ORIGINAL ARTICLES

Reproductive and Genetic Center of Integrated Traditional and Western Medicine[1], the Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, China; Department of Gynecology and Obstetrics of Traditional Chinese Medicine[2], the First Clinical College, Shandong University of Traditional Chinese Medicine, Jinan, China; School of Bioscience & Bioengineering[3], South China University of Technology, Guangzhou, China

Novel SWATH™ technology for follicular fluid metabolomics in patients with endometriosis

Zhengao Sun[1,*], Jingyan Song[2], Xingxing Zhang[2], Aijuan Wang[2], Ying Guo[1], Yi Yang[2], Xiaoming Wang[2], Kaiyue Xu[2], Jifeng Deng[3]

Received November 21, 2017, accepted February 17, 2018

**Corresponding author: Zhengao Sun, Integrative Medicine Research Centre of Reproduction and Heredity,The Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan City, 250011, Shandong Province, China*
sunzhengao77@126.com

Pharmazie 73: 318–323 (2018) *doi: 10.1691/ph.2018.7193*

Aim of the study: Sequential window acquisition of all theoretical fragment-ion spectra (SWATH™), a powerful high-resolution mass spectrometric data independent acquisition technique, was used to identify differences that relate certain metabolites to endometriosis (EMT) in follicular fluid collected from EMT patients and a control group. Methods: A case-control study was conducted to analyze the EMT-related metabolites and the IVF clinical data of 33 subjects. Subjects were divided between the observation group (17 cases, infertility due to EMT) and the control group (16 cases, infertility due to male factor, such as obstructive azoospermia). Results: Analysis revealed three metabolites including phytosphingosine, LysoPC(18:2(9Z,12Z)) and LysoPC(18:0), which were closely related to infertility associated withEMT. In the EMT group, LysoPC(18:2(9Z,12Z)) and LysoPC(18:0) were upregulated, while phytosphingosine was downregulated. Conclusions: This study employed, for the first time, the SWATH™ data acquisition mode for the metabolomics study of human follicular fluid in patients with EMT. The differential metabolite profiles of follicular fluid were identified and mapped. These differential metabolites are involved in cell proliferation and apoptosis, energy metabolism, inflammatory responses and angiogenesis. The differential metabolite profile may be a new tool for early noninvasive assessment of the developmental potential of oocytes in patients with EMT.

1. Introduction

Endometriosis is a disorder of the female human reproductive system in which the endometrium grows outside the uterus, which often occurs on the ovaries and peritoneum, and causes premenstrual pain and dysmenorrhea (Damewood 1989; Ozkan et al. 2008; Rock and Markham 1992). Endometriosis is one of the most common reasons of infertility, being diagnosed in 25-40% of infertile women (Ozkan et al. 2008). Dysfunction of a fallopian tube (Bergendal et al. 1998), subtle impairments of the oocytes and embryo development potential (Carrido et al. 2000; Lyons et al. 2002), immunological defects (Mansour et al. 2010), and anatomical dysfunctions of an ovary have been postulated to explain infertility associated with endometriosis. In addition, estrogen and inflammation have also been associated with the development of endometriosis (Bulun 2009). Even mild stage endometriosis might have a direct negative effect on oocyte quality and the potential for embryonic development and implantation (Arici et al. 1997; Barnhart et al. 2002). Oocyte quality is widely accepted as a potential major factor in infertility in those patients (Broekmans et al. 2006; Garrido et al. 2003). Cahill et al. (1997) and Xu et al. (2015) showed that ovulatory dysfunction and the associated pain of patients lead to reduced fertilization rates in women with minor endometriosis. Pauli et al. (2013) found that trans-retinoic acid plays a fundamental role in oocyte development and quality, and that reduced trans-retinoic acid synthesis may contribute to decreased fecundity in patients with endometriosis.

