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Metabolomics and lipidomics analyses by ^{1}H nuclear magnetic resonance of schizophrenia patient serum reveal potential peripheral biomarkers for diagnosis

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ABSTRACT

Using ^{1}H NMR-based metabolomics in association to chemometrics analysis, we analyzed here the metabolic differences between schizophrenia patients (SCZ) compared to healthy controls (HCs). HCs and SCZ patients underwent clinical interview using the Structured Clinical Interview for DSM Disorders (SCID). SCZ patients were further assessed by Positive and Negative Syndrome Scale (PANSS), Calgary Depression Scale, Global Assessment of Functioning Scale (GAF), and Clinical Global Impressions Scale (CGI). Using the principal component analysis (PCA) and supervised partial least-squares discriminate analysis (PLS-DA) in obtained NMR data, a clear group separation between HCs and SCZ patients was achieved. Interestingly, all metabolite compounds identified as exclusively present in the SCZ group, except for the gamma-aminobutyric acid (GABA), were never previously associated with mental disorders. Although the initial perception of an absence of obvious biological link among the different key molecules exclusively observed in each group, and no identification of any specific pathway yet, the present work represents an important contribution for the identification of potential biomarkers to inform diagnosis, as it was possible to completely separate the affected SCZ patients from HCs, with no outliers or exceptions. In addition, the data presented here reinforced the role of the modulation of glycolysis pathway and the loss of GABA interneuron/hyperglutamate hypothesis in SCZ.

1. Introduction

High complexity of schizophrenia (SCZ) clinical presentation and limited knowledge of the molecular mechanism(s) underlying this illness make the search for biomarkers that could inform the diagnosis and support indication of different treatments highly needed. Studies that can improve the comprehension of the biochemical alterations related to the disorder and the identification of diagnostic biomarkers are also of great interest. On this aspect, the concomitant quantitative analysis of several metabolites, a technique known as 'metabolomics', has the potential to contribute not only for understanding the physiopathology involved in neuropsychiatric disorders, but it may also reflect in improvements in early diagnosis, and prediction of treatment outcomes (Orešič et al., 2011; Filiou and Turck, 2012; He et al., 2012).

The metabolome has been conceptualized as the metabolic state of a given physiologic state of a cell, a tissue or an organism (Martins-de-Souza, 2014). It has been useful to reveal biochemical pathways

Abbreviations: SCZ, schizophrenia; HC, healthy control; NMR, nuclear magnetic resonance; CI, confidence interval; OR, odds ratio; PANSS, Positive and Negative Syndrome Scale; SCID, Structured Clinical Interview for DSM Disorders; DSM, Diagnostic and Statistical Manual of Mental Disorders; CDSS, Calgary Depression Scale for Schizophrenia; CGI, Clinical Global Impression; GAF, Global Assessment of Functioning; SPSS, statistical package for social sciences; PCA, principal component analysis; PLS-DA, supervised partial least-squares discriminate analysis; GABA, gamma-aminobutyric acid; NAA, *N*-acetylaspartate; PABA, *p*-aminobenzoic acid; MD, major depression; NMDAR, *N*-methyl-D-aspartate receptor; EMA, ethylmalonic acid; MOA, 3-methyl-2-oxobutinoic acid; PCP, phencyclidine; SeMet, L(+)-selenomethionine.

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involved in biological mechanisms of interest in mental illnesses as autism (West et al., 2014; Wang et al., 2016), major depression (Martins-de-Souza, 2014), bipolar disorder (Yoshimi et al., 2016) and also SCZ (Vendramini et al., 2015; Sethi et al., 2016).

Great number of studies have shown metabolic changes in SCZ individuals compared to healthy controls (HCs), demonstrating increased levels of lipids (triglycerides), amino acids (namely phenylalanine, tyrosine, proline, and glutamate) and lactic and pyruvic acids, leading thus to the suggestion of the importance of the regulators of glucose or proline metabolism in psychosis (Orešič et al., 2011). In other study, metabolic profile analysis in serum and urine of SCZ patients and HCs indicated several fatty acids (*e.g.* eicosanoic acid), ketone bodies and glycerate, β-hydroxybutyrate, pyruvate and cystine, as metabolic biomarkers with potential to contribute for the understanding of this disease (Yang et al., 2013). Xuan et al. (2011), using gas chromatography, analyzed different metabolites in the serum of SCZ patients treated with risperidone, showing that the HCs have significant higher glucose and lactate levels and lower concentration of 1,3-bisphosphoglycerate (1,3-BPG or 1,3-bisphosphoglyceric acid), which are the reaction intermediates in the glycolytic pathway. On the other hand, the analysis of 103 different metabolites in the plasma of SCZ patients and HCs suggested the amino acids (namely arginine, glutamine, histidine and ornithine) and lipids (*e.g.* PC C38:6) as potential candidates for biomarkers of SCZ (He et al., 2012).

In addition, decreased concentrations of the derivative of the amino acid aspartic acid *N*-acetylaspartate (NAA) have been reported in SCZ and also in bipolar disorder (Kraguljac et al., 2012), and it may reflect either the neuronal or axonal loss or mitochondrial dysfunction, which are commonly described in these illnesses (Meyerhoff et al., 1993; Sager et al., 2001). In general, NAA is found in neurons, but not in glial cells or blood, and therefore, it is an important neuronal marker that is thought to reflect the neuronal integrity (Kraguljac et al., 2012; Stanley, 2002).

