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ARTICLE *in* ANALYTICAL CHEMISTRY · OCTOBER 2009

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Anal. Chem. **2009,** *81,* 9755–9763

Metabolic Profiling of Human Blood Serum from Treated Patients with Bipolar Disorder Employing ^{1}H NMR Spectroscopy and Chemometrics

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Metabolic profiling employing hydrogen nuclear magnetic resonance (^{1}H NMR) spectroscopy and chemometric analysis of human blood serum samples taken from the control group ($n = 25$) and patients with bipolar disorder ($n = 25$) was performed to identify molecular changes related to the disorder and to different drug treatments: lithium ($n = 15$) versus other medications ($n = 10$). This strategy showed significant potential for exploring pathophysiological and toxicological features involved in bipolar disorder. The investigated groups (control and patients with bipolar disorder under different treatments) could be distinguished according to their metabolic profiles, and the main differential metabolites found were lipids, lipid-metabolism-related molecules (acetate, choline, and *myo*-inositol), and some key amino acids (glutamate, glutamine). Our results suggest that some of the 24 identified metabolites may be linked to lithium- and other-medication-provoked metabolic changes or may even be directly related to the disorder. Thus, these findings may contribute to paving the way for future studies aiming at identifying potential biomarkers for bipolar disorder.

Bipolar disorder, formerly known as manic-depressive psychosis, is one of the most debilitating and common psychiatric disorders worldwide. It is characterized by recurrent mood disturbances that comprise periods of depression (abnormally depressed mood, loss of interest or pleasure in activities that usually are pleasurable, decreased energy or increased fatigability, among others), mania (marked elevated mood, increased energy and activity during at least 1 week), hypomania (elevated mood, increased energy and activity during at least 4 days), and mixed states (symptoms of both mania and depression). The absence of depressive or manic episodes is called euthymia (normal mood).[1] Bipolar disorder is further categorized into subtypes that include bipolar I (one or more episodes of mania with or without major depressive episodes) and bipolar II (one or more episodes of hypomania as well as at least one major depressive episode).[2]

Diagnosing bipolar disorder can be challenging sometimes, due to the heterogeneity of the clinical presentation, the unclear boundaries with other mental disorders (hence, a skilled differential diagnosis is required), and, last but not least, a late occurrence of the first episode of mania/hypomania, after recurrent episodes of depression.[3] It is still not known what causes bipolar disorder, although a variety of biochemical, genetic, and environmental factors seem to be involved in both causing and triggering bipolar episodes. As of now, there is no independent test to confirm the disorder; diagnosis still relies on clinical expertise and judgment. Very often the individuals with bipolar disorder are misdiagnosed as unipolar depressive (because the first hypomanic or manic episode may only come later on, after one or several depressive episodes), leading to inadequate treatments and outcome. Not to mention that treatment with antidepressant drugs for patients with bipolar depression may provoke a switch into hypomania or mania. Therefore, increased accuracy in diagnosing bipolar disorder is the key to improving the mental health and treatment of patients with the disorder, which could possibly be attained by identifying differential biomolecules that reflect pathophysiologic processes in the presence of the illness.[4−6]

In this work, a metabonomics study employing ^{1}H NMR and chemometrics was performed to detect molecular changes in

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10.1021/ac901502j CCC: $40.75
Published on Web 10/28/2009

human blood serum samples by comparing the metabolic profiles of healthy subjects, patients being treated for bipolar disorder with lithium,[6−8] and patients being treated for bipolar disorder with other medications not including lithium. The medications routinely used in bipolar disorder treatment include mood stabilizers (lithium, valproic acid, carbamazepine), second-generation antipsychotics (olanzapine, risperidone, quetiapine), antidepressants (especially—and carefully—selective serotonin reuptake inhibitor drugs—the SSRIs—such as fluoxetine, paroxetine, sertraline, and citalopram), and anxiolytics (clonazepam, diazepam).[1] Although lithium is the most widely used drug in many cases for bipolar disorder treatment (both to treat current episodes and to prevent further ones), the precise neurobiological mechanisms through which lithium exerts its clinical effects are not clear, and some results found in the literature are contradictory.[5,8,9] Therefore, two groups of bipolar disorder patients (treated with lithium or not) were studied to evaluate lithium effects on blood serum metabonomics.

Metabonomics is defined as the measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification. With metabonomics, changes in endogenous metabolite levels that may result from disease processes, drug toxicity, or gene function have been evaluated in cells, tissues, or biological fluids.[10−16] Latent biochemical information obtained from metabonomics may be used for diagnostic or prognostic purposes. Such information reflects actual biological events rather than the potential for disease which gene expression data provide.[17] Different analytical platforms, based on mass spectrometry or NMR spectroscopy, are currently used for such studies. While mass spectrometry is more sensitive and specific in comparison to NMR, it relies on the separation of the analytes prior to detection using GC, HPLC, or CE. In this sense, NMR spectroscopy is more closely a universal detector in that the sample can be analyzed directly and many kinds of small metabolites can be measured at the same time.[12,14,16]