Follicular fluid comes from follicles, and contains numerous metabolites that play an important role in follicle development and oocyte maturation. Endometriosis might cause alterations in the microenvironment formed by follicular fluid for oocyte development and, therefore, negatively impact fertility. At present, there are few clear small molecular indicators (lactate, insulin, glucose, leucine, proline, etc.) of poor oocyte quality due to endometriosis (Bancsi et al. 2003; Nicholson et al. 1999; Santonastaso et al. 2017).

Metabolomics, which is the identification and quantification of a set of metabolites in biological systems, can be used to find potential biomarkers for studying the relative biochemical pathways in follicular fluid. A better understanding of this complex disorder and the identification of potential biomarkers of endometriosis may mean a significant advance for evaluating changes in oocytes caused by endometriosis.

Although high-resolution mass spectrometry (HRMS) such as time of flight (TOF) has become a conventional tool for metabolomics study (Qiang et al. 2016; Yao et al. 2016), the identification of low-level differential endogenous metabolites is still very difficult. The m/z of the precursor and product ions was recorded in MS and MS/MS spectra to provide crucial information for the elemental composition analysis and structure elucidation. The hit rates of the MS/MS spectrum played a decisive role in the identification of differential endogenous metabolites. Compared to the information-dependent acquisition (IDA) method (Lin et al. 2014; Sun et al. 2015), sequential window acquisition of all theoretical fragment-ion spectra (SWATH™) (Hopfgartner et al. 2012) significantly improved the hit rate of low-level endogenous metabolites, as it can sequentially obtain all MS/MS spectra of all mass windows across the specified mass range (Xie et al. 2017). Moreover, the MS^{ALL} technique does not require the selection of precursor ions to trigger the acquisition of fragment ion spectra (Bilbao et al. 2015). The novel SWATH technique presents various advantages in the field of absolute quantification (Bonner and Hopfgartner 2016; Wrona et al. 2005). For example, an advantage

of SWATH™ acquisition resides in the possibility of reprocessing the same data set to obtain previously unidentified features without reacquiring the sample (Gillet et al. 2012).
In our study, the SWATH technique was used, for the first time, to assess the differences between the metabolomics profile of follicular fluid collected from EMT patients and a control group. Metabolomics based on the SWATH approach could be used for providing comprehensive information about follicular fluid for the diagnosis and prognosis of endometriosis-related infertility.

2. Investigations and results

2.1. *Clinical background of subjects*

The results of basal FSH and E2 in blood are shown in Table 1. No significant changes were observed in the EMT group. Clinically, no statistical differences in the number of oocytes were seen. 2PN cleavage rate, high-grade embryo rate, cumulative pregnancy rate and live birth rate were identified between these two groups (Table 2). However, participants in the EMT group had significantly lower MII rates and fertility rates than those in the control group (Table 2).

Table 1: Clinical background of endometriosis group and control group

Clinical parameter	Control group	EMT group	T-test (P)
Age (years)	34.8±3.4	36.1±3.0	0.239
Infertility duration (years)	2.79±1.90	3.89±2.68	0.083
Body mass index (kg/m2)	23.79±4.21	23.70±3.24	0.930
Basal FSH (mIU/mL)	9.89±7.30	7.17±3.86	0.091
Basal E2 (pg/mL)	35.46±14.50	43.37±18.78	0.086
Basal AFC (N)	10.18±5.75	12.70±8.20	0.191
Duration of Gn used (days)	11.18±2.84	11.37±3.27	0.817
Dosage of Gn used (mg)	2946.7±1152.0	2802.3±1194.3	0.650

Table 2: Comparison of in vitro fertilization between EMT group and control group

Clinical parameter	Control group	EMT group	T-test (P)
Oocytes retrieved (N)	7.82±4.92	7.78±4.30	0.972
MII oocyte maturation rate (%)	89.4%	81.0%	0.015*
Fertility rate (%)	73.5%	54.3%	0.008**
2PN cleavage rate (%)	97.9%	97.4%	0.772
High-grade embryo rate (%)	55.7%	49.5%	0.331
Cumulative pregnancy rate (%)	37.0%	32.0%	0.703
Live birth rate (%)	29.6%	28.0%	0.897

