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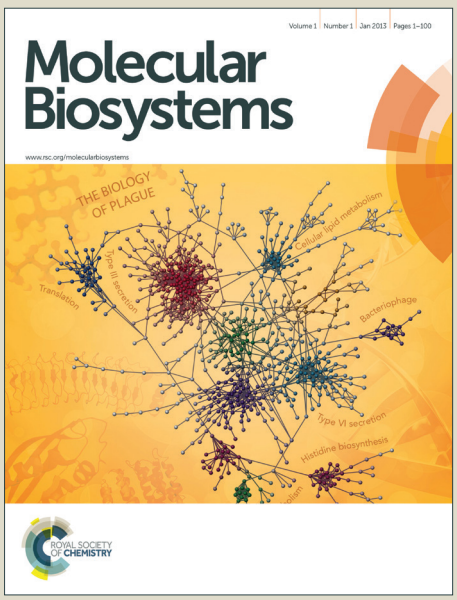

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GC-MS based metabolomics identification of possible novel biomarkers for schizophrenia in peripheral blood mononuclear cells

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Abstract

Schizophrenia is a debilitating mental disorder. Currently, the lack of disease biomarkers to support objective laboratory tests constitutes a bottleneck in the clinical diagnosis of schizophrenia. Here a GC-MS based metabolomic approach was applied to characterize the metabolic profiling of schizophrenia subjects (n=69) and healthy controls (n=85) in peripheral blood mononuclear cells (PBMCs) to identify and validate biomarkers for schizophrenia. Multivariate statistical analysis was used to visualize group discrimination and identify differentially expressed metabolites in schizophrenia subjects relative to healthy controls. The multivariate statistical analysis demonstrated that schizophrenia group was significantly distinguishable from the control group. Totally, 18 metabolites responsible for the discrimination between the two groups were identified. These differential metabolites were mainly involved in energy metabolism, oxidative stress and neurotransmitter metabolism. A simplified panel of PBMCs metabolites consisting of pyroglutamic acid, sorbitol and tocopherol-α was identified as an effective diagnostic tool, yielding an area under the receiver operating characteristic curve (AUC) of 0.82 in the training samples (45 schizophrenia subjects and 50 healthy controls) and 0.71 in the test samples (24 schizophrenic patients and 35 healthy controls). Taken together, these findings facilitate to develop diagnostic tools for schizophrenia.

Key words: schizophrenia; metabolomics; diagnosis; biomarkers; GC-MS

Introduction

Schizophrenia is a multifaceted and devastating illness affecting approximately 0.5-1% of the population worldwide.[1] It causes tremendous socioeconomic burden and negatively impacts on quality of life for patients and their families.[2] Currently, diagnosis of schizophrenia primarily relies on subjective judgments of clinical symptoms presented by patients. However, the clinical presentations of this disease are highly heterogeneous. Therefore, the current symptom-based method results in high rate of misdiagnosis or delayed diagnosis, contributing to poor outcomes in schizophrenia patients.[3] In regard of these factors, it is greatly needed to identify diagnostic biomarkers for schizophrenia.

Metabolites represent the final products of interactions among various factors including genetic, physiological and environmental factors, and may better reflect the functional status of individuals. Metabolomics enables simultaneous quantitative measurement of the global low-molecular-weight metabolites within given biosamples,[4] which is useful to uncover biological or pathological variations of diseases and facilitates to identify diagnostic biomarkers.[5] Recently, using this platform, our group has reported metabolite biomarkers for stress resilience, suicide, major depression and biopolar disorder.[6-11] GC-MS based metabolomics method, characterizing by high sensitivity, peak resolution and reproducibility,[12, 13] has been widely used to characterize metabolic status and identify novel biomarkers for numerous neuropsychiatric disorders, such as epilepsy, Huntington's disease, Alzheimer's disease, major depression and autism.[14-18]

Brain tissues and cerebrospinal fluid seem to be the most suitable biological samples theoretically for schizophrenia research. Previous schizophrenia studies have characterized the metabolic changes in postmortem brain tissues and cerebrospinal fluid, which provide valuable clues to uncover the pathogenesis of schizophrenia.[19, 20] However, it is impractical to collect human brain tissues and cerebrospinal fluid samples in living schizophrenia patients due to ethical and safety constraints, limiting the biomarker discovery in schizophrenia. In contrast, PBMCs can be easily collected in the early phases of the disorder and allow access to freshly isolated systemic cellular reactivity during the critical time frame after disease onset.[21] Previous numerous studies have shown that the brain and PBMCs show a number of parallel responses,[22-25] which suggest that PBMCs may be a useful surrogate of brain function. Such cells had been provided to be useful peripheral sources of biomarkers for neuropsychiatric disorders.[26-28] Moreover, peripheral immune dysfunction was widely implicated in the onset of schizophrenia.[29-32] Thus, we hypothesize that a diagnostic marker signature for schizophrenia may be detected in PBMC.

In this study, a GC-MS based metabolomic method was therefore used to compare the PBMCs metabolic profilings between 45 schizophrenia subjects and 50 matched healthy controls (training set) in order to preliminarily identify the potential diagnostic biomarkers for schizophrenia. Furthermore, 24 schizophrenia patients and 35 healthy controls (test set 1) were employed to independently validate the diagnostic performance of these PBMCs metabolite biomarkers. To validate the reliability of metabolomics results, schizophrenic biomarkers were quantified in

PBMCs of 20 schizophrenia subjects and 20 healthy controls (test set 2).

Materials and Methods

Ethics Statement

The Ethical Committee of Chongqing Medical University reviewed and approved the protocol of this study and the procedures employed for sample collection. All participants signed a written informed consent before any study procedure was carried out. All procedures were performed according to the Helsinki Declaration.