Hydrogen nuclear magnetic resonance (^{1}H NMR) spectroscopy already enabled a large number of biofluid constituents to be identified and catalogued.[10−12] ^{1}H NMR has an exceptional reproducibility and is quantitative to the extent that a given peak area is directly proportional to the concentration of the corresponding metabolite, which has allowed it to become a well-established technique used in metabonomics studies.[13] Various alterations in the metabolite levels present in the brain of patients with psychiatric disorders using NMR spectroscopy have been reported.[18] For bipolar disorder investigations, in vivo hydrogen nuclear magnetic resonance spectroscopy (^{1}H MRS),[19] hydrogen magnetic resonance imaging (^{1}H MRSI),[20] and ^{1}H NMR spectroscopy-based metabonomics have been applied.[21]

In this rather exploratory work, metabolic profiling has been performed for searching molecular changes in human blood serum related to bipolar disorder and the lithium treatment (the treatment that has the longest record of efficacy[6−8]). Blood serum samples were chosen for being obtained by a minimally invasive method and for showing spectroscopic profiles robust to small variations in sample collection and handling relative to biological differences.[22] Our results pointed out that the three investigated groups (the control and bipolar disorder patients under two different treatments) can be distinguished according to their metabolic profiles and the identified metabolite alterations could guide future studies on biomarker discovery for this disorder.

MATERIALS AND METHODS

Serum Collection and Storage. This study was approved by the local Ethics Committee (Hospital de Clínicas, University of Campinas, Brazil), and the subjects gave their written informed consent before sample collection. All blood samples were taken in the afternoon (between 14 and 16 h). Blood was drawn into Vacutainer tubes, immediately placed on ice, allowed to clot for at least 30 min, and centrifuged at 1500*g* for 15 min. The obtained serum was aliquoted, transferred into polypropylene tubes containing 0.01% (m/v) sodium azide, and stored at − 80 °C until assayed. The maximum period of storage was two weeks.

Fifty serum samples were collected and classified into three groups: the control group, constituted by 25 samples of subjects without bipolar disorder, bipolar disorder patients currently under treatment with lithium group, constituted by 15 samples, and bipolar disorder patients under treatment with other drugs than lithium group, constituted by 10 samples. Bipolar disorder patients were all in the euthymic state, previously identified as bipolar I, and under treatment in the psychiatric outpatient clinic (Hospital de Clínicas, University of Campinas, Brazil). No participants have other concomitant diseases such as cancer, AIDS, or hepatic, endocrinological, or metabolic diseases. The summary of the collected sample characteristics is presented in Table 1, and the description of the medications used for the treatment of bipolar disorder patients is described in Table 2. Bipolar disorder is associated with significantly higher prevalences of tobacco smoking behavior compared with the general population.[23] Such higher prevalence of tobacco smoking was found in our sample of bipolar patients, which means that this variable was not controlled in our study.

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Table 1. Collected Sample Characteristics[a]

	control	bipolar patients using lithium	bipolar patients not using lithium
sample size	$n = 25$	$n = 15$	$n = 10$
age, years (mean ± sd)	28 ± 5	40 ± 13	42 ± 17
no. of each gender (female/male)	14/11	9/6	7/3
duration of illness, years	NA	1−28	1−20
duration of current treatment, months	NA	2−240	1−120
no. with treatment with antipsychotics	NA	5	3
no. with treatment with mood stabilizers	NA	15	10
no. with treatment with antidepressants	2	1	2
no. with treatment with anxiolytics	NA	5	5
lithium doses, mg (mean ± sd)	NA	911 ± 325	NA
fraction smoking, %	0	26.7	20

[a] Abbreviations: sd, standard deviation; NA, not applicable.

Table 2. Description of the Medications Used for the Treatment of Bipolar Disorder Patients

sample no.	bipolar patients using lithium	bipolar patients not using lithium
1	lithium and carbamazepine	risperidone, clonazepam, fluoxetine, and diazpeam
2	lithium and olanzapine	carbamazepine, valproic acid, and diazepam
3	lithium and valproic acid	valproic acid
4	lithium and valproic acid	valproic acid
5	lithium, olanzapine, and clonazepam	valproic acid
6	only lithium	quetiapine
7	lithium and risperidone	valproic acid and lamotrigine
8	only lithium	valproic acid and clonazepam
9	lithium, fluoxetine, and clonazepam	valproic acid, clonazepam, and olanzapine
10	only lithium	valproic acid and citalopram
11	lithium, diazepam, and chlorpromazine	
12	lithium and clonazepam	
13	lithium and lorazepam	
14	only lithium	
15	lithium, risperidone, and valproic acid	

