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Serum metabolic profiling study of endometriosis by using wooden-tip electrospray ionization mass spectrometry

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Abstract

A high throughput metabolite fingerprinting tool based on wooden-tip electrospray ionization mass spectrometry (WT-ESI-MS) has been established for serum metabolic profiling study of endometriosis with little sample pre-treatment, no chromatography and instrument cycle times of less than 5 min. Serum samples from endometriosis patients and healthy controls were analyzed by direct WT-ESI-MS with a high resolution ESI-Q-TOF-MS. The resulting data were analyzed by multivariate data analysis. MS/MS experiments were carried out to identify potential biomarkers. The global metabolic profiling and subsequent multivariate analysis clearly distinguished endometriosis patients from healthy controls. The total of ten metabolites, up-regulated or down-regulated, were identified and contributed to endometriosis progress. These promising identified biomarkers underpinning the metabolic pathway including steroid hormone biosynthesis, glycerophospholipid metabolism, sphingolipid metabolism, pyruvate metabolism, bile acid biosynthesis, androgen and estrogen metabolism. Considering that a much higher throughput can be obtained without a chromatographic step, the present WT-ESI-MS method could be developed as a fast prognostic or diagnostic method for endometriosis.

key words: WT-ESI-MS, endometriosis, metabolomics, biomarkers

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Introduction

Metabolomics, based on the dynamic changes of low molecular weight metabolites in organisms, indicates the overall physiological status in responding to pathophysiological stimuli or genetic, environmental, or lifestyle factors.[1, 2] Metabolic profiling has attracted an interest for biomarker discovery and for investigating the pathogenesis of diseases. The development of metabolomics requires high resolution analytics.[3] No single analytical platform can offer a fully comprehensive survey of the chemical diversity representing the metabolome.[4] Of various analytical techniques, mass spectrometry (MS) method has been widely used, because of their high sensitivity, good specificity, and high accuracy.[5]

There is a trade-off between comprehensive sample analysis and high sample throughput in MS-based metabolomics.[6] Traditional approaches for metabolomics analysis by mass spectrometry involve the use of liquid (LC–MS) or gas (GC–MS) chromatography to first attempt to separate metabolites before detection. Although being beneficial to comprehensive analysis, the chromatographic step limits the throughput, especially when the number of sample sets is large.[7] Furthermore, these metabolite "profiling" methods demand careful control over the chromatographic process to ensure reproducibility and require significant time, effort and expertise for data pre-processing in order to deconvolve, align and annotate peaks correctly. Unfortunately, any chromatography column matrix will undergo gradual detoriation with repetative use, resulting in significant changes in data characteristics after a period of constant operation in larger (>200 samples) profiling experiments.[8] Therefore, development a spectrometric "fingerprint" without recourse to any chromatographic separation is highly beneficial to capture information relating to total metabolite.

Electrospray ionization mass spectrometry (ESI-MS) is a useful analytical tool for the analysis of complex mixtures, providing information on the molecular weights and chemical structures of the analytes. In conventional ESI, a sample solution is introduced into a capillary and usually with the assistance of gas. In the late 1990s, use of a copper wire as solid-substrate ESI emitter was firstly introduced by Shiea et

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al. [9] Recent non-capillary ESI techniques with solid substrates, such as metal needle,[10] wooden tip,[11] paper,[12] aluminium foil,[13] and other solid materials[14-17] have been successfully developed as emitters for ESI and applied for direct analysis of complex solid materials or liquid aerosols without the need for sample pre-treatment or extraction.

Endometriosis is a common, chronic gynaecological disease affecting up to 10% of women in their reproductive years.[18] The diagnosis of endometriosis can be suspected in women with pelvic pain and/or subfertility, although endometriosis may be completely asymptomatic.[19] Clinical detection of abdominal or pelvic pain can be suggestive of endometriosis. Vaginal ultrasound is an adequate diagnostic method to detect ovarian endometriotic cysts and deeply infiltrative endometriotic noduli, but does not rule out peritoneal endometriosis or endometriosis-associated adhesions. The gold standard for the diagnosis of endometriosis is laparoscopic inspection, ideally with histological confirmation [19, 20]. Development of a non-invasive diagnostic test for endometriosis would have a groundbreaking impact on the patients' quality of life, on the efficacy of available treatment as well as on the cost of endometriosis.[21] However, non-invasive approaches such as ultrasound, magnetic resonance imaging or blood tests have not yielded sufficient power for the diagnosis of endometriosis.[22, 23] A noninvasive diagnostic test in easily accessible fluid (i.e., plasma, serum, urine) would be beneficial to both physicians and patients but does not exist.[24, 25] Once diagnosed, the options for treatment of endometriosis include non-invasive medical therapy and surgery.[26-28] The use of a biomarker may help to reduce the need for diagnostic surgery in some women, enable monitoring of the disease progression by non-surgical methods, and potentially allow for better pre-operative assessment of women with endometriosis.[29]

In this study, a simple wooden-tip ESI-MS (WT-ESI-MS) was adopted for serum metabolic profiling study of endometriosis patients and healthy controls (Figure 1). The resulting data were analyzed by multivariate data analysis. MS/MS experiments were carried out to identify potential biomarkers, the aims are to develop a novel strategy for rapid distinguish endometriosis patients from healthy controls, to identify

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120 potential biomarkers and to explore the potential mechanisms of endometriosis.