* (p<0.05), ** (p<0.01)

2.2. *Reproducibility of the LC-MS system*

In total, six quality control (QC) samples (one QC after each four follicular fluid samples) were prepared by mixing equal volumes of different individual follicular fluid samples. These were used to assess the reproducibility and reliability of the UPLC-MS system. The QC samples were tightly clustered together (Fig. 1). The reproducibility of the main background ions and the internal standard ions, such as midazolam (m/z 326.0860), was assessed using the relative standard deviation (RSD). The RSD of these background ions and the internal standard ions for all QC samples was less than 13.5%. This indicates good reproducibility and reliability of the UPLC-MS system. The internal standard ion (m/z 326.0860) was detected at m/z 326.0866, and was less than 1 mDa by comparison with the theoretical mass. This shows that the accuracy of the method was adequate for detecting the unknown samples. The chromatography residue was also assessed using the internal standard peak area in the blank sample, following the follicular fluid sample. The internal standard peak was not detected in the blank sample, which indicates that there was no chromatography residue. These results indicate that the analytical method was adequate for use in the metabolomics study.

2.3. *Comparison of EMT group and control group*

The EMT group could be separated completely from the control group, as shown in Fig. 1 (PCA score plot) and Fig. 2 (loading plot), which indicated significant differences between them. The contribution list of metabolites was produced based on p-values below 0.05. The metabolites were validated based on accurate mass, isotope patterns, and mass spectrometric fragmentation patterns, and the results are shown in Table 3.

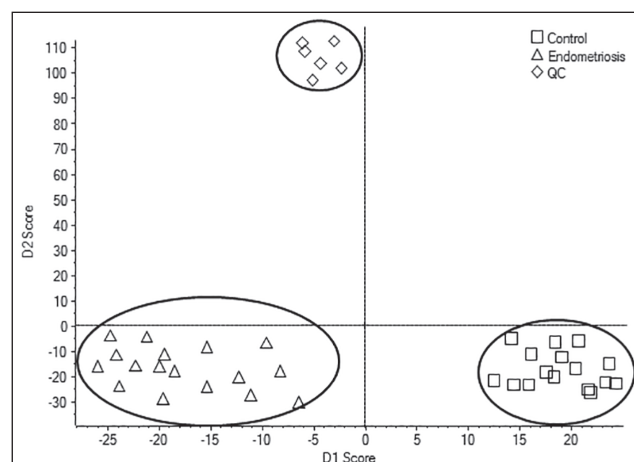

Fig.1: Score plots obtained from non-targeted UPLC-TOF MS analysis.

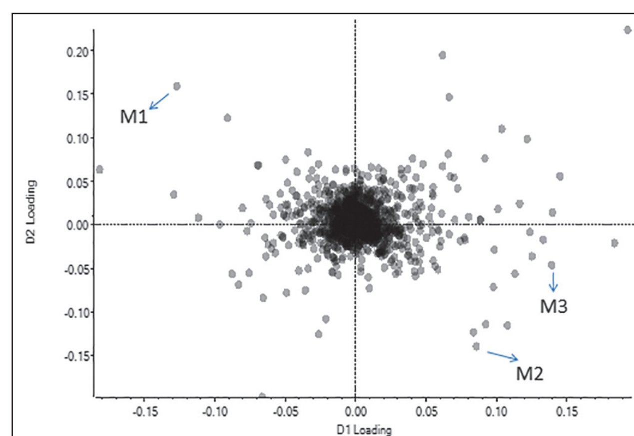

Fig. 2: Loading plot obtained from non-targeted UPLC-TOF MS analysis.