Sample Preparation and Acquisition of ^{1}H 1D (T_2-Edited) and 2D NMR (TOCSY) Data. For NMR spectroscopic analyses, serum samples were thawed and centrifuged at 12300*g* for 10 min at 4 °C to separate any precipitate. Aliquots of 250 μL of the supernatants were diluted with 350 μL of D_2O and placed in 5.0 mm diameter NMR tubes. A simple dilution procedure was employed to avoid additional sample preparation steps because one of the intentions of the proposed methodology is to be applied in bipolar disorder diagnosis and/or lithium treatment monitoring. All the NMR experiments were carried out without spinning at 499.89 MHz and 25 °C on an INOVA-500 (B_0 = 11.7 T) spectrometer (Varian, Palo Alto, CA) equipped with a 5 mm inverse triple-resonance probe. Standard one-dimensional (1D) PRESAT spectra were acquired using a 90° pulse sequence, with 128 scans, a pulse length of about 10 μs, and a recycle delay of 2 s. The 1D spin−echo spectra were recorded using the CPMG (Carr−Purcell−Meiboom−Gill) sequence of $D-[-90°-(\tau-180°-\tau)n-\text{ACQ}]$, where a fixed total spin−spin relaxation delay $2n\tau$ of 100 ms was used to attenuate the broad NMR signals from slowly tumbling molecules such as proteins and retain those from low molecular weight compounds and some lipid components. Typically, 64 transients and 32K data points were collected with a spectral width of 12 kHz, an acquisition time of 1.64 s, and a relaxation delay of 4 s. The free induction decay (FID) was zero-filled to 64K, and an exponential line-broadening function of 0.3 Hz was applied to the FID prior to Fourier transformation. All spectra were carefully phase and baseline corrected and referenced to the methyl peak of lactate at 1.33 ppm (3 H, d, $^3J = 7$ Hz), present in all spectra, since TSP is not a suitable reference for the serum samples due to interaction-induced line broadening. All spectra were processed with VNMR software (Varian). To confirm the assignments made from 1D ^{1}H NMR spectra, some blood serum samples were also examined using 2D ^{1}H−^{1}H TOCSY spectra with solvent suppression. The spectra were acquired with a 1.5 s relaxation delay, 1.5 s water signal suppression, and 6030.5 Hz spectral width for the ^{1}H dimensions. For each 2D spectrum, 256 increments with 64 transients per increment were collected and extended to 4K data points using linear prediction and zero filling approaches. The TOCSY experiments used an MLEV-17 spin-lock scheme for ^{1}H−^{1}H transfers with a mixing time of 90 ms at a spin-lock strength of 8 kHz. The signal assignments were based on the literature and/or Madison Metabolomics Consortium Database[24,25] and are indicated on the T_2-edited spectrum and confirmed by the 2D fully assigned ^{1}H−^{1}H TOCSY spectrum.

Chemometrics Analyses of ^{1}H NMR Spectral Data. ^{1}H NMR data were transported to a data matrix, and chemometrics analyses, based on interval principal component analysis (iPCA) and partial least-squares discriminant analysis (PLS-DA), were performed using MATLAB 6.5 software (The Mathworks, Natick, MA). iPCA was performed for the spectral region of chemical shifts ranging from −0.5 to +4.4 ppm. The principle

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of this algorithm is to split the spectra into smaller equidistant regions and, afterward, calculate PCA models for each interval and present the results in multiple score plots.[26] PLS-DA is intended to give a data overview and can be helpful in exploratory studies and interpretation (e.g., when looking for clustering among the samples). It can also be used as a supervision method of classification. The analyzed spectral area ranged from −0.5 to +4.4 ppm, where information and reduced noise were obtained. The chosen preprocessing method was the orthogonal signal correction (OSC), and unnecessary information was eliminated. In the OSC procedure, the **X** matrix was corrected by a subtraction of variation orthogonal to the **y** vector calibration.[27] Considering this work, **y** was a vector containing the class corresponding to each sample. This vector was used for both preprocessing OSC and PLS-DA algorithms. All variables were mean-centered, the spectra were normalized, and a "leave one out" cross-validation was performed. After that, another validation of the model was done by splitting at random the samples into calibration and validation sets and building a new PLS-DA model using the calibration set and the same conditions of the previous one. Then a class prediction of the validation samples was done.

Chemicals. All chemical reagents were of analytical grade. Deuterium oxide (D_2O; 99.9% D) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA), and sodium azide was purchased from Sigma (St. Louis, MO).

RESULTS

^{1}H NMR Spectral Data Analysis Using Chemometrics. The chemometrics tools were combined with NMR spectroscopy in the analyses of human blood serum samples to evaluate changes in the metabolic profiles in the presence of bipolar disorder and to compare drug treatments with and without lithium. The spectral region between 4.5 and 4.8 ppm was not considered because of the residual water signal (HDO, 4.7 ppm). The region above 4.8 ppm was also not considered for further analyses because it did not show important differences among the groups.

To explore all the potential differences in the metabolic profiles of the studied groups, the NMR spectra were first pretreated with the standard normal variate (SNV) for correcting spectral noise and background effects that caused baseline shifting and tilting in the spectra, then segmented into 0.6 ppm intervals, and finally subjected to iPCA. On the basis of the iPCA results, it was possible to observe a distinction of the samples into two groups: the control and bipolar disorder patients (independently of the drug treatment). Some samples presented a different behavior than the majority of the group, but it is a difficult task to determine exactly the reason for the metabolic profile differentiation, as we do not have the means for accounting for such outliers. One interesting point concerns the subject from sample 18, a male control classified together with the bipolar disorder patient group. This subject does not take any medications, nor has he had a psychiatric history, but his younger sister has bipolar disorder.