121 **Experimental**

122 **Sample collection and preparation**

123 Serum samples during proliferative phase were obtained from 22 patients (range 124 31-48 years) who had been diagnosed with endometriosis by laparoscopy and 125 confirmed by pathologic examination (stages I-IV) at the Jiangxi Provincial Maternal 126 And Child Health Hospital. Another 25 age matched fertile women who were selected 127 from the health examination center of the Jiangxi Provincial Maternal And Child 128 Health Hospital were regarded as the control group. The sampling conditions between 129 endometriosis patients and healthy controls are identical. These control women were 130 healthy (with neither clinical symptoms of endometriosis nor any abnormality 131 detected during the clinical examination) according to physical examination, 132 transvaginal ultrasound, and biochemical blood tests. Irregular cycling, amenorrheic 133 postmenopausal women and those who had received steroid hormone therapy in the 134 last 6 months were excluded from the study. All patients signed a written informed 135 consent form and agreed to the collection of tissues for research. This study was 136 approved by the local ethics committee of Jiangxi Provincial Maternal And Child 137 Health Hospital.

138 Overnight fasting blood samples were obtained by venipuncture, and the samples 139 were centrifuged at 2000 g for 10 min. Serum aliquots of approximately 500 μl were 140 then transferred into sterile cryovials, frozen and stored at -80 °C.

141 **Wooden-tip ESI-MS analysis**

142 In this study, a simple wooden-tip ESI-MS (WT-ESI-MS) was adopted for analysis 143 of serum. The wooden toothpicks (purchased from Nanchang supermarket) were 144 manufactured by SunChua wood and bamboo company (Zhejiang, China). The 145 wooden toothpicks have a length of ～5cm, one end with an o.d. of ～2mm, the 146 other sharp-end with an o.d. of ～0.2mm. The disposable wooden tips used are cheap, 147 are readily available, and can be directly mounted on commercial nano-ESI ion source 148 device; the angle between wooden tip and the MS inlet was ninety degrees like 149 previous methods.[11, 30-32] Briefly, 2 μl serum samples were loaded to the sharp tip-end

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150 by pipetting. Upon application of a high voltage (+3.5 kV) to the wooden tip, spray
151 ionization was generated and mass spectrum was observed by a quadrupole
152 time-of-flight mass spectrometer (Q-TOF-MS) (Waters, Manchester, U.K.). The mass
153 scan range of Q-TOF-MS is m/z: 10-1000, scan rate is 0.2 second per spectrum. The
154 positive ion mode was used because more compounds can be ionized in this mode and
155 because it is more widely used in serum metabolite profiling.

156 According to the previous studies and our experimental investigation,[11, 30-32] in
157 general, when wooden tips were used for sample loading and ionization, no
158 significant background noises and interfering mass peaks were observed, probably
159 because of wooden tip is inert material. The chemical noises might become more
160 significant when the sample solution is approaching completely consumed, but this
161 usually did not remarkably affect the spectral quality after averaging of the whole
162 mass spectrum. To further reduce chemical noises in this study, the experimental data
163 were averaged within the first two minutes. Wooden-tip ESI-MS not only allows less
164 sample consumption, but also to avoid the clogging problem in conventional capillary
165 ESI and for more convenient sample loading. Longer duration of ion signals could be
166 obtained by introducing a larger volume of sample solution on the tip. In this study, 2
167 μl serum sample is sufficient for MS analysis, and the rest of sample, for other
168 purposes, e.g. proteomics research, is being investigated . To avoid the
169 cross-contamination, a blank run was inserted between sample runs.

170 **Date processing**

171 After data acquisition by using WT-ESI-MS, the data mining algorithms were
172 followed by using the program of MassLynx Workstation software (Version 4.1,
173 Waters). Firstly, background subtract algorithm was utilized to reduce the chemical
174 noise. The algorithm fitted well with a polynomial of specified order to a spectrum: a
175 specified percentage (usually 30–50%) of the data points was positioned below the
176 polynomial. Then, Spectra Smooth algorithm was used to reduce the high-frequency
177 noise present in a spectrum. In averaging the data, the smoothing method was sliding
178 a window along the data as to produce a point in the smoothed spectrum. After that, a
179 peak centering process was used either to label each peak with the calculated mass, or

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180 to produce a single bar from each peak in a continuum spectrum. The feature
181 detection performed a combination of background subtraction, smoothing and
182 centering all in one command. Then, all the ions above the MS threshold (200 counts)
183 were found and extracted as directed features. The ion list was exported to an EXCEL
184 file, which included information of the m/z and intensity. Each run, including blanks,
185 was set as a dataset.

186 Metabolomic data normalization was usually necessary and had been done prior to
187 statistical analysis and pathway analysis by means of MetaboAnalyst (a web service
188 for metabolomic data analysis). There are two major types of data normalization
189 provided by the MetaboAnalyst to make the data follow the Gaussian distribution as
190 closely as possible. The parameters of data normalization could be adjusted timely,
191 since the results of data normalization can be visualized with kernel density plots and
192 box plots of the data distributions. Row-wise normalization (samples in row) aims to
193 reduce systematic bias from samples, while column-wise normalization (variables in
194 column) attempts to make each variable comparable to others from the same sample.

