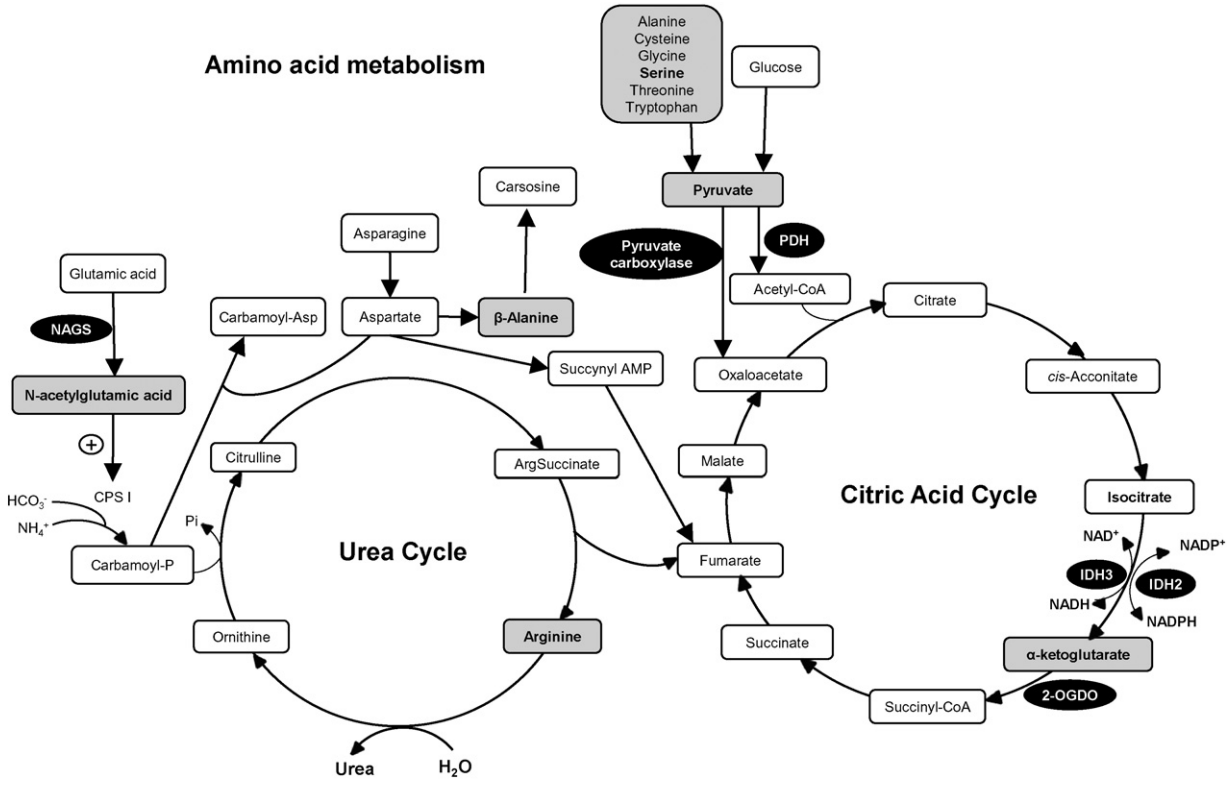

Fig. 1. Metabolic pathway of the citric acid cycle, urea cycle, and amino acid metabolism Amino acid metabolism is biochemical processes by which the various amino acids are produced from other substances. Pyruvic acid (pyruvate) is prepared from glucose through glycolysis. Pyruvate is metabolized to oxaloacetate by pyruvate carboxylase. Acetyl-CoA is also from the breakdown of sugars by glycolysis that yield pyruvate that in turn is decarboxylated by the enzyme pyruvate dehydrogenase (PDH). The citric acid cycle (also known as the tricarboxylic acid (TCA) cycle or the Krebs cycle) begins with the transfer of a two-carbon acetyl group from acetyl CoA to the four-carbon acceptor substance oxaloacetate to form six-carbon substance (citrate). In the citric acid cycle, α-ketoglutarate is synthesized from isocitric acid (isocitrate) by isocitrate dehydrogenase (IDH2/3), and is metabolized to succinyl-CoA by 2-oxoglutarate-dependent dioxygenase (2-OGDO). *N*-acetylglutamic acid is synthesized from glutamic acid and acetyl-CoA by *N*-acetylglutamate synthase (NAGS). Arginine is synthesized from citrulline in the urea cycle, and plays a role in the production of nitric oxide (NO). β-alanine is synthesized from aspartate by aspartate 1-decarboxylase, and is also the rate-limiting precursor of carnosine. Substances in the dark box are significantly altered in the serum from bipolar disorder (BD) patients.

Table 3
Independent predictor in serum samples of BD patients by logistic regression.

Parameter	Odds ratio (95% confidence interval)	P value
Pyruvic acid	0.940 (0.896–0.987)	0.0128
N-acetylglutamic acid	>999.999 (>999.999–>999.999)	0.0042
α-Ketoglutarate	1.683 (1.218–2.324)	0.0016
β-alanine	0.264 (0.109–0.638)	0.0031
Serine	0.947 (0.918–0.977)	0.0007
Arginine	1.045 (1.009–1.082)	0.0149

BD: Bipolar disorder
Logistic function, $P = 1/\{1 + \exp(-1.0826 + 0.0614X_1 - 40.1927X_2 - 0.5203X_3 + 1.3329X_4 + 0.0544X_5 - 0.0436X_6)\}$.
P: Probability of being statistically-discriminated as BD
X1, Pyruvic acid; X2, N-acetylglutamic acid; X3, α-Ketoglutarate; X4, β-alanine; X5, Serine; X6, Arginine

4. Discussion