Participants

Totally, 89 schizphrenic subjects were recruited from the psychiatric center in the First Affiliated Hospital of Chongqing Medical University. All diagnoses were performed according to the Structured Psychiatric Interview using DSM-IV-TR criteria.[33] The diagnosis of schizophrenia was made by two senior psychiatrists. 46 of the 89 schizophrenia subjects were first-episode and drug-naïve, while the remaining schizophrenia subjects (n=43) were being treated with various antipsychotics. Exclusion criteria for schizophrenia subjects included any pre-existing physical or other mental disorders or illicit drug use. During the same time period, 105 healthy controls were recruited from the medical examination center in the First Affiliated Hospital of Chongqing Medical University. Healthy controls were required to have no current or previous lifetime history of neurological, DSM-IV Axis I/II, or systemic medical illness.

The recruited schizopherenia subjects and healthy controls were divided into a

training set, test set 1 and test set 2. The training set, including 45 schizophrenia subjects and 50 matched healthy controls, was used to identify disturbed metabolic pathways and the potential diagnostic markers for schizophrenia. The test set 1, including 24 schizophrenia subjects and 35 healthy controls, were used to independently validate the diagnostic generalizability of these PBMCs metabolite markers. The use of independent samples to validate the identified biomarkers is an essential step to assess the clinical value of these biomarkers.[34] The remaining subjects were used to construct the test set 2 to quantify levels of schizophrenic biomarkers in PBMCs. The detailed clinical characteristics of the recruited subjects are presented in Table 1 and supplemental Table 1. The schizopherenia group and healthy control group matched for age and gender in the training set and test set 2, but not in the test set 1.

Sample Preparation and GC/MS Acquisition

Fasting blood samples were collected into 10 mL EDTA-coated tubes. Plasma was obtained by centrifugation at 3000 rpm for 15 minutes at 4℃, then overlaid onto Ficoll-Paque Plus (GE Healthcare Bio-sciences AB, Sweden). After centrifugation (2000 rpm, 20 minutes), PBMCs were harvested and washed three times in phosphate-buffered saline. Each PBMC sample was divided into equal aliquots, transferred into liquid nitrogen for 3 minutes and then stored at −80°C until undergoing GC-MS analysis.

For GC/MS analysis, each PBMC sample was added with 1ml chromatographic grade methanol and 10μL L-leucine-13C6 (0.5 mg/mL) as an internal standard. After

vortexing for 30s and standing overnight at 4°C, the mixture was sonicated for 15 min and subsequently centrifuged at 14000 rpm for 15 min at 4°C. A 200ul aliquot of supernatant was evaporated to dryness under a stream of nitrogen gas. The dried metabolic extract was derivatized first with 30 µl of methoxamine (20 mg/mL) for 90 min at 37°C with continuous shaking. Subsequently, 30 µl of BSTFA with 1% TCMS was added to the mixture and heated for 1 h at 70°C to form trimethylsilyl (TMS) derivatives. After derivatization and cooling to room temperature, 1 µl of this derivative was injected in the GC/MS for analysis.[6]

GC/MS Analysis

Each 1 µl of the derived sample was injected into an Agilent 7890A GC system (Agilent Technologies Inc., USA). An HP-5 MS fused silica capillary column (30 m × 0.25 mm × 0.25 µm, Agilent, USA) within which helium carrier gas flowed at a rate of 1 ml/min, was applied for metabolite separation. The injector temperature was set at 280°C. The column temperature was initially kept at 80°C for 2 min and then increased to 320°C at 10°C /min, where it was maintained for 6 min. The column effluent was introduced into the ion source of an Agilent 5975 mass selective detector (Agilent Technologies). The MS quadrupole temperature was set at 150°C, and the ion source temperature was set at 230°C. Data acquisition was performed in the full scan mode from m/z 50 to 550.

Metabolomic Data Analysis

GC/MS metabolite profiles were processed after conversion into a NetCdf file format using TagFinder.[35] This processing enabled deconvolution, alignment, and data

reduction to produce a list of mass and retention time pairs with corresponding intensities for all detected peaks from each data file in the data set. The resulting three-dimensional data set - including peak index (RT-m/z pair), sample names (observations), and normalized peak area percentages - were imported into SIMCA-P 11.0 (Umetrics, Umeå, Sweden) for statistical analysis.

Orthogonal partial least-squares discriminant analysis (OPLS-DA), a supervised multivariate approach, was performed on the unit variance-scaled spectral data to visually discriminate schizophrenic subjects from healthy controls.[36, 37] The quality of the OPLS-DA models was evaluated by three parameters (R^2X, R^2Y, and Q^2Y), which were calculated by the default leave-one-out procedure. R^2X and R^2Y were used to quantify the goodness-of-fit; Q^2Y was applied to assess the predictability of the model. Then, a 300-iteration permutation test was performed to avoid overfitting of the model. If the values of Q^2 and R^2 resulting from the original model were higher than the corresponding values from the permutation test, the model was considered valid.[38] The discriminating metabolites were identified based on a statistically significant threshold of variable influence on projection (VIP) values obtained from the OPLS–DA model (VIP>1) and two-tailed Student's *t* test (*p*-value<0.05). In addition, the corresponding fold change was calculated to show the degree of variation in metabolite levels between groups.

Identification of PBMC metabolite biomarkers for schizophrenia

Given that a small number of metabolite biomarkers would be more feasible and convenient in clinical practice, a stepwise optimization algorithm based on Akaike's

information criterion (AIC) was performed to optimize the metabolite biomarker combination.[10, 11, 39] To further evaluate the diagnostic performance of the simplified schizophrenia biomarker panel, a receiver-operating characteristic (ROC) curve analysis was carried out to quantify the ability of this metabolite biomarker panel to discriminate schizophrenia subjects from healthy controls in both the training and test sets.[40]

Quantitative analysis of schizophrenic biomarkers

Pyroglutamic acid, sorbitol and tocopherol-α were quantified by GC/MS target metabolomics method according to our previous study.[7] The procedures of quantitative analysis were the same as metabolomics section except preparing calibration curves and changing mass data acquisition mode. In this study, the internal standard ($^{13}C_5$-Glutamine) and standards of pyroglutamic acid, sorbitol and tocopherol-α were purchased from Sigma–Aldrich (Shanghai, China). All standards and samples (test set 2) were analyzed first in the full-scan mode of GC-MS, then in selected ion monitoring (SIM) mode for quantification. The characteristic fragment ions and retention times of metabolites were summarized in supplemental Table 2. Calibration curves were generated by linear regression of the peak area ratio of standard metabolites to the internal standard at eight concentration levels. The quality control sample, pooled from a representative PBMCs sample of each group, was added in each batch of analyses in order to monitor the analysis quality.