Table 3: Characterization of the biomarkers between endometriosis group and control group in follicular fluid by UPLC-Q-TOF MS

Compound	T_R (min)	m/z	Molecular Formula	Identity (Endometriosis group vs Control group)	Error (mDa)	Fold change (E/C)	T-test (p)
M1	4.78	318.3007	$C_{18}H_{39}NO_3$	Phytosphingosine	-0.1	0.15	<0.05
M2	5.38	520.3396	$C_{26}H_{50}NO_7P$	LysoPC(18:2(9Z,12Z))	-0.7	10.2	<0.01
M3	7.22	524.3722	$C_{26}H_{54}NO_7P$	LysoPC(18:0)	0.6	3.6	<0.01

The differential metabolite M1 showed the $[M+H]^+$ ion at m/z 318.3007. The elution time of M1 was 4.78 min in the UPLC chromatogram. Its molecular formula was inferred as $C_{18}H_{39}NO_3$, according to its accurate mass and isotope patterns. A series of characteristic product ions were observed at m/z 300.2893, 256.2648, 212.2387, 102.0948, and 88.0780 by successive loss of H_2O, $C_2H_6O_2$, $C_4H_{10}O_2$, $C_{13}H_{28}O_2$ and $C_{14}H_{30}O_2$. The structure of M1 was inferred as phytosphingosine, based on the MS and MS2 information (Fig. 3). The differential metabolite M2 showed the

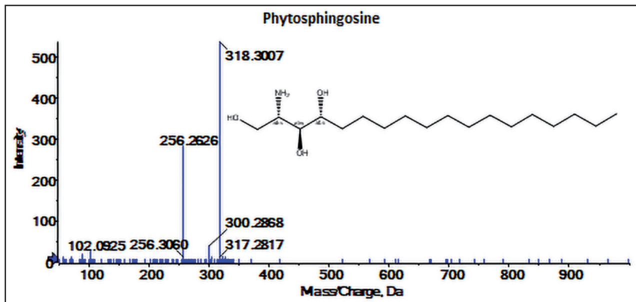

Fig. 3: The product ion spectra and structure of M1

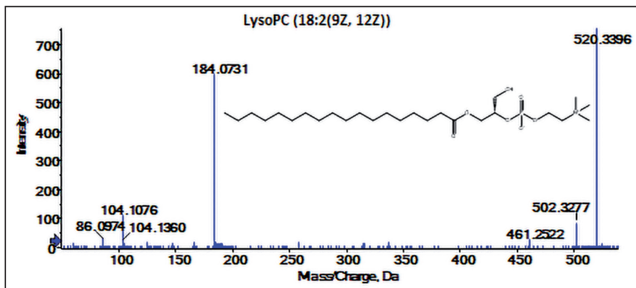

Fig. 4: The product ion spectra and structure of M2

$[M+H]^+$ ion at m/z 520.3396. The elution time of M2 was 5.38 min in the UPLC chromatogram. Its molecular formula was inferred as $C_{26}H_{50}NO_7P$, based on its accurate mass and isotope patterns. A series of characteristic product ions were observed at m/z 502.3277, 461.2522, 184.0731, 104.1076 and 86.0974 by successive loss of H_2O, C_3H_9N, $C_{21}H_{36}O_3$, $C_{21}H_{37}O_6P$ and $C_{21}H_{39}O_7P$. The structure of M2 was inferred as LysoPC (18:2(9Z, 12Z)), based on the MS and MS2 information (Fig. 4). Differential metabolite M3 showed the $[M+H]^+$ ion at m/z 524.3729. The elution time of M3 was 7.22 min in the UPLC chromatogram. Its molecular formula was inferred as $C_{26}H_{54}NO_7P$, according to its accurate mass and isotope