This is the first study showing increased serum levels of pyruvate, *N*-acetylglutamic acid, α-ketoglutarate, and arginine, as well as decreased serum levels of β-alanine in BD patients compared with controls. Chronic (4-weeks) treatment of Li or VPA did not alter serum levels of pyruvate, *N*-acetylglutamic acid, α-ketoglutarate, β-alanine, serine, or arginine in rats, suggesting that the mood stabilizers Li or VPA do not affect the synthesis and metabolism of these substances in the blood. Thus, it is unlikely that the use of these mood stabilizers explain altered serum levels of these metabolites in BD patients, although some metabolites were associated with clinical variables and medications.

Accumulating evidence supports that mitochondrial dysfunction plays a key role in the pathogenesis of BD [11–18]. Pyruvate, the end-product of glycolysis, is derived from additional sources in the cellular cytoplasm (Fig. 1), and is ultimately destined for transport into mitochondria as a master fuel input undergirding the citric acid cycle carbon flux [47]. In addition, pyruvate is critical for mitochondrial ATP generation and for driving several major biosynthetic pathways intersecting the citric acid cycle [47]. Pyruvate is also converted to acetyl-CoA by pyruvate dehydrogenase complex (PDH), and to oxaloacetate by pyruvate carboxylase (Fig. 1). In this study, serum levels of pyruvate in BD patients were significantly higher than those of healthy controls, while serum levels of acetyl-CoA and oxaloacetate were not altered in BD patients. We recently found that CSF levels of pyruvate were higher in BD patients than controls, even though the logistic regression analysis did not reach to statistical significance [Table S2 in 31]. Although the reasons underlying increased pyruvate levels in BD patients are currently unknown, it is likely that increased pyruvate levels plays a role in the pathogenesis of BD. Since pyruvate supplies energy to living cells through the citric acid cycle, abnormality in the citric acid cycle in the mitochondria might play a role in the pathogenesis of BD. It was recently reported that pyruvate was increased in serum and CSF from patients with ASD, suggesting a possible mitochondrial dysfunction also in ASD [48]. The precise mechanisms underlying pyruvate pathway alterations in psychiatric disorders, including BD, needs to be further explored.