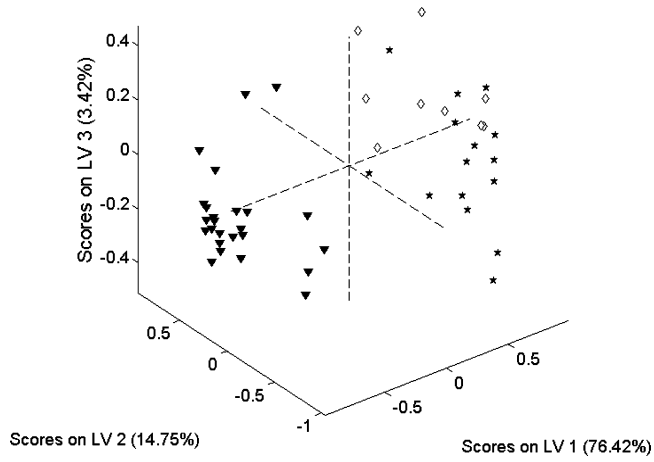

Figure 1. PLS-DA score plot for the chemical shift interval from −0.5 to +4.4 ppm. Samples from the control group, bipolar patients treated with lithium, and bipolar patients not treated with lithium are represented with the following symbols: ▼, ★, and ◊, respectively.

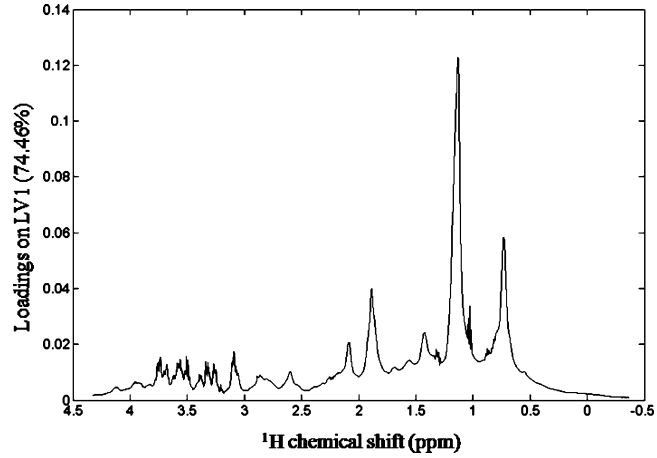

Figure 2. PLS-DA loading plot on the first latent variable for the chemical shift interval ranging from −0.5 to +4.4 ppm.

This observation can be in agreement with previous researches that evidence a genetic characteristic of the disease.[28]

Considering the iPCA results and the previous observations about the spectral regions not relevant for analyses, PLS-DA was performed. For such analyses, the spectra were first normalized and then processed with OSC[29] preprocessing, used to remove information within the NMR data not correlated to the target variables, by applying restricted principal component analysis. This data-filtering method is particularly important to human metabonomic studies like this one, because of the great variability in human populations, when compared to studies involving laboratory-controlled animals.

Figure 1 shows the PLS-DA score plots for the best model built, which was the one using 10 latent variables (LVs) with a variance of 98.74% in the X-block (spectra) and 62.05% in the Y-block (classes). The score plot allowed the visualization of the relations among the samples in the plane model to estimate whether there are any clustering, trends, and/or outliers.[30] On the basis of the PLS-DA results, the three groups, i.e., the control

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Table 3. Chemical Shift Assignments for Metabolites Identified in ^{1}H NMR Spectra (500 MHz) of Serum Samples[a]

peak[b]	chemical shift/ppm	molecule	assignment
1	0.99	valine/lipids/ lipoproteins/mobile lipids	CH_3
2	1.04	valine/broad peak underneath proteins	CH_3
3	1.33	lactate, lipids, lipoproteins, mobile lipids	CH_2
4	1.48	alanine	CH_3
5	1.57	lipids (mainly vldl's[c])	CH_2CH_2CO
6	1.72	lipids	$CH_2CH_2CH{=}CH$
7	**1.93**	**lipids**	**$CH_2CH{=}CH$**
8	**1.95**	**glycoprotein lipids**	**$CH_2CH_2CH{=}CH$**
9	1.99	proline	β-H
10	2.04	glutamine	β-H
11	**2.12**	**acetate**	**CH_3**
12	2.24	valine	β-H
13	2.29	proline	β-H
14	2.30	glutamate	γ-H
15	2.44	asparagine	β-H
16	**2.6 and 2.64**	**lipids**	**$CH{=}CHCH_2CH{=}CH$**
17	2.81, 2.83, and 2.85	albumin lysyl	ε-CH_2
18	3.03	lysine/creatine	δ-H (lysine)
19	**3.21**	**choline**	**δ-CH_2**
20	3.24	arginine/glucose	δ-H (arginine)
21	3.37	proline/glucose	δ-H (proline)
22	**3.54**	***myo*-inositol**	**H1, H3**
23	3.77	arginine	α-H
24	**3.79**	**lysine**	**α-H**

[a] In bold are highlighted the metabolites with higher loading values (see Figure 2). [b] According to Figure 4d. [c] Very low density lipids.