Statistical Analysis

As appropriate, the parametric Student's t test or the chi-square test were applied

to compare the clinical characteristics between two groups. All the statistical analyses were carried out with SPSS software (version 19.0). A *p*-value of less than 0.05 was considered to be statistically significant.

Result

Metabolomics analysis of PBMCs samples from schizophrenia patients and healthy controls

OPLS-DA analysis was applied to explore the metabolic differences between schizphrenia subjects and healthy controls in the training set. The OPLS-DA score plots showed a clear discrimination between schizophrenia patients and healthy controls (R^2X=0.55, R^2Y=0.54, Q^2Y=0.29; Fig. 1a). The parameters (R^2X, R^2Y, and Q^2Y) quantifying the OPLS-DA model were all positive, demonstrating that the OPLS-DA model was robust. Moreover, a permutation test with 300 iterations confirmed that the constructed OPLS-DA model was valid and not overfitted, as the original Q^2 and R^2 values to the right were significantly higher than the corresponding permutated values to the left (Fig. 1b).

To assess the homogeneity of metabolic phenotype between the non-medicated and medicated schizophrenic patients, the OPLS-DA model was constructed with 26 unmedicated schizophrenia patients and 50 healthy controls (Fig. 1c). Then, the reconstructed OPLS-DA model was used to predict the class membership of the 19 medicated schizophrenic patients. The T-predicted scatter plot from the reconstructed OPLS-DA model demonstrated that 15 of 19 medicated schizophrenic patients were

correctly predicted (Fig. 1d). These findings demonstrated that the psychotolytic drugs may not significantly influence the metabolic phenotypes of individuals with episode schizophrenia.

18 differential metabolites responsible for discriminating schizophrenia subjects from healthy controls were identified (VIP＞1, p＜0.05) (Table 2). Compared to healthy controls, schizophrenia subjects were characterized by higher levels of hydroxylamine, octanoic acid, glycerol, aspartic acid, 2-hydroxyethyl palmitate, benzoic acid, homoserine, and lower levels of methyl phosphate, valine, fumaric acid, pyroglutamic acid, creatinine, sorbitol, inositol, dopamine, maltose, tocopherol-γ, tocopherol-α. Functional analysis showed that these identified differential metabolites were primarily involved in energy metabolism, oxidative stress and neurotransmitter metabolism.

Biomarker panel for diagnosis of schizophrenia

To identify a simplified diagnostic biomarker panel for schizophrenia, a step-wise optimization algorithm based on AIC was performed. A panel of PBMCs metabolite biomarkers contributing to the most maximum classification between schizophrenia subjects and healthy controls was identified. These metabolite biomarkers were pyroglutamic acid, sorbitol and tocopherol-α. A ROC analysis was further performed to quantify the diagnostic performance of this panel in both the training and test sets. The AUC of this panel was 0.82 (95% confidence interval: 0.74-0.91) in the training set and 0.71 (95% confidence interval: 0.56-0.85) in the test set (Fig. 2a-b). As an AUC of 0.5 indicates a weakly discriminating test and an AUC

of 1 indicts a strongly discriminating test, these results demonstrated the efficacy of this simplified PBMC metabolite panel in schizophrenia detection.

To validate reliability of the results, these biomarkers were quantified in PBMCs of schizophrenia subjects and demographic–matched healthy controls (test set 2, n=20 for each group). Calibration curves indicated good linear regression for these biomarkers, with R^2 being within the range 0.996–0.998 (supplemental Table 2). The concentrations of schizophrenic biomarkers in the individual sample were presented in supplemental Table 3. Quantitative analysis showed that pyroglutamic acid, sorbitol and tocopherol-α were all significantly decreased in schizophrenia subjects relative to healthy controls (supplemental Fig.1). These findings were consistent with aforementioned metabolomics results.

Discussion

Schizophrenia is a widespread and debilitating psychiatric disorder. Currently, no biomarkers are available to aid clinicians in diagnosing this disorder. Here we applied a GC-MS based metabolomic approach to identify potential metabolite biomarkers for schizophrenia in PBMCs. A simplified biomarker panel consisting of pyroglutamic acid, sorbitol and tocopherol-α was established using logistic regression. This biomarker panel was a ‘good’ classifier for diagnosing schizophrenia, yielding an AUC of 0.82 in the training sets and 0.71 in the test sets 1. In particular, the fact that schizophrenia patients and controls in the test set 1 were not matched in gender and age suggested an unbiased discrimination of this set of biomarkers in the general population. These findings suggest that this PBMCs metabolite biomarker panel may

serve as an effective diagnostic approach for schizophrenia.