195 Following normalization, Principal Component Analysis (PCA) and Partial Least
196 Squares Discriminant Analysis (PLS-DA) were performed using web-based
197 metabolomic data processing tool MetaboAnalyst 2.0 .[33] In MetaboAnalyst, statistical
198 computing and visualization operations are performed using functions from the R and
199 Bioconductor packages. PCA is used to detect intrinsic clusters and outliers within the
200 data set, while PLS-DA maximizes class discrimination. In PLS-DA model,
201 metabolite ions with VIP (variable importance in the projection) > 1.0 were selected
202 as differential ions beneficial to the phenotype classification in the study, and a 1000
203 permutation test was implemented to validate the reliability of the model because of
204 its propensity to overestimation of the separation performance, which could be
205 inspected by permutation tests, but not always by cross-validation .[34]

206 **Metabolites identification**

207 The identities of the metabolites were confirmed by the library search and the
208 available confirmation standard based on the information of mass spectra. The Mass
209 Fragment application manager (Waters MassLynx v4.1, Waters corp., Milford, MA)

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was used to facilitate the MS/MS fragment ion analysis process by way of chemically intelligent peak-matching algorithms. This information was then submitted for database searching, either in-house or using the online ChemSpider database (www.chemspider.com), data source. Moreover, the identified metabolites were retrieved in HMDB (Human Metabolome Database, http://www.hmdb.ca/) for auxiliary confirmation of structures and the biological functions.

Metabolic pathway analysis

Detailed analysis of the most relevant metabolic pathways and networks in women with endometriosis was performed by Metaboanalyst that combines results from powerful pathway enrichment analysis involved in the conditions under study. Metaboanalyst uses high-quality HMDB metabolic pathways as the back end knowledge base. It integrates many well-established methods and novel algorithms and concepts with pathway analysis.

Results and discussion

Analytical performance of serum metabolic profiling

Having wooden-tip ESI-MS to become a potential high-throughput ionization method for metabolomics analysis without sample cleanup or chromatography, it must be able to meet some important and stringent requirements. These requirements include reproducibility, sensitivity, linearity, and cross contamination or memory effects.[35] Ion formation in wooden-tip ESI-MS did not result in serious cross contamination and memory effects, because of the use of disposable wooden tips for loading and ionization of samples. Meanwhile, previous study demonstrated the sensitivity and linearity of wooden-tip ESI-MS were high and acceptable.[15] In metabolomics analysis, a subtle, gradual change in the performance of the system will lead to a result which may be closely related to the run order, rather than any differences in the samples, so false negative results were more acceptable than false positive results;[36] therefore, reproducibility should be considered first and fully validated, even if sensitivity is sacrificed. A reliable MS method with adequate reproducibility is critical for metabolomics analysis.[37] One example of the reproducibility test was shown in Figure 2. In this test, a total of nine injections of an

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identical serum sample were analyzed by wooden-tip ESI-MS. Then, three representative ions of serum were chosen for evaluation the reproducibility of this method. The coefficients of variance of their peak height were between 11.46% to 14.48%, which was acceptable for the metabolomics analysis. Hence, the reproducibility and stability of global experimental performances were high and acceptable, and that the variances from the samples were likely the real reflection of metabolic differences underlying the biological systems rather than the artifactual biases from instrumental analysis.

Metabolic Profiling Analysis

Representative mass spectra of serum collected from endometriosis patients and healthy controls in positive modes were shown in Figure 3. A total of 423 ions in serum samples at positive mode were detected from endometriosis patients and healthy controls. The unsupervised principal component analysis (PCA) model was used to separate serum samples into two blocks between endometriosis patients and healthy controls. As can been shown in Figure 4 A and B, it was evident in PCA model that endometriosis patients samples could separated from healthy control samples. PCA scores plots showed clear clustering of endometriosis patients samples versus healthy control samples.

To improve the separation and find the differentially produced metabolites, a partial least squares discriminant analysis (PLS-DA) was used to process the datasets. The score plots are shown in Figure 4 C and D. The endometriosis patients are clearly distinguished from healthy controls using their serum metabolites. Leave-one-out cross-validation (LOOCV) was used, from which Q^2 and R^2 values representing predictive capability and explained variance, respectively, were extracted. The model with $R^2 = 0.67$ and $Q^2 = 0.43$ showed a reasonable predictive ability. Moreover, using 1000 permutation test, it was obvious that B/W ratio (ratio of sum of squares between groups to that within group) of the original classes was markedly different from the distribution of permuted data ($p < 0.001$), indication that the good separation performance was achieved by analyzing real metabolic signals but not random noises, and the results of cross validation were reliable (Figure 5 A). Prediction accuracy

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during training using 1000 permutation, the p value is reported as $p < 0.001$, denoting that none of the results are better than the original one (Figure 5 B).

A staging system has been developed by the American Society of Reproductive Medicine. The stage of the endometriosis does not relate to the level of pain experienced, risk of infertility, or symptoms present. For example, it is possible for a woman in stage I to be in tremendous pain, while a woman in stage IV may be asymptomatic.[38] In this study, the global metabolic profiling and subsequent multivariate analysis did not distinguish endometriosis patients of different stages.

Identification of potential biomarkers

As an example, we took m/z 546.6878 to illustrate the marker identification process. At first, an accurate mass of the marker ($[M + Na]^+$ at m/z 546.6878) was found from the mass spectrum. Secondly, particular MS/MS information about fragmentation pattern of the marker was acquired from the Q–TOF system. Under positive ion mode, MS/MS figure contains fragment ion 487.6833([M +Na -N(CH3)3]+), 279.9414 ([Palmitic acid +Na]+). It can be inferred that this marker might be a lysophosphatidylcholine (LPC). Finally, the database of Human Metabolome was searched based on the clues we got from the above process. As a result, the marker was identified as LysoPC (18:0).

According to the protocol detailed above, ten endogenous metabolites, contributing to the separation between endometriosis patients and healthy controls, were identified (Table 1). The precise molecular mass was determined within measurement errors (<5 ppm) by Q-TOF. Compared with healthy controls, there are up regulated metabolites in endometriosis patients, including CE (16:0), CE (18:2(9Z,12Z)), SM (d18:1/16:0), TAG (52:3), TAG (54:4). While the significantly down regulated ones were LysoPC (16:0), LysoPC (18:0), PC (34:2), PC (36:2), PC (38:8).