(group I), bipolar treated with lithium (group II), and bipolar treated with other medications not including lithium (group III), showed trends to form distinct groups, as illustrated in Figure 1. The samples from group I had scores below zero in LV1, staying apart from the other samples, which had scores above zero. Despite the fact that the samples from group II and group III had not formed individual groups, it is possible to note different trends of each set. Samples from group II had a trend to have negative values in LV2, while samples from group III had a trend to have positive values in LV2.

The loading plot (Figure 2) highlights the most significant variables by describing the influence and relation among the variables in the model plane.[30] Therefore, it is possible to conclude that the separation among the groups is due mainly to the peaks with chemical shifts of 0.99, 1.04, 1.33, and 1.93 ppm. Such peaks refer to valine, lactate, lipids, and lipoproteins. Other very important chemical shifts are listed in Table 3.

To validate the model, the samples were split at random into calibration and validation sets. Five, three, and two samples were selected and respectively correlated to the control, bipolar treated with lithium, and bipolar treated with other medications not including lithium for validation sets. A new PLS-DA model was built using the calibration set applying the same conditions of the previous model. To build it, the spectral calibration set was first corrected with OSC. Once the correction was done, validation or prediction data could be adjusted through the function $\mathbf{NEWX} = \mathbf{X} - \mathbf{X}(\mathbf{NW})\ \mathrm{inv}[[(\mathbf{NP}')(\mathbf{NW})](\mathbf{NP}')]$, where **NEWX** is the OSC-corrected validation or prediction matrix, **X** is the validation or prediction original matrix, **NW** is the weights matrix, **NP′** is the loads matrix, and **NT** is the scores matrix that were used in making the correction. Inv is the inverse of the square matrix $[(\mathbf{NP}')(\mathbf{NW})](\mathbf{NP}')$. The OSC algorithm[31] gives all these parameters.

The new model was built using the same number of latent variables as the previous model after the normalization of all spectra. The variance was 98.90% in the X-block (spectra) and 93.94% in the Y-block (classes). A leave one out cross-validation was performed, with errors of 0%, 20.24%, and 15.62%, respectively, for the three groups. Figure 3 shows the predictions for the three classes of the studied groups. It can be seen that the predictions were precise to classify samples among the bipolar disorder patient groups and the control group. Two mistakes occurred in the prediction of group II, where sample 43, which actually belongs to group I, was predicted and sample 47 was not predicted.

Differential Metabolite Identification. Figure 4 shows the ^{1}H NMR spectra for a sample of each one of the studied groups. The same pattern was observed in all spectra, but specific peaks appeared with different intensities for each group. These results were also demonstrated by the PLS-DA analysis. The NMR spectra contain broad peaks from molecules with high molecular mass, such as lipids and proteins, which resulted in a rugged baseline that caused overlapping of some low molecular mass compounds and disabled their identification. The chemical shift is perhaps the most important parameter of an NMR spectrum. It is directly proportional to the electron density surrounding the nucleus and is used to obtain structural information about the molecules present in a sample.[32] In Figure 4d, the marked peaks refer to the metabolites that differed between the groups studied. These metabolites were identified as lipids, lipid-metabolism-related molecules, and amino acids (Table 3) by comparing their chemical shifts to those previously reported in the literature.[33,34] Combining the results from the loading plot (Figure 2) with the information obtained from the