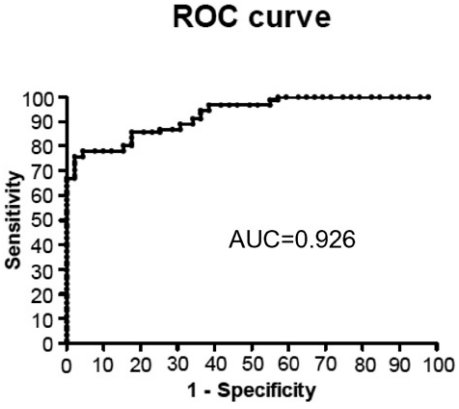

Fig. 2. A receiver-operating characteristics (ROC) curve and area under the curve (AUC) In this model, after cross-validation testing using the jack-knife procedure, the model's sensitivity was 85.2% and specificity was 76.9%. The Hosmer–Lemeshow goodness-of-fit statistic (the internal validation of the logistic regression model) was 3.6123 with 8 DF (P = 0.8903), indicating a good fit of the model.

BD patients had higher serum levels of α-ketoglutarate than controls. α-Ketoglutarate (2-oxoglutarate) is a key metabolite in the citric acid cycle, but also an obligatory substrate for 2-oxoglutarate-dependent dioxygenases (2-OGDO) (Fig. 1). The 2-OGDO enzyme family includes the major enzymes of DNA and histone methylation [49]. It is possible that altered α-ketoglutarate levels in BD lead to epigenetic changes. Epigenetic modifications have been suggested to play an important role in the pathogenesis of a number of psychiatric disorders including BD [50,51]. However, serum levels of α-ketoglutarate in rats were significantly increased after chronic Li (but not VPA) treatment. It is therefore possible that Li medication may affect serum levels of α-ketoglutarate in BD patients despite that Li medication was not associated with serum levels of α-ketoglutarate in BD patients.

Recently, we reported that CSF levels of isocitrate were significantly higher in BD patients than in healthy control, and that the expression of the isocitrate dehydrogenase (IDH) subtype IDH3A was lower in BD postmortem brain samples than in control samples [31]. These findings supported the mitochondrial dysfunction hypothesis in BD. However, we did not find alteration in the serum levels of isocitrate in the same BD patients (Table 2). Thus, these changes might be specific for the central nervous system and not discernible in peripheral samples.

N-acetylglutamate is biosynthesized from glutamic acid (glutamate) and acetyl-CoA by the enzyme *N*-acetylglutamate synthase (NAGS)(Fig. 1). Mammalian NAGS is found primarily in the mitochondrial matrix of cells of the liver and intestines. *N*-acetylglutamate is also known to activate carbamoyl phosphate synthetase (CPSI) in the mitochondrial matrix of urea cycle (Fig. 1)[52,53]. In this study, we found increased serum levels of *N*-acetylglutamate in BD patients. Given the crucial role of *N*-acetylglutamate in the mitochondrial matrix of cells, it is likely that an alteration in the *N*-acetylglutamate levels may cause mitochondrial dysfunction, supporting a mitochondrial dysfunction hypothesis in BD. In CSF, however, we have previously found that *N*-acetylglutamate is under the detection limit [31]. Therefore, it is unknown whether *N*-acetylglutamate levels in the brain are altered in BD patients.

Arginine is a conditionally essential amino acid for adult mammals. This amino acid is not only metabolically interconvertible with the amino acids proline and glutamate, but also it serves as a precursor for the biosynthesis of proteins, creatine, polyamine, nitric oxide (NO), agmatine, and urea. Arginine is mainly metabolized by the enzymes, arginase and NO synthase (NOS) to produce urea and L-ornithine, and NO and citrulline, respectively (Fig. 1)[54,55]. In this study, we found that serum levels of arginine in BD patients were significantly higher than in healthy controls, suggesting an abnormality in the urea cycle (or arginine metabolism) in BD. We also found that serum levels of arginine were associated with anxiolytic and antipsychotic medications. By contrast, we previously found that CSF levels of arginine did not differ between BD patients and healthy controls [31]. One previous study showed that NOS activity in the platelets from BD patients (n = 28) was significantly lower than that of healthy controls (n = 10), suggesting an impaired NO generation in BD patients [56]. A meta-analysis showed that NO levels were altered in patients with BD [57]. Taken together, the arginine-NO pathway may play a role in the pathogenesis of BD [58].