It should be noted that most of the detected species in serum by WT-ESI-MS are lipids, and their distribution gives sufficient information to distinguish between endometriosis patients and healthy controls. Similar to the results obtained by WT-ESI-MS for animal tissue analysis, lipids were predominantly observed in the spectrum.[15] Lipids are very abundant in biological systems, constitute ~50% of the

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mass of most animal cell membranes, and exhibit crucial roles in cellular energy storage, structure and signaling.[39] Lipid homeostasis is fundamental to maintain health, and lipid defects are central to the pathogenesis of important and devastating diseases.[40] WT-ESI-MS metabolite fingerprinting may be utilized in situations where there is a need for a high-throughput screening method with comprehensive coverage of metabolite diversity that allows sample classification or discrimination according to their origin or biological relevance. With an analytical cycle time of typically 3-5 min per sample, it is considered that WT-ESI-MS metabolite fingerprinting in particular is an ideal choice for high throughput "first pass" screening analysis. Following "first pass" screening stage more time consuming chromatographic separation by LC or CE can be used to obtain a more complete metabolic profiling if necessary.

Metabolic pathway and function analysis

It was found from pathway analysis that metabolic regulations associated with endometriosis were chiefly involved in steroid hormone biosynthesis, glycerophospholipid metabolism, sphingolipid metabolism, pyruvate metabolism, bile acid biosynthesis, androgen and estrogen metabolism (Figure 6). The pathogenesis of endometriosis associated with metabolic disturbance can been shown figure 7 based on our researches and literatures. It was implied that the pathway of steroid hormone biosynthesis, glycerophospholipid metabolism, sphingolipid metabolism, pyruvate metabolism, bile acid biosynthesis, androgen and estrogen metabolism in serum were disturbed. In addition, thirteen endogenous metabolites, contributing to the separation between endometriosis patients and healthy controls were identified. These promising biomarkers candidates verified that the pathogenesis of endometriosis is closely related to multiple etiology and pathogenesis. On the basis of these findings, further studies have to be performed in order to validate the changes and targeted metabolites.

Conclusion

A high throughput metabolite fingerprinting tool based on WT-ESI-MS has been established for serum metabolic profiling study of endometriosis. This approach is employed without recourse to chromatographic separation make it particularly attractive when dealing with large sample sets. The global metabolic profiling and

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subsequent multivariate analysis clearly distinguished endometriosis patients from healthy controls. WT-ESI-MS offers a very powerful "first pass" screening of large sample populations. Considering that a much higher throughput can be obtained without a chromatographic step, the present WT-ESI-MS method could be developed as a fast prognostic or diagnostic method for endometriosis.

Conflict of interest

The authors declare that they are no conflicts of interest.

Acknowledgments

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References

1. J. K. Nicholson, J. C. Lindon and E. Holmes, *Xenobiotica*, 1999, **29**, 1181-1189.
2. O. Fiehn, *Plant Mol. Biol.*, 2002, **48**, 155-171.
3. J. Szpunar, *Analyst*, 2005, **130**, 442-465.
4. R. Goodacre, S. Vaidyanathan, W. B. Dunn, G. G. Harrigan and D. B. Kell, *Trends Biotechnol.*, 2004, **22**, 245-252.
5. K. Dettmer, P. A. Aronov and B. D. Hammock, *Mass Spectrom. Rev.*, 2007, **26,** 51-78.
6. L. Lin, Q. Yu, X. Yan, W. Hang, J. Zheng, J. Xing and B. Huang, *Analyst*, 2010, **135**, 2970-2978.
7. W. B. Dunn, S. Overy and W. P. Quick, *Metabolomics*, 2005, **1**, 137-148.
8. J. Draper, A. J. Lloyd, R. Goodacre and M. Beckmann, *Metabolomics*, 2013, **9**, S4-S29.
9. C. M. Hong, C. T. Lee, Y. M. Lee, C. P. Kuo, C. H. Yuan and J. Shiea, *Rapid Commun. Mass Sp.*, 1999, **13**, 21-25.
10. K. Hiraoka, K. Nishidate, K. Mori, D. Asakawa and S. Suzuki, *Rapid Commun. Mass Sp.*, 2007, **21**, 3139-3144.
11. B. Hu, P.-K. So, H. Chen and Z.-P. Yao, *Anal. Chem.*, 2011, **83**, 8201-8207.
12. J. Liu, H. Wang, N. E. Manicke, J.-M. Lin, R. G. Cooks and Z. Ouyang, *Anal. Chem.*, 2010, **82**, 2463-2471.
13. B. Hu, P. K. So and Z. P. Yao, *Anal. Chim. Acta*, 2014, **817**, 1-8.
14. J. J. Liu, H. Wang, R. G. Cooks and Z. Ouyang, *Anal. Chem.*, 2011, **83**, 7608-7613.
15. B. Hu, Y.-H. Lai, P.-K. So, H. Chen and Z.-P. Yao, *Analyst*, 2012, **137**, 3613-3619.
16. H. K. Chen, C. H. Lin, J. T. Liu and C. H. Lin, *Int. J. Mass Spectrom.*, 2013, **356**, 37-40.
17. B. Hu, L. Wang, W. C. Ye and Z. P. Yao, *Sci. Rep.-Uk*, 2013, **3,** 2104-2111
18. L. C. Giudice and L. C. Kao, *Lancet*, 2004, **364,** 1789-1799.
19. S. Kennedy, A. Bergqvist, C. Chapron, T. D'Hooghe, G. Dunselman, R. Greb, L. Hummelshoj, A. Prentice and E. Saridogan, *Hum. Reprod.*, 2005, **20,** 2698-2704.