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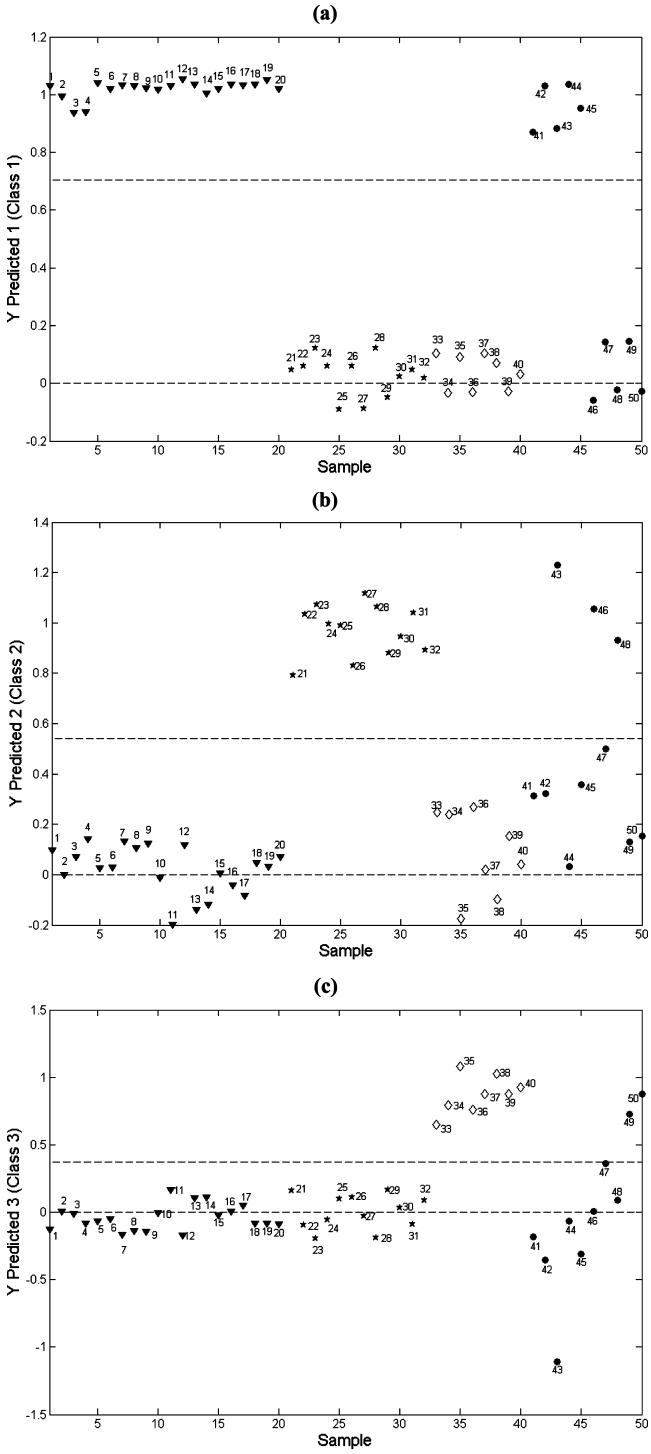

Figure 3. Predictions for (a) class 1 (control group), (b) class 2 (bipolar patients treated with lithium), and (c) class 3 (bipolar patients not treated with lithium). Samples 1−40 are from the calibration set: class 1 (▼), class 2 (★), and class 3 (◊). All the samples marked with a solid circle are from the validation set. Samples 41−45 belong to class 1, 46−48 to class 2, and 49 and 50 to class 3. The dashed line above zero (a−c) is the threshold. A color code can be seen in Figure S-2 in the Supporting Information.

NMR spectra (Figure 4 and Table 3), significant potential biomarkers for distinguishing bipolar disorder patients from healthy controls and also bipolar patients under treatment using lithium from those not undergoing lithium treatment are possible to assign. Such molecules, shown in bold (Table 3), are glycoprotein lipids, mono- and polyunsaturated lipids, acetate, choline, and *myo*-inositol.

DISCUSSION

A metabonomic approach was employed to evaluate the metabolic profile of blood serum from patients with treated bipolar disorder. Those with the disorder were readily distinguished from control subjects. Moreover, bipolar patients under treatment using lithium were distinguished from those not treated with this drug by comparing ^{1}H NMR metabolic profiles (illustrated in Figure 1). The metabolic profiles of healthy subjects (control group) and bipolar disorder patients under treatment using lithium (15 subjects) or not using lithium (10 subjects) were compared. For that purpose, two different control groups were considered: one age-matched with the bipolar patients (data shown in Figure S-1, Supporting Information) and another with a higher sample number as described in Table 2. The characteristics of the first group were age average of 31 ± 5 years (age averages among groups not differing with statistical significance at 5% probability as indicated by the Tukey test[35]), sample number of 15 (9 female and 6 male), and all nonsmokers. The results showed that age was a nonrelevant parameter considering bipolar disorder metabolic profile changes in the present case.

As mentioned before, the loading plot (Figure 2) highlighted the most significant variables with the highest loading values, which enabled the identification of potential biomarkers for bipolar disorder: glycoprotein lipids, mono- and polyunsaturated lipids, acetate, choline, glutamate, and *myo*-inositol, mainly.

Also using ^{1}H NMR spectroscopy-based metabonomics analysis, Lan et al.[21] identified molecular changes in postmortem brain tissue of bipolar disorder patients and in rat brain tissue after chronic treatment with lithium or valproate. Glutamate levels were increased in postmortem brains of bipolar patients, while the glutamate/glutamine ratio was decreased following valproate treatment, and γ-aminobutyric acid levels were increased after lithium treatment. Creatine and *myo*-inositol levels were increased in the postmortem brain, but decreased in the presence of the medications.