Initially, 18 differentially expressed metabolites were identified to discriminate schizophrenia subjects from healthy controls. To develop deeper insight into the underlying molecular mechanisms of schizophrenia, these differential metabolites were comprehensively analyzed in terms of *in vivo* metabolic activity. These metabolites were found to be mainly involved in i) energy metabolism, ii) oxidative stress, iii) neurotransmitter metabolism, which are discussed in detail below. These findings consistently confirm that schizophrenia is a multifactorial disease.[41-44]

Energy metabolism. In this study, schizophrenia subjects showed higher levels of octanoic acid and lower levels of maltose, valine, inositol, sorbitol, creatinine and fumaric acid relative to healthy controls in PBMCs (Fig. 3). Octanoic acid is the substrate for lipoic acid synthesis. Lipoic acid acts as an essential cofactor of several multi-subunit complexes in the mitochondrial matrix involving energy metabolism, including pyruvate dehydrogenase, α-ketoglutarate dehydrogenase. The increased octanoic acid level found here, in conjunction with a previous study showing the majority (87.5%) of glucose metabolic enzymes were significantly increased in PBMCs of schizophrenia subjects, likely indicated activation of energy metabolism in schizophrenia.[45] Other groups have also reported higher levels of ATP and increased expression of enzymes involving glycolysis and tricarboxylic acid (TCA) cycle in schizophrenia,[46, 47] further supporting our findings. We deduced that lower levels of metabolic precursors (maltose and valine) and bypass products (inositol and sorbitol) of glucose metabolism identified in this study may resulted from the underlying

increased carbon flux through glycolysis and TCA cycle in schizophrenia. Excess ATP mainly produced from TCA cycle can convert creatine to phosphocreatine as a rapidly mobilizable reserve of energy. Creatinine can be metabolized from creatine by a non-enzymatic cyclization. The significantly decreased creatinine level in schizophrenia patients implied increased energy production and energy conversion, which was consistent with the aforementioned increased TCA cycle flux in schizophrenia subjects. Fumaric acid is the intermediate of TCA cycle and key precursor for proline, tyrosine and aspartate metabolism. Currently, numerous studies have reported activation of these amino acid metabolism pathways in schizophrenia.[48-50] Therefore, we deduced that the decreased level of fumaric acid may reflected the increased flux into amino acid metabolism in schizophrenia, which need further research. These speculations concord with the well-established theory that disturbance of energy metabolism contributes to the pathogenesis of schizophrenia.[19, 51-56]

Oxidative stress. Levels of four oxidative stress related metabolites - hydroxylamine, pyroglutamic acid, tocopherol-γ and tocopherol-α - were significantly perturbed in schizophrenia subjects as compared to healthy controls in this study. Hydroxylamine is an intermediate of ammonia oxidation as a product of normal cell metabolism. The autooxidation of hydroxylamine leads to the formation of reactive oxygen species (ROS). In this study, a significantly higher level of hydroxylamine was observed in schizophrenia subjects relative to healthy controls, suggesting excess ROS accumulation. GSH serves as an important scavenger of ROS, preventing

excessive oxidation of sensitive cellular components, and is a substrate for various antioxidant enzymes as well. Pyroglutamic acid, a glutathione metabolism by-product, was downreglulated in schizophrenia patients compared to healthy controls. The decreased pyroglutamic acid level may reflect the lower level of GSH. Besides, tocopherol-γ and tocopherol-α, the peroxyl radical scavengers, were significantly decreased in schizophrenia patients. These findings indicated that schizophrenia is involved in decreased antioxidative defense capability and increased ROS production. In agreement with this speculation, increased oxidative stress was implicated in the development of schizophrenia in previous studies.[55, 57-65]

Neurotransmitter metabolism. Here, aspartic acid and homoserine were significantly upregulated and dopamine was significantly downregulated in schizophrenia patients compared to healthy controls in PBMCs. In the mammalian CNS, both aspartic acid and dopamine function as primary excitatory neurotransmitters. Furthermore, aspartic acid can be metabolized to homoserine via the intermediacy of aspartate semialdehyde. It is reported that peripheral and central dopamine systems may have a common regulation.[66] Thus, the decreased level of peripheral dopamine likely indicated decreased central dopamine level in schizophrenia patients. Previous studies have also revealed abnormal expressions of neurotransmission and its receptors in animal models and human postmortem brains of schizophrenia.[67-69] These combined findings highlight the potential involvement of disturbed neurotransmission metabolism in the development of schizophrenia.

The results and conclusions of this study should be cautiously interpreted on

account of the following limitations. Firstly, the sample size was relatively small. Thus, multicenter, large-scale trials are required to validate our findings. Secondly, all recruited subjects were of the same ethnicity and from the same site; Ethno- and site-specific biases cannot be ruled out. Finally, the diagnostic performance of the PBMC metabolite biomarker panel was confirmed solely by discriminating schizophrenia subjects from healthy controls. Future studies should recruit patients with other psychiatric disorders such as major depression and bipolar disorder to validate the diagnostic generalizability of these biomarkers.

Conclusion

In this study, using a GC-MS based metabolomic method, a biomarker panel composing of pyroglutamic acid, sorbitol and tocopherol-α was identified, which enabled differentiating schizophrenia patients from healthy controls with high accuracy in both training and test sets. These findings lay the groundwork for the future development of clinical available diagnostic biomarkers for schizophrenia.

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Conflict of interest

All authors declared no financial or other relationship relevant to the subject of this article.

References

1. S. Saha, D. Chant, J. Welham and J. McGrath, *PLoS medicine*, 2005, 2, e141.
2. R. Freedman, *N Engl J Med*, 2003, 349, 1738-1749.
3. W. R. Keller, B. A. Fischer and W. T. Carpenter Jr, *CNS neuroscience & therapeutics*, 2011, 17, 83-88.
4. J. K. Nicholson, J. C. Lindon and E. Holmes, *Xenobiotica*, 1999, 29, 1181-1189.
5. K. K. Pasikanti, P. Ho and E. Chan, *Journal of Chromatography B*, 2008, 871, 202-211.
6. W.-H. Shao, S.-H. Fan, Y. Lei, G.-E. Yao, J.-J. Chen, J. Zhou, H.-B. Xu, H.-P. Liu, B. Wu and P. Zheng, *Metabolomics*, 2013, 9, 433-443.
7. X.-J. Xu, P. Zheng, G.-P. Ren, M.-L. Liu, J. Mu, J. Guo, D. Cao, Z. Liu, H.-Q. Meng and P. Xie, *Molecular BioSystems*, 2014,10, 813-819
8. P. Zheng, H. C. Gao, Q. Li, W. H. Shao, M. L. Zhang, K. Cheng, D. Y. Yang, S. H. Fan, L. Chen and L. Fang, *Journal of proteome research*, 2012, 11, 1741-1748.
9. P. Zheng, H.-C. Gao, Z.-G. Qi, J.-M. Jia, F. F. Li, J. J. Chen, Y. Wang, J. Guo, N. Melgiri and P. Xie, *Metabolomics*, 2013, 9, 688-696.
10. P. Zheng, Y. Wang, L. Chen, D. Yang, H. Meng, D. Zhou, J. Zhong, Y. Lei, N. Melgiri and P. Xie, *Molecular & Cellular Proteomics*, 2013, 12, 207-214.
11. P. Zheng, Y.-D. Wei, G.-E. Yao, G.-P. Ren, J. Guo, C.-J. Zhou, J.-J. Zhong, D. Cao, L.-K. Zhou and P. Xie, *Metabolomics*, 2013, 9, 800-808.