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368 20. S. Kennedy, A. Bergqvist, C. Chapron, T. D'Hooghe, G. Dunselman, R. Greb, L. Hummelshoj, A. Prentice, E. Saridogan and E. S. I. G. Endomet, *Hum. Reprod.*, 2005, **20,** 2698-2704.
370 21. E. Somigliana, P. Vigano, A. Tirelli, I. Felicetta, E. Torresani, M. Vignali and A. Di Blasio, *Hum. Reprod.*, 2004, **19,** 1871-1876.
372 22. K. Ballard, K. Lowton and J. Wright, *Fertil. Steril.*, 2006, **86,** 1296-1301.
373 23. A. Mihalyi, O. Gevaert, C. Kyama, P. Simsa, N. Pochet, F. De Smet, B. De Moor, C. Meuleman, J. Billen and N. Blanckaert, *Hum. Reprod.*, 2010, **25,** 654-664.
375 24. K. Kinkel, K. A. Frei, C. Balleyguier and C. Chapron, *Eur. Radiol.*, 2006, **16,** 285-298.
376 25. E. Janssen, A. Rijkers, K. Hoppenbrouwers, C. Meuleman and T. D'Hooghe, *Hum. Reprod. Update*, 2013, **19,** 570-582.
378 26. K. Vouk, N. Hevir, M. Ribic-Pucelj, G. Haarpaintner, H. Scherb, J. Osredkar, G. Moeller, C. Prehn, T. L. Rizner and J. Adamski, *Hum. Reprod.*, 2012, **27,** 2955-2965.
380 27. M. Dutta, M. Joshi, S. Srivastava, I. Lodh, B. Chakravarty and K. Chaudhury, *Mol. Biosyst.*, 2012, **8,** 3281-3287.
382 28. S. K. Jana, M. Dutta, M. Joshi, S. Srivastava, B. Chakravarty and K. Chaudhury, *BioMed Res. Int.*, 2013, **2013,** 329058-329058.
384 29. T. L. Rizner, *Expert Rev. Mol. Diagn.*, 2014, **14,** 633-633.
385 30. B. Hu, P.-K. So and Z.-P. Yao, *J. Am. Soc. Mass Spectrom.*, 2013, **24,** 57-65.
386 31. P.-K. So, T.-T. Ng, H. Wang, B. Hu and Z.-P. Yao, *Analyst*, 2013, **138,** 2239-2243.
387 32. B.-c. Yang, F.-y. Liu, J.-b. Guo, L. Wan, J. Wu, F. Wang, H. Liu and O.-p. Huang, *Anal. Methods-UK*, 2015, **7,** 2913-2916.
389 33. J. Xia, R. Mandal, I. V. Sinelnikov, D. Broadhurst and D. S. Wishart, *Nucleic Acids Res.*, 2012, **40,** 127-133.
391 34. J. A. Westerhuis, H. C. Hoefsloot, S. Smit, D. J. Vis, A. K. Smilde, E. J. van Velzen, J. P. van Duijnhoven and F. A. van Dorsten, *Metabolomics*, 2008, **4,** 81-89.
393 35. G.-Z. Xin, B. Hu, Z.-Q. Shi, Y. C. Lam, T. T.-X. Dong, P. Li, Z.-P. Yao and K. W. Tsim, *Anal. Chim. Acta*, 2014, **820,** 84-91.
395 36. K. Lan and W. Jia, *Curr. Drug Metab.*, 2010, **11,** 105-114.
396 37. Y.-Q. Huang, J.-Q. You, B.-F. Yuan and Y.-Q. Feng, *Analyst*, 2012, **137,** 4593-4597.
397 38. G. D. Adamson, *Curr. Opin. Obstet. & Gyn.*, 2013, **25,** 186-192.
398 39. M. Oresic, V. A. Hanninen and A. Vidal-Puig, *Trends Biotechnol.*, 2008, **26,** 647-652.
399 40. Z. Takats, J. M. Wiseman and R. G. Cooks, *J. Mass Spectrom.*, 2005, **40,** 1261-1275.

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Figure legends

Figure 1 WT-ESI-MS-based metabolomic platform.

Figure 2 Reproducibility test of nine repeated injections of serum sample from endometriosis patients by wooden-tip ESI-MS, the average % coefficient of variation (CV) of three representative peak heights is 12.57%.

Figure 3 Representative mass spectra of serum from (A) healthy control (B) endometriosis patient at positive ESI mode.

Figure 4 PCA and PLS-DA model results of MS data between endometriosis patients and healthy controls in positive modes. (A) 2-D PCA scores plot; (B) 3-D PCA scores plot; (C) 2-D PLS-DA scores plot; (D) 3-D PLS-DA scores plot.

Figure 5 Permutation test statistics at 1000 permutations with observed statistic at $P<0.001$. (A) separation distance (B/W); (B) Prediction accuracy during training.

Figure 6 Pathway analysis of based on pathway-matched identified differential metabolites.

Figure 7 Proposed pathogenesis of endometriosis with metabolic disturbance. sphingosine-1-phosphate (S-1P), lysophosphatidylcholine (LPC), lysophosphatidic acid (LPA), cyclo-oxygenase-2 (COX-2), androstenedione (A), estrone (E_1), estradiol (E_2), vascular endothelial growth factor (VEGF), steroidogenic acute regulatory protein (StAR), 17β-Hydroxysteroid dehydrogenase type 1 (17-βHSD1), 17β-Hydroxysteroid dehydrogenase type 2 (17-βHSD2).