In the present study, we found that serum levels of total serine (L-serine and D-serine) were significantly lower in BD patients than in controls, which is consistent with a recent paper of ours [26]. Our metabolomics analysis did not distinguish the two enantiomers of serine, although moderate levels of D-serine are known to exist in the human blood [59,60]. Whereas serum levels of D-serine were higher in BD

patients, serum levels of L-serine were lower in BD patients than controls [26], thus suggesting an aberrant serine metabolism in BD patients. Interestingly, we reported the opposite pattern in schizophrenia, with increased L-serine and decreased D-serine serum levels [59,60]. The latter findings suggest that hypofunctioning NMDA receptors in schizophrenia may be due to decreased D-serine levels [61,62]. Serum level of total serine, including both L-serine and D-serine, significantly decreased in BD patients (this study) although both enantiomers of serine in depressed patients were higher than those on healthy subjects [63]. Considering the different roles of NMDA receptor neurotransmission in the pathogenesis of schizophrenia and BD, measurements of serine enantiomers (D- and L-serine) may represent diagnostic peripheral biomarkers for these disorders [64]. Serine hydroxymethyltransferase 1 is an enzyme in the reversible interconversion between glycine and L-serine. L-serine is also synthesized from 3-phosphoglycerate by 3-phosphoglycerate dehydrogenase, and D-serine is synthesized from L-serine by serine racemase and is metabolized by D-amino acid oxidase [62]. Taken together, further studies on the role of biosynthesis and metabolism of serine in BD are warranted.

This is the first report showing decreased levels of β-alanine in BD patients. A previous study found that CSF levels of β-alanine were not altered in BD [31]. β-alanine is a naturally occurring non-essential amino acid. β-alanine can be released during the breakdown of histidine dipeptide, such as carnosine or anserine. Or it can be formed as a secondary byproduct of a reaction that converts L-alanine to pyruvate. In addition, β-alanine can be produced during digestion, when intestinal microbes remove a carbon atom from L-aspartate, releasing both β-alanine and carbon dioxide (Fig. 1). The precise mechanisms underlying the role of β-alanine synthesis/metabolism in the pathogenesis of BD are currently unknown. Supplementation of β-alanine has been widely used as the nutrition in sports [65,66]. Speculatively, it would be interesting to examine whether β-alanine supplementation can affect symptoms in BD patients.

There are some limitations to this study. First, only male subjects were enrolled in this study. There are some reports showing gender differences in the treatment of BD patients [67]. It remains to be examined whether serum levels of these metabolites are also altered in female BD patients. Second, it cannot be excluded that long-term medication with mood stabilizers, antidepressants, and antipsychotics may affect serum levels of metabolites even though a 4-week treatment with Li or VPA did not alter serum levels of metabolites (except α-ketoglutarate) in rats. Therefore, further studies using a larger sample size of medication-free patients will be needed.

5. Conclusion

The metabolomics analyses showed alterations in the serum levels of pyruvate, *N*-acetylglutamic acid, β-alanine, serine and arginine in BD patients, suggesting that abnormalities in the citric acid cycle, the urea cycle, and the amino acid metabolism may play a role in the pathogenesis of BD. It is possible that these metabolites could serve as peripheral biomarkers for BD, although further studies using a larger sample size are needed to confirm the feasibility.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbacli.2016.03.008.

Transparency document

The Transparency document associated with this article can be found, in online version.

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