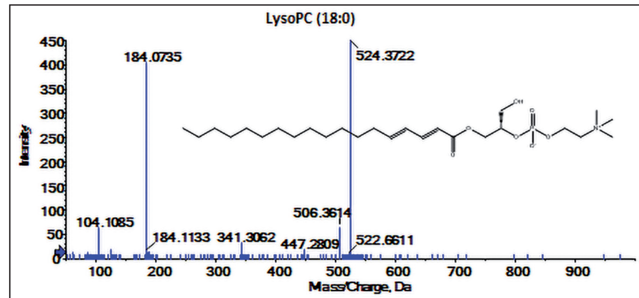

Fig. 5: The product ion spectra and structure of M3

patterns. A series of characteristic product ions were observed at m/z 506.3614, 341.3062, 184.0735, 104.1085 and 86.0955, by successive loss of H_2O, $C_5H_{14}NO_4P$, $C_{21}H_{40}O_3$ and $C_{21}H_{43}O_7P$. The structure of M3 was inferred as LysoPC (18:0), based on the MS and MS2 information (Fig. 5). The complete results are listed in Table 3. As seen in Fig. 6, differences in the three metabolites between the EMT group and the control group were displayed with Graph Pad Prism. The names of the metabolites are shown in the box plot. When accounting for outliers, the whiskers extended to a maximum of 1.5 times the inter-quartile range.

3. Discussion

SWATH™ is a new on-line data acquisition method, used for the assessment of independent parameters of compounds. The SWATH™ method enables the detection of all peaks and the corresponding MS/MS spectra. Some small indicators such as lactate, insulin, glucose, leucine and proline were identified in previous studies (Bancsi et al. 2003; Santonastaso et al. 2017; Nicholson et al. 1999). In our study, new differential metabolites between two groups were obtained by the SWATH™ method.

Oocyte quality directly reflects the intrinsic developmental potential and is responsible for normal fertilization/embryonic development during IVF (Harlow et al. 1996). The rate of fertilization was reduced during IVF/ICSI cycles in mice with endo-