Lipids. Lipid level changes associated with bipolar disorder were previously reported in the literature. As an example, Atmaca et al.[36] observed decreased serum cholesterol and leptin levels in bipolar disorder patients with manic episodes and in patients with bipolar disorder I in full remission. More recently, Ozbulut et al.[37] evaluated cholesterol, leptin, and ghrelin levels in euthymic patients with bipolar disorder that received lithium maintenance monotherapy and found that decreased serum ghrelin and increased total cholesterol levels in the patients under lithium treatment were detected when compared with those of the controls. Ozbulut et al.[37] suggest that ghrelin and total cholesterol might be associated with lithium treatment and lithium-induced improvement of symptoms such as food intake and sleep−wake regulation, but not with weight gain. Schwarz et al.[38] used a high-

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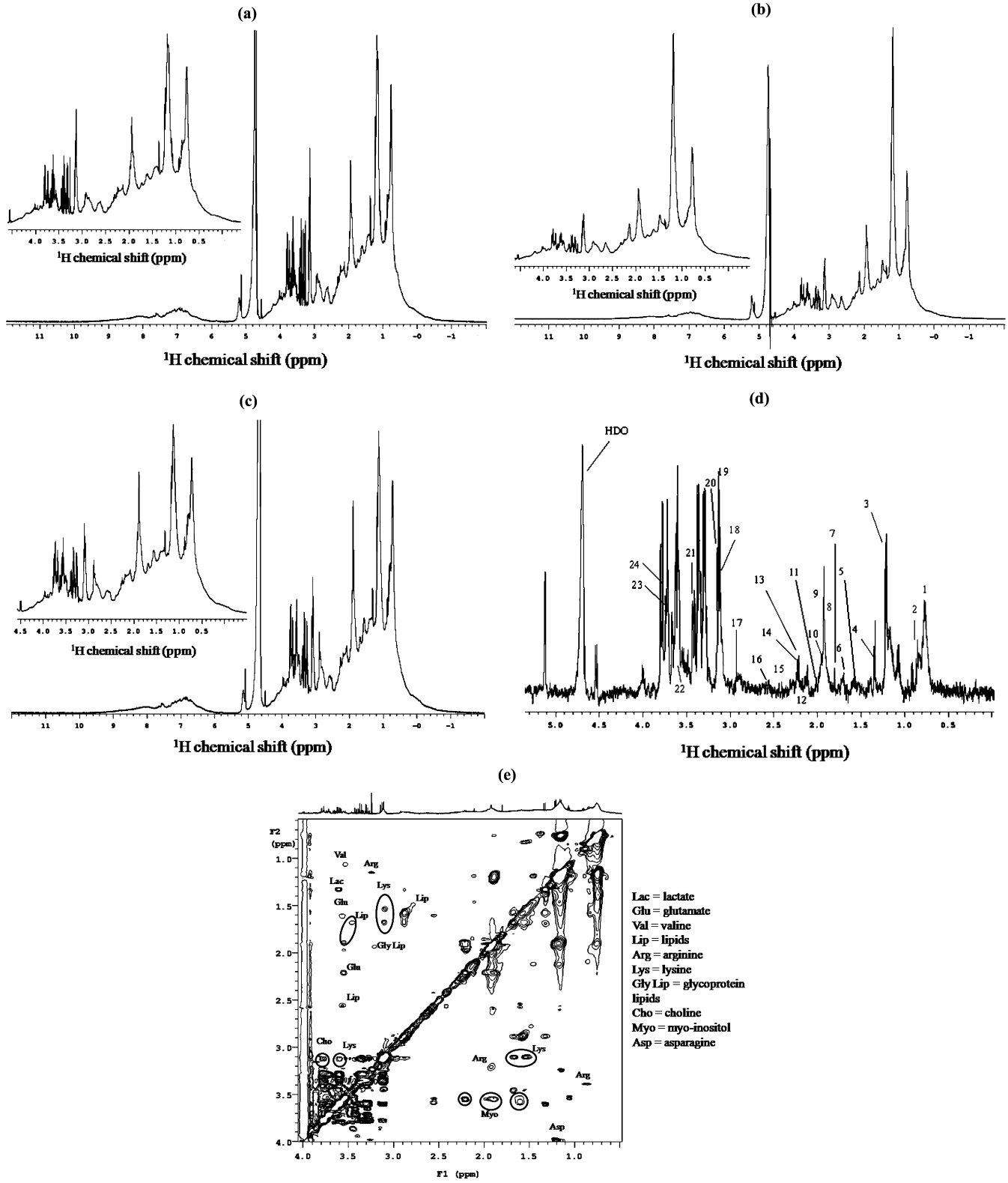

Figure 4. ^{1}H NMR spectra for a blood serum sample of (a) a control subject, (b) a bipolar disorder patient treated with lithium, and (c) a bipolar disorder patient not treated with lithium. The expanded −0.5 to +4.4 ppm region used in chemometrics is shown in the upper left corners (a−c). The peak assignments described in Table 3 are shown in (d) on the T_2-edited spectrum of a control sample. (e) ^{1}H−^{1}H TOCSY NMR spectrum of human blood serum recorded (control sample) using MLEV-17 as a spin-locking scheme with a mixing time of 90 ms. Some important metabolites are indicated.

throughput mass spectrometry approach (UPLC−MS) to analyze samples of gray and white matter and red blood cells and compared subjects with schizophrenia and bipolar disorder to control subjects. Significant alterations in the levels of free fatty acids and phosphatidylcholine were detected. Such differences suggest that lipid abnormalities may be an intrinsic feature of both

schizophrenia and bipolar disorder that is reflected by significant changes in the central nervous system as well as in peripheral tissues. In addition, a recent study from some of the authors of this paper (in preparation), using independent proteomic profiling techniques on the same samples, showed a consistent and significant alteration in the levels of apolipoprotein A-I, which is a component of the high-density lipid fraction. Therefore, these current studies support the hypothesis of lipids as potential biomarkers related to bipolar disorder and/or treatment using lithium.