12. M. F. Almstetter, P. J. Oefner and K. Dettmer, *Analytical and bioanalytical chemistry*, 2012, 402, 1993-2013.

13. R. J. Mishur and S. L. Rea, *Mass spectrometry reviews*, 2012, 31, 70-95.

14. K. L. Chang, L. S. New, M. Mal, C. W. Goh, C. C. Aw, E. R. Browne and E. C. Chan, *Journal of proteome research*, 2011, 10, 2079-2087.

15. P. Emond, S. Mavel, N. Aïdoud, L. Nadal-Desbarats, F. Montigny, F. Bonnet-Brilhault, C. Barthélémy, M. Merten, P. Sarda and F. Laumonnier, *Analytical and bioanalytical chemistry*, 2013, 405, 5291-5300.

16. G. Paslakis, M. Deuschle, M. Hartmann and S. Wudy, *Experimental and Clinical Endocrinology & Diabetes*, 2013, 121, P77.

17. D.-C. Wang, C.-H. Sun, L.-Y. Liu, X.-H. Sun, X.-W. Jin, W.-L. Song, X.-Q. Liu and X.-L. Wan, *Neurobiology of Aging*, 2012, 33, 1057-1066.

18. C. Wei, Y. Li, H. Yao, H. Liu, X. Zhang and R. Guo, *Molecular BioSystems*, 2012, 8, 2197-2204.

19. E. Holmes, T. M. Tsang, J. T.-J. Huang, F. M. Leweke, D. Koethe, C. W. Gerth, B. M. Nolden, S. Gross, D. Schreiber and J. K. Nicholson, *PLoS medicine*, 2006, 3, e327.

20. A. Y. Taha, Y. Cheon, K. Ma, S. I. Rapoport and J. S. Rao, *Journal of psychiatric research*, 2013, 47, 636-643.

21. N. J. van Beveren, G. H. Buitendijk, S. Swagemakers, L. C. Krab, C. Röder, L. de Haan, P. van der Spek and Y. Elgersma, *PloS one*, 2012, 7, e32618.

22. A. Gladkevich, H. F. Kauffman and J. Korf, *Progress in*

Neuro-Psychopharmacology and Biological Psychiatry, 2004, 28, 559-576.

23. C.-C. Liew, J. Ma, H.-C. Tang, R. Zheng and A. A. Dempsey, *Journal of Laboratory and Clinical Medicine*, 2006, 147, 126-132.

24. B. Rollins, M. V. Martin, L. Morgan and M. P. Vawter, *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, 2010, 153, 919-936.

25. P. F. Sullivan, C. Fan and C. M. Perou, *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, 2006, 141, 261-268.

26. D. F. Moore, H. Li, N. Jeffries, V. Wright, R. A. Cooper, A. Elkahloun, M. P. Gelderman, E. Zudaire, G. Blevins and H. Yu, *Circulation*, 2005, 111, 212-221.

27. R. Segman, T. Goltser-Dubner, I. Weiner, L. Canetti, E. Galili-Weisstub, A. Milwidsky, V. Pablov, N. Friedman and D. Hochner-Celnikier, *Molecular psychiatry*, 2010, 15, 93-100.

28. Y. Yao, J. Schröder and H. Karlsson, *Journal of psychiatric research*, 2008, 42, 639-643.

29. E. J. Gardiner, M. J. Cairns, B. Liu, N. J. Beveridge, V. Carr, B. Kelly, R. J. Scott and P. A. Tooney, *Journal of psychiatric research*, 2013, 47, 425-437.

30. H.-G. Maxeiner, M. T. Rojewski, A. Schmitt, H. Tumani, K. Bechter and M. Schmitt, *Brain, behavior, and immunity*, 2009, 23, 134-142.

31. U. Meyer, M. J. Schwarz and N. Müller, *Pharmacology & therapeutics*, 2011, 132, 96-110.

32. B. J. Miller, P. Buckley, W. Seabolt, A. Mellor and B. Kirkpatrick, *Biological*

psychiatry, 2011, 70, 663-671.

33. J. Cooper, RCP, 2001.

34. C. M. Micheel, S. J. Nass and G. S. Omenn, *Evolution of translational omics: lessons learned and the path forward*, National Academies Press, 2012.

35. A. Luedemann, K. Strassburg, A. Erban and J. Kopka, *Bioinformatics*, 2008, 24, 732-737.

36. M. Bylesjö, M. Rantalainen, O. Cloarec, J. K. Nicholson, E. Holmes and J. Trygg, *Journal of Chemometrics*, 2006, 20, 341-351.

37. J. Trygg and S. Wold, *Journal of Chemometrics*, 2002, 16, 119-128.

38. S. Mahadevan, S. L. Shah, T. J. Marrie and C. M. Slupsky, *Analytical chemistry*, 2008, 80, 7562-7570.

39. K. Oikonomopoulou, L. Li, Y. Zheng, I. Simon, R. Wolfert, D. Valik, M. Nekulova, M. Simickova, T. Frgala and E. Diamandis, *British journal of cancer*, 2008, 99, 1103-1113.