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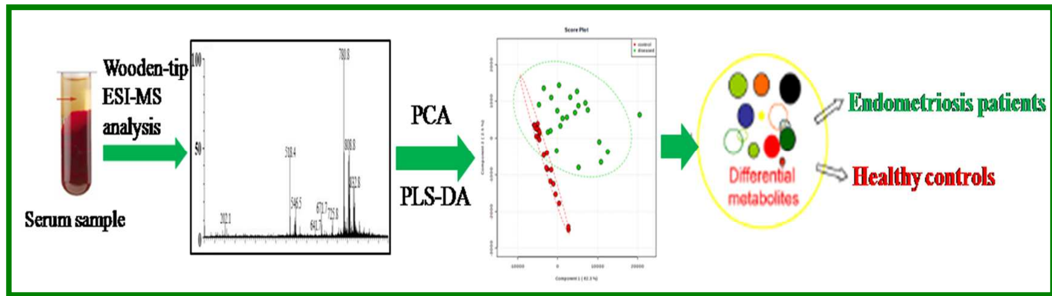
1 2 3 447 Figure 1



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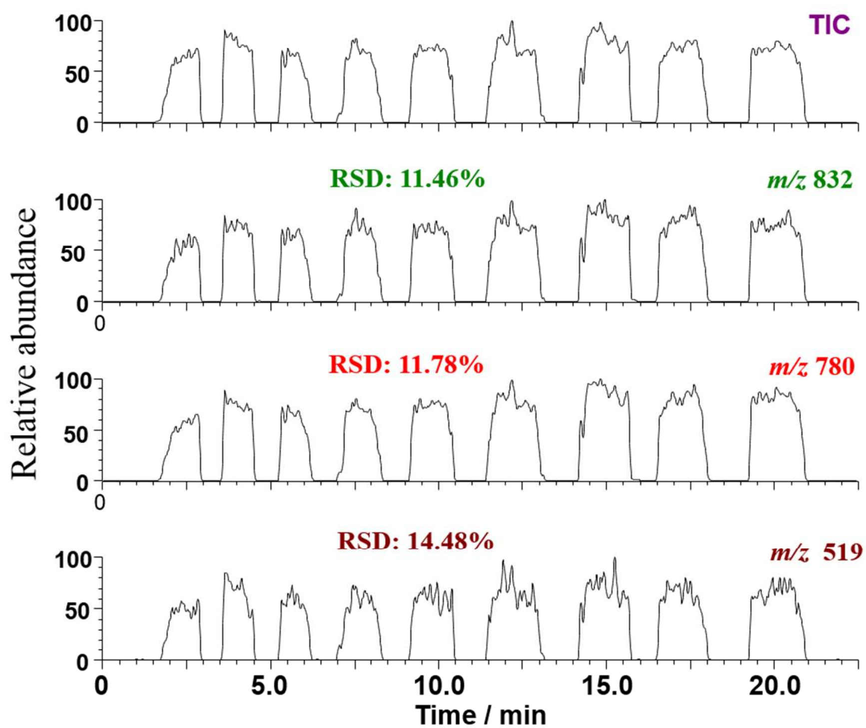
484 Figure 2