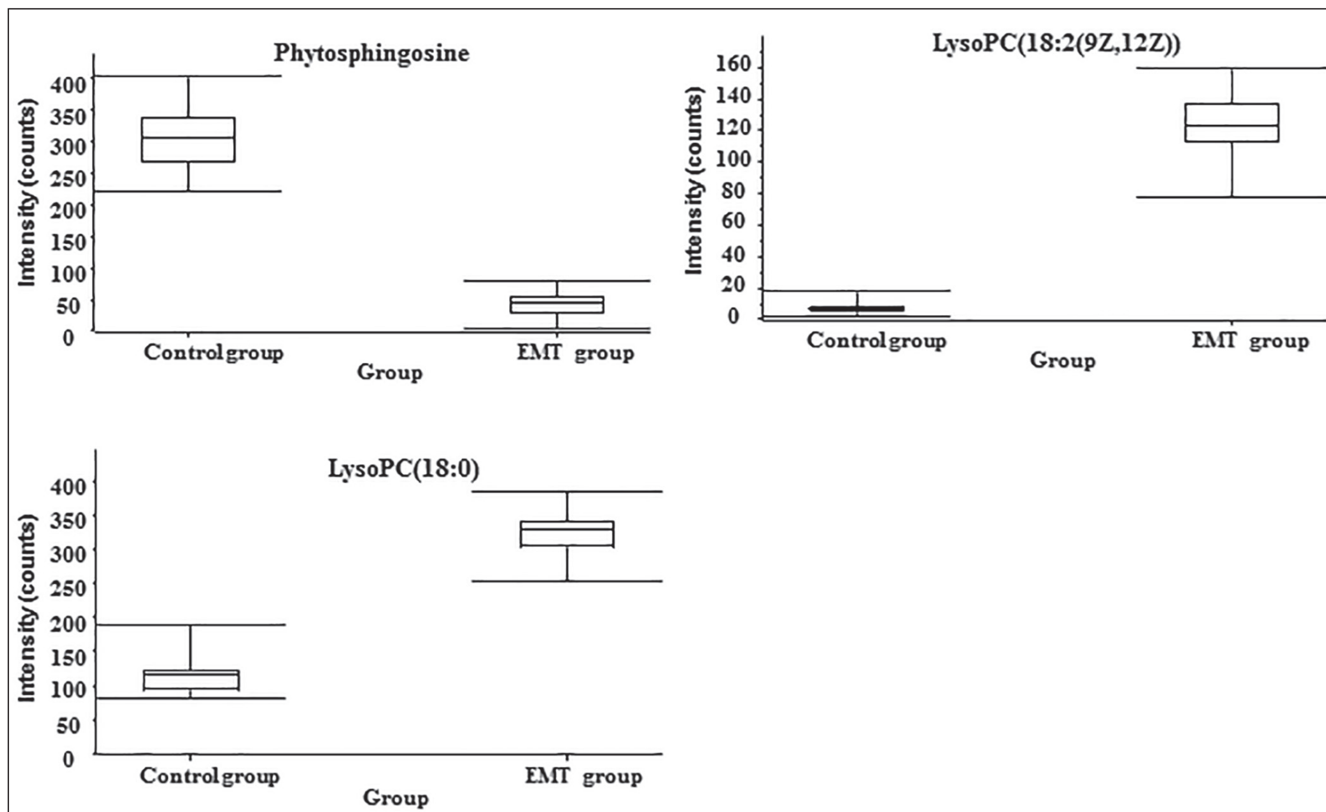

Fig. 6: Metabolite profiles of the 3 candidate biomarkers obtained from the quantitative analysis of the subjects ($p < 0.05$).

metriosis, in a previous study (Mansour et al. 2010). Poor oocyte quality could be the main factor in adverse pregnancy outcomes during IVF/intracytoplasmic sperm injection (ICSI) cycles in women with endometriosis. The proliferation of uterine endometrial cells outside the uterine cavity significantly increases the demand of biosynthesis and biological energy. Fatty acids are esterified to phospholipids as the sources of signaling molecules and energy supply, to support the rapid proliferation of ectopic endometrial cells (Marei et al. 2010). Furthermore, endometriosis may be associated with altered endogenous lipid metabolism (Toya and Hiroi 2000). Vouk et al. (2012) and Lee et al. (2014) indicated that the signaling pathway of endogenous lipids related to sphingolipids, ethers and lysophospholipids is influenced in the endometrial tissues of EMT patients. In our study, lysoPC(18:0) and lysoPC(18:2(9Z,12Z)) showed higher levels in the EMT group compared to the control group. LysoPC can induce the acrosome reaction (AR) of spermatozoa in different species, including humans, enhancing fertility (De Lamirande et al. 1998; Ohzu and Yanagimachi 1982). Dutta et al. (2012) also identified three differential metabolites such as monoacylglycerol (MAG), lysophosphatidylcholine (lysoPC) and phytosphingosine (PHS). Their results indicated that lysoPC and PHS are secreted by cumulus cells during *in vitro* fertilization, and can participate in the induced AR process. However, the capacitation of the sperm may be affected by the high concentration of LysoPC. Acrosomal loss was also caused by high concentration of LysoPC, which may affect the combination of egg cells and sperm (Byrd and Wolf 1986). Therefore, a higher level of lysoPC in follicular fluid may be one of the reasons for low conception rate in endometriosis patients. Our study also found that the level of phytosphingosine in the EMT group was significantly lower than that in the control group. Phytosphingosine was involved in the pathway of sphingolipid metabolism (Fig. 7), which indicates that sphingolipid metabolism was abnormal in the patients with EMT. Sphingolipids are bioactive molecules that participate in diverse functions, controlling fundamental cellular processes such as cell division, differentiation, and cell death (Rao et al. 2013). Furthermore, the decreased level of phytosphingosine in patients with EMT could increase the risk of type 2 diabetes mellitus (Floegel et al. 2013).

4. Experimental

4.1. Chemicals and reagents

Gemfibrozil (purity: > 98.5%) and isotope-labeled d3-palmitic acid (purity: > 99%), as internal standard, were purchased from Sigma (St. Louis, MO, USA). Chromatographic grade acetonitrile and formic acid were obtained from Merck & Co., INC (Darmstadt, Germany).