40. A. P. Bradley, *Pattern recognition*, 1997, 30, 1145-1159.

41. M. Ayalew, H. Le-Niculescu, D. Levey, N. Jain, B. Changala, S. Patel, E. Winiger, A. Breier, A. Shekhar and R. Amdur, *Molecular psychiatry*, 2012, 17, 887-905.

42. S. H. Fatemi and T. D. Folsom, *Schizophrenia bulletin*, 2009, 35, 528-548.

43. P. H. Patterson, *Behavioural brain research*, 2009, 204, 313-321.

44. O. Pogarell, W. Koch, S. Karch, S. Dehning, N. Müller, K. Tatsch, G. Poepperl and H. Möller, *Pharmacopsychiatry*, 2012, 45, S36.

45. M. Herberth, D. Koethe, T. Cheng, N. Krzyszton, S. Schoeffmann, P. Guest, H. Rahmoune, L. Harris, L. Kranaster and F. Leweke, *Molecular psychiatry*, 2011, 16, 848-859.

46. D. Martins-de-Souza, G. Maccarrone, T. Wobrock, I. Zerr, P. Gormanns, S. Reckow, P. Falkai, A. Schmitt and C. W. Turck, *Journal of psychiatric research*, 2010, 44, 1176-1189.

47. J. E. Jensen, J. Miller, P. C. Williamson, R. W. Neufeld, R. S. Menon, A. Malla, R. Manchanda, B. Schaefer, M. Densmore and D. J. Drost, *The British Journal of Psychiatry*, 2004, 184, 409-415.

48. M. Orešič, J. Tang, T. Seppänen-Laakso, I. Mattila, S. E. Saarni, S. I. Saarni, J. Lönnqvist, M. Sysi-Aho, T. Hyötyläinen and J. Perälä, *Genome Med*, 2011, 3, 19.

49. C. L. Clelland, L. L. Read, A. N. Baraldi, C. P. Bart, C. A. Pappas, L. J. Panek, R. H. Nadrich and J. D. Clelland, *Schizophrenia research*, 2011, 131, 139-145.

50. L. Sun, J. Li, K. Zhou, M. Zhang, J. Yang, Y. Li, B. Ji, Z. Zhang, H. Zhu and L. Yang, *PloS one*, 2013, 8, e60598.

51. E. A. Hazlett, M. S. Buchsbaum, E. Kemether, R. Bloom, J. Platholi, A. M. Brickman, L. Shihabuddin, C. Tang and W. Byne, *American Journal of Psychiatry*, 2004, 161, 305-314.

52. W. S. Stone, S. V. Faraone, J. Su, S. I. Tarbox, P. Van Eerdewegh and M. T. Tsuang, *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, 2004, 127, 5-10.

53. T. Fujimoto, K. Takeuch, T. Matsumoto, K. Kamimura, R. Hamada, K. Nakamura and N. Kato, *Psychiatry Research: Neuroimaging*, 2007, 154, 49-58.

54. P. Bubber, V. Hartounian, G. Gibson and J. Blass, *European Neuropsychopharmacology*, 2011, 21, 254-260.

55. D. Martins-de-Souza, L. W. Harris, P. C. Guest and S. Bahn, *Antioxidants & redox signaling*, 2011, 15, 2067-2079.

56. J. E. Jensen, J. Miller, P. C. Williamson, R. W. Neufeld, R. S. Menon, A. Malla, R. Manchanda, B. Schaefer, M. Densmore and D. J. Drost, *Psychiatry Research: Neuroimaging*, 2006, 146, 127-135.

57. J. F. Wang, L. Shao, X. Sun and L. T. Young, *Bipolar disorders*, 2009, 11, 523-529.

58. S. Chittiprol, G. Venkatasubramanian, N. Neelakantachar, S. Babu, N. A. Reddy, K. T. Shetty and B. N. Gangadhar, *Journal of psychiatric research*, 2010, 44, 310-313.

59. J. Flatow, P. Buckley and B. J. Miller, *Biological psychiatry*, 2013, 74, 400-409.

60. M. Raffa, F. Atig, A. Mhalla, A. Kerkeni and A. Mechri, *BMC Psychiatry*, 2011, 11, 124.

61. X. F. Li, Y. L. Zheng, M. H. Xiu, D. C. Chen, T. R. Kosten and X. Y. Zhang, *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 2011, 35, 1064-1067.

62. Z. Wu, X. Y. Zhang, H. Wang, W. Tang, Y. Xia, F. Zhang, J. Liu, Y. Fu, J. Hu and Y. Chen, *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 2012, 36, 34-38.

63. X. Y. Zhang, D. C. Chen, M. H. Xiu, W. Tang, F. Zhang, L. Liu, Y. Chen, J. Liu, J. K. Yao and T. A. Kosten, *Schizophrenia research*, 2012, 139, 66-72.

64. J. Q. Wu, T. R. Kosten and X. Y. Zhang, *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 2013, 46, 200-206.

65. Y. Albayrak, C. Ünsal, M. Beyazyüz, A. Ünal and M. Kuloğlu, *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 2013, 45, 144-149.

66. B. Rubí and P. Maechler, *Endocrinology*, 2010, 151, 5570-5581.

67. L. Kristiansen, R. Cowell, M. Biscaia, R. McCullumsmith and J. Meador-Woodruff, in *Handbook of Neurochemistry and Molecular Neurobiology*, Springer, 2009, pp. 443-492.

68. J. Hirvonen and J. Hietala, *CNS neuroscience & therapeutics*, 2011, 17, 89-96.

69. O. D. Howes and S. Kapur, *Schizophrenia bulletin*, 2009, 35, 549-562.

Figure Legends

Fig. 1. Metabolomic analysis of PBMCs samples from schizophrenia subjects and healthy controls. (a) The score plots of the OPLS-DA model showing a clear discrimination between 45 schizophrenia subjects (green dots) and 50 healthy controls (red triangles) in the training set. (b) Statistical validation of the OPLS-DA model by permutation testing. (c-d) The OPLS-DA model was reconstructed with data from 26 unmedicated schizophrenia subjects (green dots) and 50 healthy controls (red triangles). This regenerated OPLS-DA model was used to classify 19 medicated schizophrenia subjects (blue dots), 15 of 19 schizophrenia subjects were accurately classified.