The lipid-metabolism-related molecules found were acetate, glutamate, choline, and *myo*-inositol. Acetate is formed in the body by the metabolism of certain substances, particularly in the liver in the oxidation of lipids. Glutamate is the most common precursor of the brain neurotransmitter (GABA) and is always excitatory usually due to simple receptors that increase the flow of positive ions by opening ion channels. As reported in the literature for postmortem brain tissue samples,[21] glutamate levels increased in bipolar patient serum samples independent of the lithium treatment.

It is known that lipid concentrations in serum are altered in smokers compared with nonsmokers.[39] Nevertheless, a constant behavior of lipid levels in the bipolar disorder patient groups (containing some smokers) in relation to the control group (containing only nonsmokers) is possible to assign. As mentioned in the Materials and Methods, this variable was not controlled in the present study, but it was shown to be a nonrelevant factor, since the lipid levels changed in the same way inside each investigated group.

Choline. Choline is a natural amine found in the lipids that make up cell membranes, and it is the precursor of the neurotransmitter acetylcholine, which acts in the cholinergic neurotransmission.[40] Choline, lithium, and bipolar disorder are linked by interactions at several levels. Clinically, there is evidence that the choline precursor lecithin (phosphatidylcholine) is moderately effective in some patients with mania. Furthermore, lithium exerts a potent and specific inhibitory effect on human choline transport.[41]

***myo*-Inositol.** *myo*-Inositol is a sugar involved in the regulation of neuronal osmolarity, the metabolism of membrane-bound phospholipids, and the phosphoinositide secondary messenger pathway.[42] Changes in *myo*-inositol levels may reflect increased inositol monophosphatase (IMPase) activity, which would lead to an increase in the levels of *myo*-inositol containing compounds in patients treated with lithium.[43] In the present work, *myo*-inositol was found with an increased level in the serum of bipolar patients treated with lithium compared to the control group, and with a lower level in the serum of bipolar patients treated with drugs not including lithium. In the recent literature,[21] this molecule was also found with an increased level in postmortem brain tissue of bipolar disorder patients, but with a decreased level in the brain tissue of rats treated with lithium. Comparing these results, a discrepancy between *myo*-inositol levels found in human samples (increased) and animal samples (decreased) when taking into account the lithium treatment effects is possible to observe.

Amino Acids. Differential amino acid (proline, glutamine, valine, asparagine, arginine, and lysine) levels were also found when comparing control subjects to bipolar patients under the different treatments. This suggests an alteration in the patients' amino acid metabolism. The metabolites *N*-acetylaspartate, choline, *myo*-inositol, glutamate/glutamine, and creatine separately were reported in the literature for euthymic, maniac, and depressed adult and child/adolescent bipolar patients by ^{1}H MRS analyses in specific cerebral regions.[42] This further supports our results in that we found such metabolites also differing in blood serum samples.

CONCLUSIONS

Metabolic profiling using ^{1}H NMR spectroscopy and chemometrics analyses proved to be an innovative strategy to differentiate healthy subjects from bipolar ones and also to distinguish bipolar patients according to treatment (using lithium or not). A possible limitation to this study could be the small sample size. However, even with this limitation a clear distinction among the studied groups was observed, where those samples belonging to the same group closely showed the same results. To the best of our knowledge, this is the first time that such methodology was used with these purposes employing human blood serum samples. The results found in this work for blood serum samples (systemic level) corroborate those found in the recent literature[21] for postmortem brain tissue samples (local level), especially in terms of glutamate and *myo*-inositol level changes. The proposed methodology allows the detection of multiple differential metabolites simultaneously, providing a general pattern for the disease and for a specific treatment, with the advantage of requiring serum samples only, which are obtained through a minimally invasive strategy. A distinctive pattern in the metabolic profile of serum from bipolar disorder patients could even come to play a diagnostic role in the future, and the differential patterns comparing the patients treated with lithium or not could possibly indicate relevant drug action pathways.

It is important to note that the mechanism of metabolic changes in human blood serum of bipolar patients, considering treatment with lithium or not, should be further studied. ^{1}H NMR spectroscopy analysis provides only a static picture of the metabolites measured, and it does not allow the determination of rates of metabolism or changes in the metabolic pathways that may be altered in the presence of the disease and with lithium treatment. The differential metabolites addressed in this work could also guide further studies on the pathophysiology of bipolar disorder and mechanisms of action of lithium treatment and also on biomarker discovery.

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ACKNOWLEDGMENT

Prof. Dr. Fred Y. Fujiwara is acknowledged for very valuable help involving the NMR spectrometer software and acquiring the T_2-edited spectra. We also thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, São Paulo, Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasília, Brazil), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasília, Brazil) for financial support and fellowships.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review July 7, 2009. Accepted October 12, 2009.

AC901502J