4.2. Subjects

All subjects were recruited from the Integrative Medicine Research Centre of Reproduction and Heredity, of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine, from January to December 2015. The study was approved by the Health Authorities and Ethics Committees of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine. All study participants signed an informed consent form before the start of the study. The diagnosis of endometriosis was done *via* laparoscopy and requires the presence of one or more typical bluish or black lesions, according to guidelines for diagnosis and treatment of endometriosis (Department of Endometriosis of the Chinese Medical Association 2015). We recruited 17 endometriosis patients and 16 age- and BMI-matched unaffected women as controls, and participant information was listed in Table 1. All controls had a normal menstrual cycle, and none had clinical and/or biochemical hyperandrogenism. The age of the subjects was between 31 and 40 years. Exclusion criteria for both groups included (1) having received hormonal therapy in the last three months; (2) inability to support pregnancy due to severe diseases; (3) suffering from severe mental diseases, acute urogenital system inflammation or sexually transmitted diseases; (4) being affected by hereditary diseases that prohibit having a baby; harmful addictions, including drugs

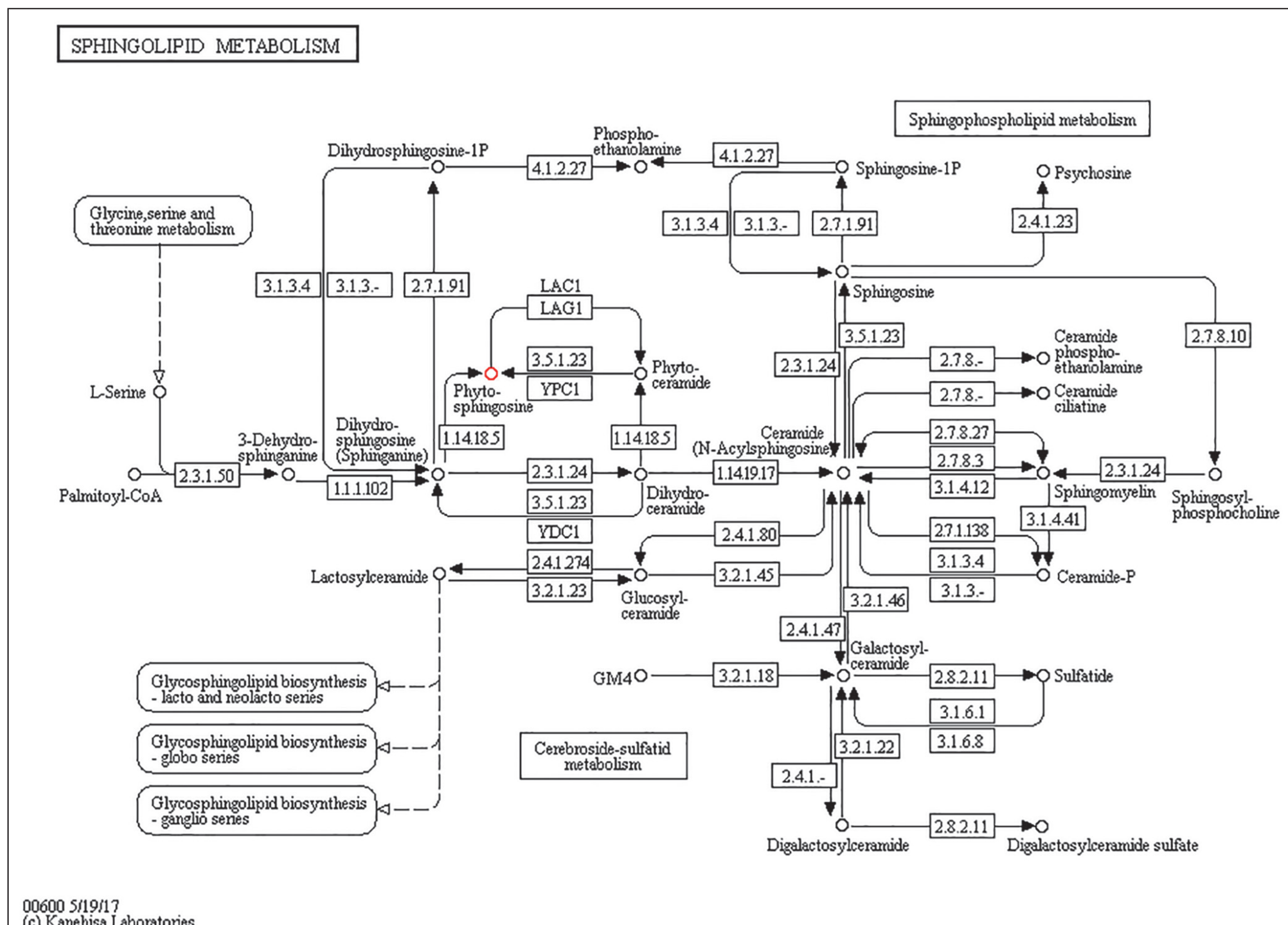

Fig. 7: The pathway of sphingolipid metabolism

and alcohol; being exposed to radiation, toxins and/or drugs within the action period that could cause malformations in the fetus.

4.3. Study design

Prior to entering the trial, 33 women signed informed consents. On the basis of established protocols, all patients underwent controlled ovarian hyperstimulation (COH). When the mean diameter of at least three leading follicles reached more than 18 mm, 10,000 IU human chorionic gonadotropin (hCG) (Choriomon, IBSA, Switzerland) was administered intramuscularly, 34-38 h after hCG injection under ultrasound guidance. The follicles (the maximum size < 20 mm) were aspirated using a 17-gauge Cook needle. Subsequently, oocytes were retrieved. After oocyte isolation, follicular fluid from three mature follicles was pooled and centrifuged at 14,000× g for 20 min, to remove cells and insoluble