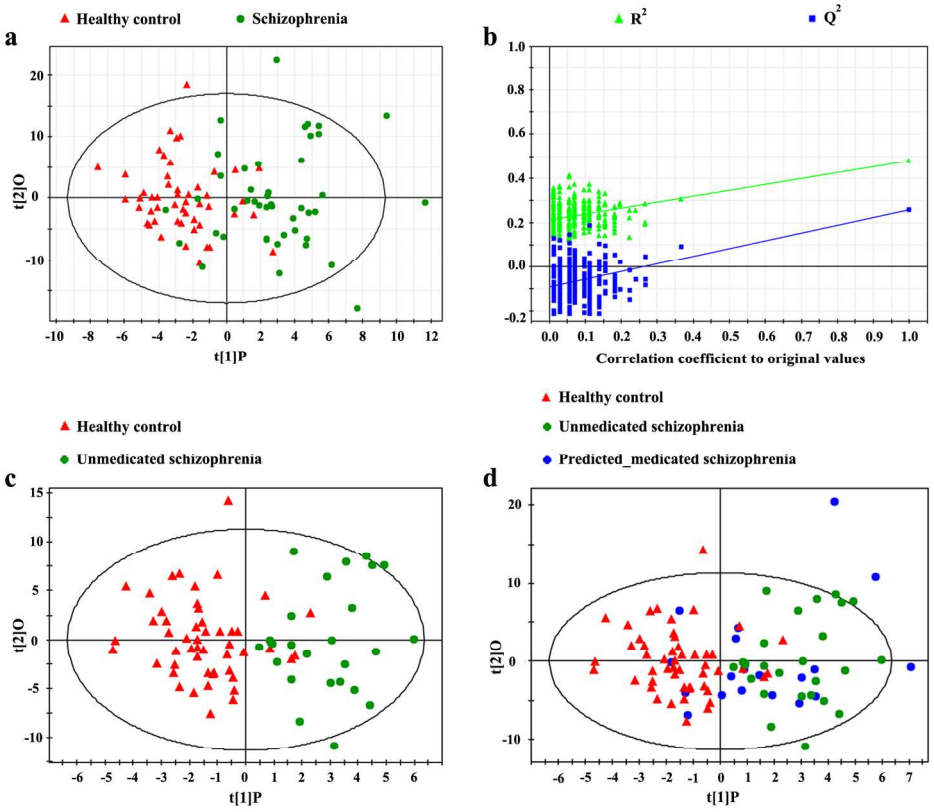
Fig. 2. Assessment of the diagnostic performance of the metabolite biomarkers. (a-b) ROC analysis was performed to quantify the diagnostic performance of biomarkers, yielding an area under the curve of 0.82 in the training set and 0.71 in test set.

Fig. 3. Differential metabolites and enzymes involved in energy metabolism. Red indicates increased metabolites in schizophrenia; green indicates decreased metabolites in schizophrenia; purple indicates upregulated metabolic enzymes in PBMCs of schizophrenia subjects as previous proteomic study reported.[45]

Abbreviations: SZ, schizophrenia; ALDOC, aldolase C; TPIS, triosephosphate isomerase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; PGK1, phosphoglycerate kinase 1; PGAM2, phosphoglycerate mutase 2; PKL, pyruvate kinase; LDHB, lactate dehydrogenase B.

Supplemental Fig.1. (a-c) The concentrations of diagnostic biomarkers in

schizophrenia and healthy control groups. * indicates P＜0.001.



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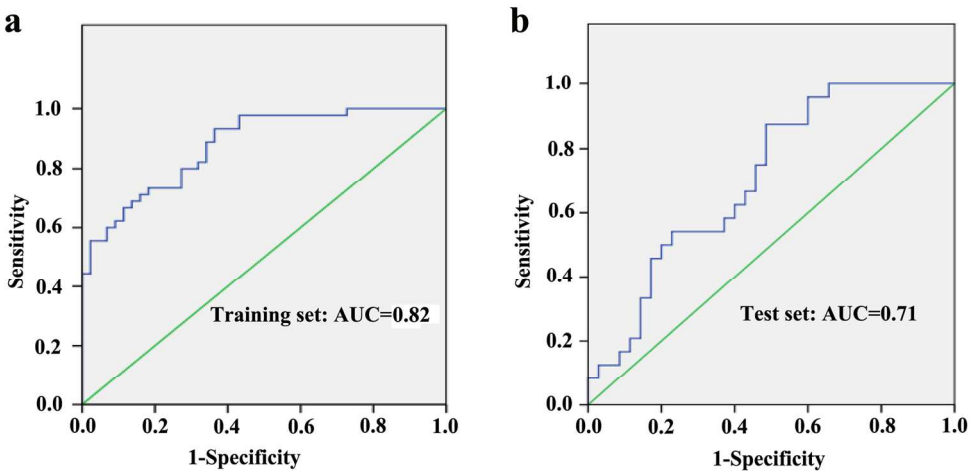
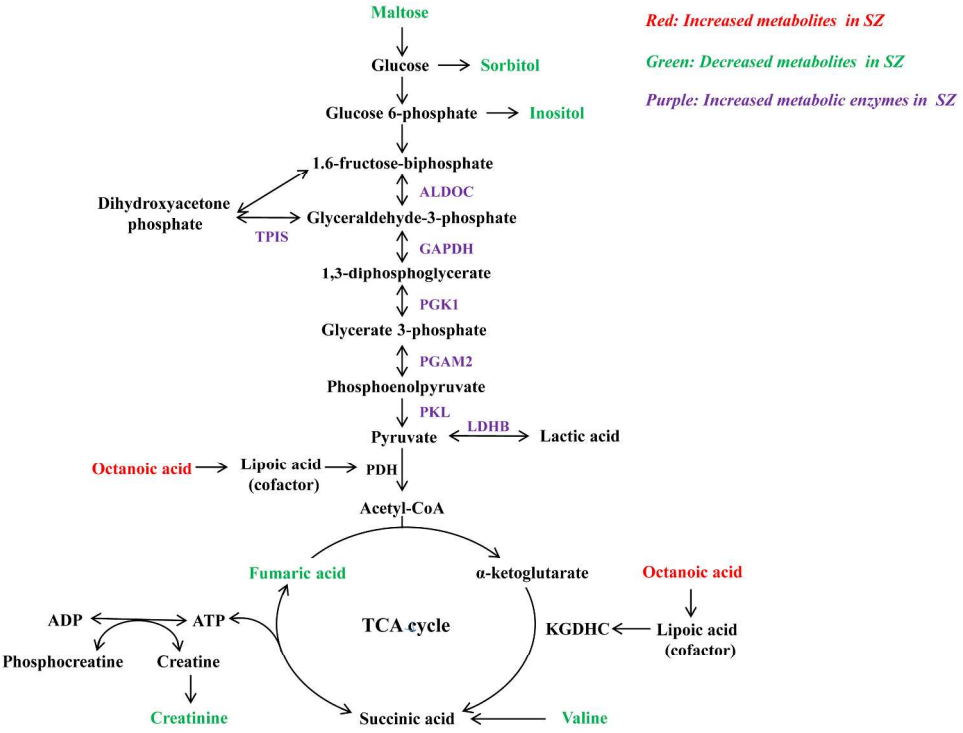