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508 Figure 3

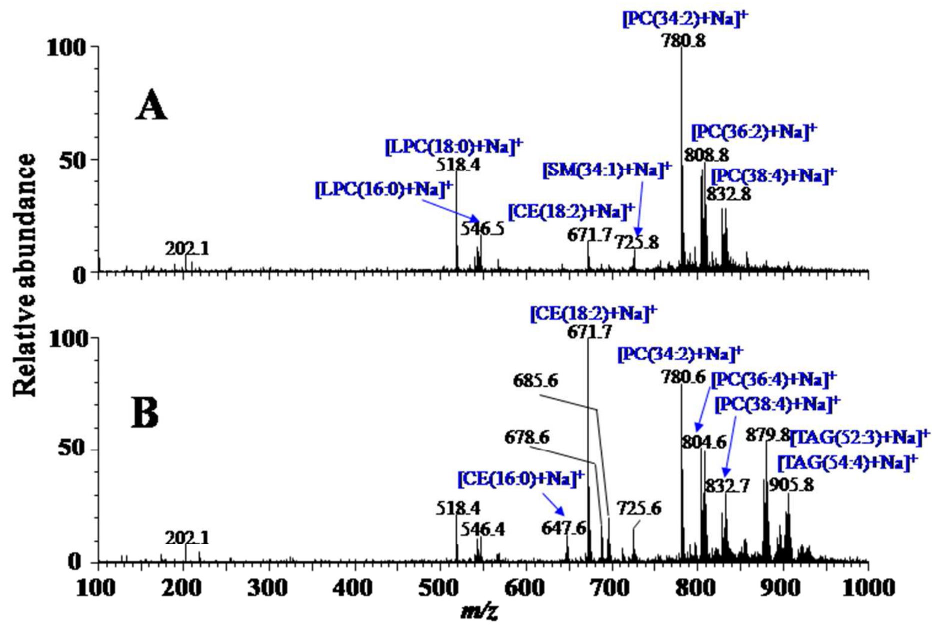

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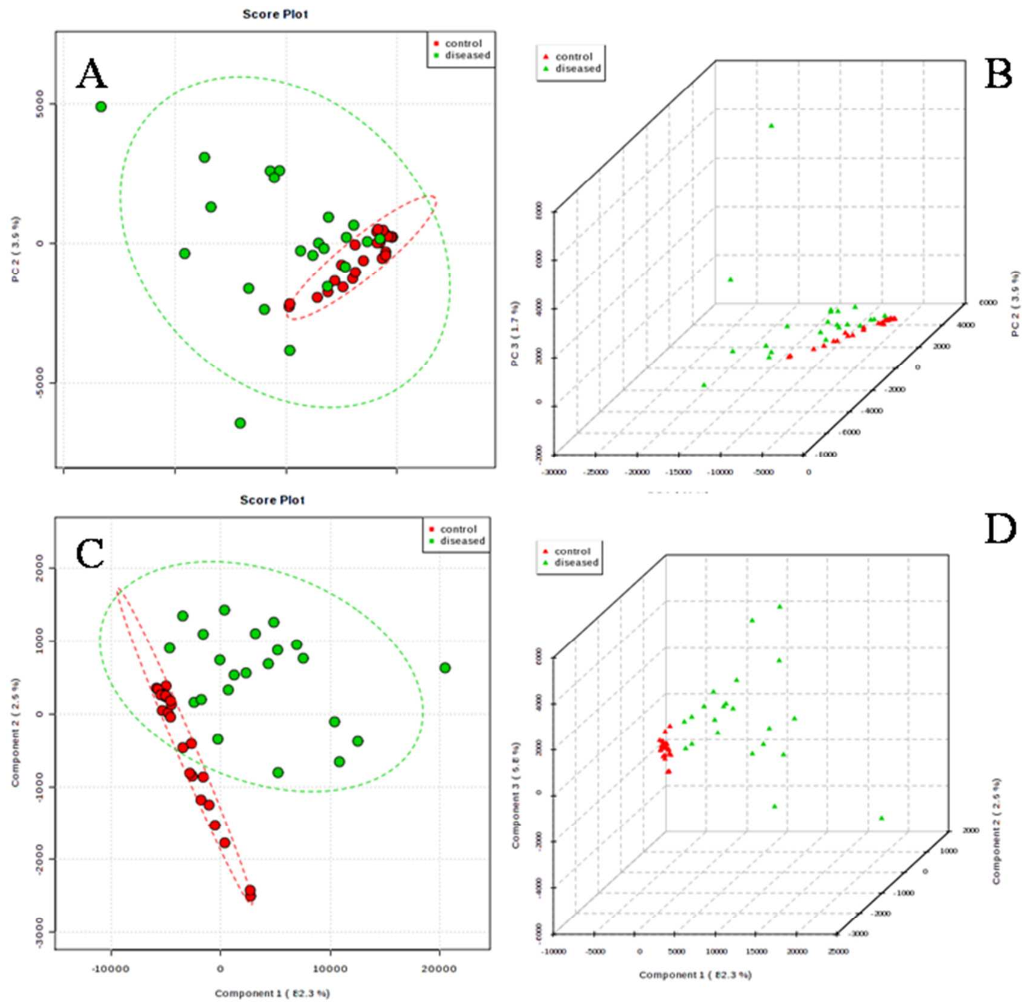
Figure 4



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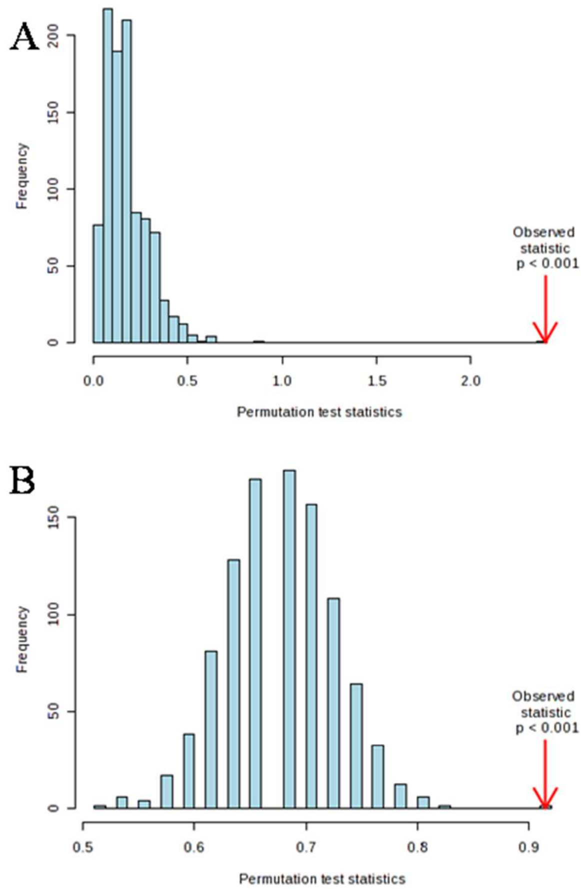
549 Figure 5