Although metabolomics studies are employed in psychiatric illnesses, such as SCZ, the use of nuclear magnetic resonance (NMR), specifically proton NMR (^{1}H NMR) associated to chemometrics, was insufficiently explored so far. The principal component analysis (PCA) and supervised partial least-squares discriminate analysis (PLS-DA) in NMR data treatment may aid in comparative analysis and increase the chance of identifying important biomarkers to support the diagnosis.

Serum samples from subjects with DSM-IV SCZ as assessed with the Structured Clinical Interview for DSM-IV (SCID-IV) were collected. Severity of symptoms was evaluated by using the Positive and Negative Syndrome Scale (PANSS), Calgary Depression Scale for Schizophrenia (CDSS), Global Assessment of Functioning Scale (GAF), and Clinical Global Impression Scale (CGI). Metabolome measures were analyzed here to demonstrate that NMR-based method in combination with chemometrics analysis is able to distinguish SCZ patients from HCs. Moreover, the metabolites that allowed the separation of affected (SCZ patients) from HC group were identified and the results are described herein.

2. Methods

This study was approved by the Research Ethics Committee of UNIFESP [CEP No. 0305/12], and a written informed consent was obtained from all recruited participants prior to inclusion. Clinical and laboratory investigations were strictly conducted according to the principles expressed in the Declaration of Helsinki.

2.1. Studied population

In this study, 26 patients with schizophrenia (SCZ) and 27 healthy control (HC) volunteers were enrolled. SCZ individuals were recruited from the Schizophrenia Program (PROESQ) of Universidade Federal de São Paulo (UNIFESP/EPM), which is an outpatient unit dedicated to provide medical care for adults with SCZ. Trained psychiatrists confirmed the diagnoses by applying the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders (SCID-IV). Any other available information, including medical records relevant for the diagnosis, was considered. Inclusion criteria were as follows: (i) diagnosis of DSM-IV SCZ or schizoaffective disorder; (ii) at least one year of follow-up; (iii) age between 16 and 65 years; (iv) ability to read and write. Exclusion criteria were: (i) clinical instability with need of hospitalization; (ii) suicide risk; (iii) severe or unstable general medical comorbidity; (iv) diagnosis of a current substance use disorder (except nicotine dependence); (v) pregnancy or post-partum period. The clinical assessment also included the Positive and Negative Syndrome Scale (PANSS) (Higuchi et al., 2014), the Calgary Depression Scale for Schizophrenia (CDSS) (Bressan et al., 1998), the Global Assessment of Functioning (GAF) and the Clinical Global Impression (CGI) (Lima et al., 2010).

HCs were selected from a governmental unemployment office and the inclusion criteria were as follows: (i) age between 16 and 65 years old; (ii) no current or lifetime axis I psychiatric diagnosis, as assessed with SCID-NP; (iii) absence of known family (checked up to second-degree relatives) member with history of psychosis, mood disorders or suicide; (iv) ability to read and write. Exclusion criteria were: (i) severe or unstable general medical comorbidity; (ii) pregnancy or post-partum period.

The blood samples were collected into dry vacuum tubes (BD Vacutainer, BD, NJ, USA), in the morning (from 8 to 10 AM), from all subjects fasted for at least 8 h, and the serum was processed essentially as previously described (Gadelha et al., 2013). In short, the blood samples were immediately processed, and the serum was carefully recovered after centrifugation at 2000 × *g* for 10–15 min, at room temperature. Serum aliquots (250 μL each) were stored at −80 °C, in sterile plastic microtubes (Axygen Inc., CA, USA) until use. The maximum storage period before the NMR measurements was 2 weeks.

2.2. NMR experimentation and preprocessing

The human serum samples were defrosted in ice bath and they were immediately mixed with 250 μL of deuterium oxide (D_2O) or phosphate buffered saline (PBS with 10% of D_2O), before they were transferred into a 5 mm NMR tube for analyses.

All ^{1}H NMR spectra were recorded on a Bruker 600 MHz spectrometer coupled with a 5 mm probe (TBI) at 25 °C. The ^{1}H NMR spectra (1D, 600.173 MHz) were obtained by using water suppression method-*Watergate* (p3919gp), with 64 transient scans, 32 k data points, and bandwidth of 12 kHz. All spectra analyses were performed on samples from 3 independent experiments performed on different days, after correction of the phase and baseline, with the methyl lactate at 1.33 ppm (3H, d, $^3J = 7.0$ Hz) as reference.

Employing the Carr-Purcell-Meiboom Gill (CPMG) pulse sequence, the T_2-edited spectra of ^{1}H NMR were obtained. The ^{1}H NMR signals for larger molecules, including proteins and/or some lipid components, were eliminated from the T_2-edited like spectra, which was helpful for the identification of metabolites of interest. In addition, the 2D HSQC and HMBC {^{13}C-^{1}H} were used for the identification of the biomarkers, attributing expected coupling patterns for the identified key molecules.

2.3. Metabolite analysis and quantification

Data pre-processing, including data organization, removal of undesired areas and binning, as well as data processing were performed with MATLAB® (Mathworks Inc.) and Pirouette® (Infometrix Inc.). Chemometrics analyses employing PCA and PLS-DA were performed, and the two groups, SCZ and HCs were successfully separated into two classes using ^{1}H NMR 1.0–4.4 ppm spectral region. The loading graphs and T_2-NMR, HSQC, HBMC and databases search were employed to identify the biomarkers from the ^{1}H NMR metabolites profiles.

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