Fig. 2. Assessment of the diagnostic performance of the metabolite biomarkers. (a-b) ROC analysis was performed to quantify the diagnostic performance of biomarkers, yielding an area under the curve of 0.82 in the training set and 0.71 in test set.
180x88mm (300 x 300 DPI)



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247x190mm (300 x 300 DPI)

Table 1. The detailed clinical characteristics of the recruited subjects

	Variables	SCZ	HC	Statistics	dF	P^b
Training set	Sample Size	45	50	_	_	_
	Medication(Y/N)	19/26	N	_	_	_
	Sex(M/F)	18/27	22/28	χ^2=0.16	1.00	0.69
	Age(year)[c]	33.22±12.93	37.26±8.67	t=-1.77	75.70	0.08
Test set 1	Sample Size	24	35	_	_	_
	Medication(Y/N)	Y	N	_	_	_
	Sex(M/F)	5/19	19/16	χ^2=6.60	1.00	0.01
	Age(year)[c]	24.46±10.52	38.86±15.61	t=4.23	56.99	0.00
Test set 2	Sample Size	20	20	_	_	_
	Medication(Y/N)	N	N	_	_	_
	Sex(M/F)	10/10	10/10	χ^2=0.00	1.00	1.00
	Age(year)[c]	31.95±10.22	32.60±3.32	0.27	38	0.79

[a]Abbreviations: SCZ, schizophrenia; HC, healthy controls; Y/N, yes/no; M/F, male/female;

[b]Two-tailed student test for continuous variables (age)

Chi-square analyses for categorical variables (sex)

[c]Values expressed as the mean ± SDs

Table 2. Key metabolites responsible for the discrimination of schizopohrenia subjects and healthy controls

Metabolites	Metabolic pathway	RT(min)	m/z	VIP[a]	t	dF	*p*-value [b]	Fold Change[c]
Octanoic acid	Energy metabolism	8.24	201	2.14	-3.34	65.59	1.39E-03	-0.85
Fumaric acid	Energy metabolism	9.38	245	1.75	3.18	92.98	1.98E-03	0.24
Valine	Energy metabolism	7.66	144	1.18	2.77	93.00	6.82E-03	0.16
Creatinine	Energy metabolism	12.14	115	1.96	2.85	93.00	5.32E-03	0.35
Inositol	Energy metabolism	17.33	318	1.42	2.17	93.00	3.26E-02	0.28
Sorbitol	Energy metabolism	16.39	319	2.51	4.87	93.00	4.65E-06	0.31
Maltose	Energy metabolism	23.35	361	1.40	2.21	56.54	3.12E-02	0.51
Hydroxylamine	Oxidative stress	6.26	133	1.47	-2.22	77.92	2.93E-02	-0.29
Pyroglutamic acid	Oxidative stress	11.7	156	0.98	2.58	92.51	1.15E-02	0.13
Tocopherol-γ	Oxidative stress	24.73	223	1.85	2.89	93.00	4.75E-03	0.34
Tocopherol-α	Oxidative stress	25.71	237	1.57	3.65	93.00	4.39E-04	0.19
Aspartic acid	Neurotransmitter metabolism	10.43	160	1.19	-2.13	73.46	3.67E-02	-1.05
Homoserine	Neurotransmitter metabolism	10.63	218	1.59	-2.39	74.92	1.92E-02	-0.50
Dopamine	Neurotransmitter metabolism	20.93	174	1.53	2.00	93.00	4.89E-02	0.31
Benzoic acid	Others	8.06	179	1.61	-2.57	65.82	1.23E-02	-0.50
2-hydroxyethyl palmitate	Others	19.7	239	0.81	-2.01	72.34	4.86E-02	-0.39
Glycerol	others	8.52	205	1.49	-2.75	67.63	7.64E-03	-0.35
Methyl phosphate	others	7.15	241	1.40	2.68	93.00	8.73E-03	0.22

[a] Variable importance in the projection (VIP) was obtained from OPLS-DA with a threshold of 1.0.

[b] *p*-values were derived from two-tailed Student's t test.

[c] Values greater than 0 indicate lower levels in schizophrenia patients relative to healthy controls; values less than 0 indicate higher levels in schizophrenia patients relative to healthy controls.