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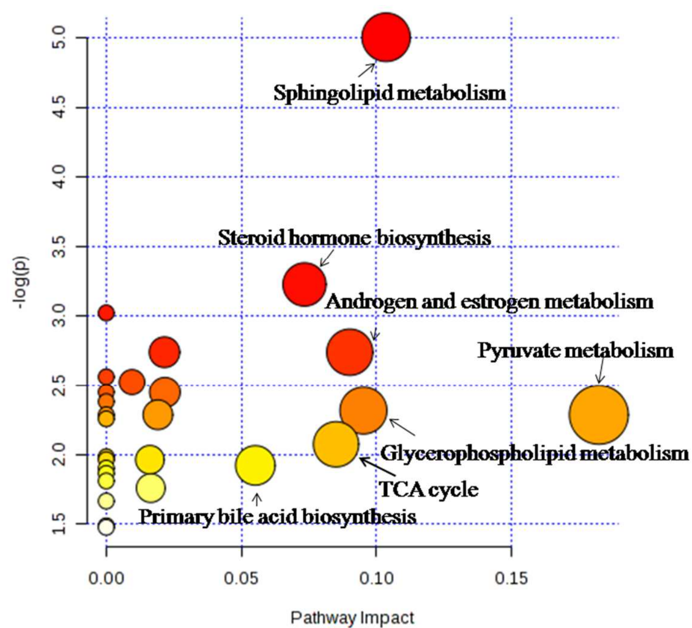
564 Figure 6



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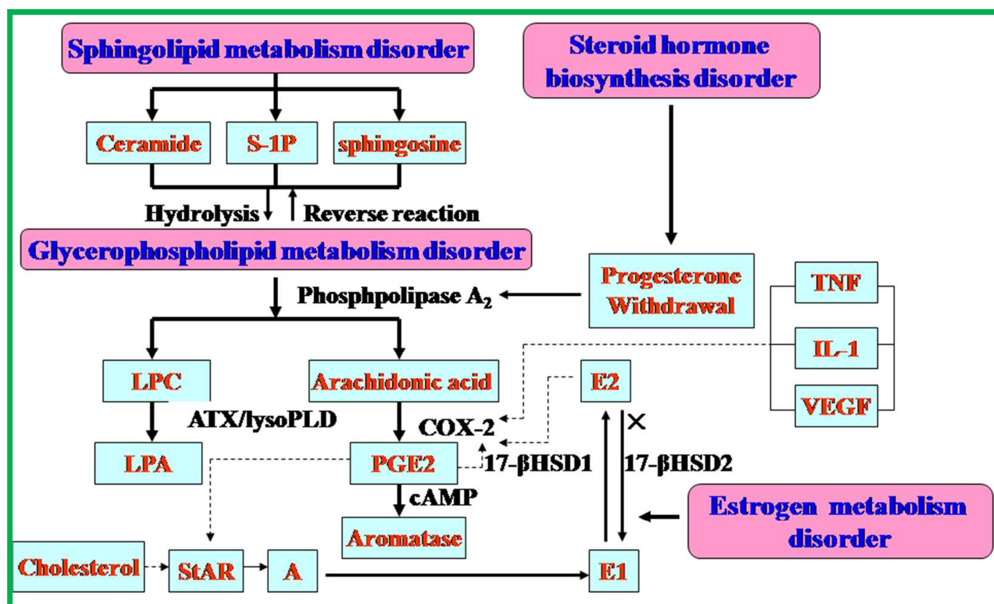

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612 Table.1

Table.1 Identified differential metabolites accountable for the discrimination between endometriosis and healthy controls in serum

m/z	Main fragment ions of MS/MS	Metabolites	VIP[a]	Content variance[b]
647.6285	279.9150 ([Palmitic acid +Na]$^+$)	CE (16:0)	5.18	↑
671.6627	303.0562 ([Linoleic acid+Na]$^+$)	CE(18:2)	4.67	↑
518.3471	458.9773([LysoPC (16:0) +Na -N(CH$_3$)$_3$]$^+$) 279.9414 ([Palmitic acid +Na]$^+$)	LysoPC (16:0)	2.36	↓
546.6878	487.6833([LysoPC (18:0) +Na -N(CH$_3$)$_3$]$^+$),279.9414 ([Palmitic acid +Na]$^+$)	LysoPC (18:0)	3.77	↓
725.5577	666.4474([SM (d18:1/16:0) +Na -N(CH$_3$)$_3$]$^+$) 524.4174([SM (d18:1/16:0) +Na – 183(polar head group)]$^+$)	SM (d18:1/16:0)	12.12	↑
780.6434	721.5334 ([PC (34:2) +Na -N(CH$_3$)$_3$]$^+$), 597.5134 ([PC (34:2) +Na -183(polar head group)]$^+$), 575.5236 ([PC (34:2) +H -183(polar head group)]$^+$)	PC (34:2)	23.54	↓
808.6724	749.5624 ([PC (36:2) +Na -N(CH$_3$)$_3$]$^+$), 625.5324 ([PC (36:2) +Na -183(polar head group)]$^+$), 601.5426 ([PC (36:2) +H -183(polar head group)]$^+$)	PC (36:2)	8.84	↓
832.6912	773.5812 ([PC (38:4) +Na -N(CH$_3$)$_3$]$^+$), 649.5512 ([PC (38:4) +Na -183(polar head group)]$^+$), 627.5614 ([PC (38:4) +H -183(polar head group)]$^+$)	PC (38:4)	5.12	↓
879.8475	6234.4234([TAG (54:4)+Na –palimtic acid)]$^+$), 597.3861([TAG (54:4)+Na –oleic acid)]$^+$), 599.4020 ([TAG (54:4)+ Na –linoleic acid]$^+$), 305.4512([Oleic acid+Na]$^+$)	TAG (52:3)	8.21	↑
905.8365	623.3751 ([TAG (54:4)+Na –oleic acid)]$^+$),625.3910 ([TAG (54:4)+ Na –linoleic acid]$^+$), 305.4512([Oleic acid+Na]$^+$)	TAG (54:4)	17.32	↑

[a] Variable importance in the projection (VIP) values were obtained from PLS-DA models. [b] ↑, content increased, ↓, content decreased.

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