particles. The supernatant was transferred to sterile cryovials and stored at -80 °C for further study. Specimens with blood contamination were discarded. Blood samples were also acquired during the early follicular phase (days 3-5), from all subjects. The concentrations of follicle stimulating hormone in blood were detected using the enzyme-linked immunosorbent assay (ELISA) (Lucas et al. 1995; Li and Li 2000; Mickova et al. 2003).

4.4. Sample preparation

Follicular fluid samples of 100 µL were mixed with 300 µL of methanol containing 4 µM of gemfibrozil and isotope-labeled d3-palmitic acid. The mixture was vortexed for 5 min and then centrifuged at 14000× g for 30 min, at 4 °C. The supernatant was then transferred to an autosampler plate for analysis.

4.5. Method condition

Aliquots of 2 µL supernatant were injected into the ultra-performance liquid chromatography tandem Triple TOF 5600 system (AB SCIEX, CA, USA) in random order, to avoid complications caused by artifacts related to injection order and occasional changes in instrumental efficiency. The liquid chromatography system consisted of a reverse-phase 2.1*100 mm ACQUITY UPLC® BEH C18 1.7 µm column (Waters Corp., USA), with a gradient mobile phase composed of 0.1% formic acid solution (A) and acetonitrile containing 0.1% formic acid solution (B). The gradient was kept at 95% A for 1 min, increased to 100% B over the next 6 min, and then returned to 95% A from 9 min to 9.2 min. The total run time was 12 min. The optimized mass parameters were as follows: nebulizing gas (GAS1): 60 psi; TIS gas (GAS2): 60 psi; source temperature: 550 °C; ion spray voltage: 5500 V with 30 psi curtain gas in positive mode and -4500 V with 30 psi curtain gas in negative mode. The declustering potential and collision energy were set at 60 eV and 25 V in positive mode (-60 eV and -25 V in negative mode), respectively. The SWATH method analysis with 15 variable isolation windows was performed in full-scan mode and in product ion scan mode at m/z 100 – 1200 using the Analyst TF 1.7.1 software. Data processing was performed using MarkerView 2.0.

4.6. Data analysis

In total, 33 follicular fluid samples were analyzed in replicates using the SWATHTM technique on UPLC-TOF MS. Data was processed using the PeakView software (AB SCIEX, CA, USA) for qualitative analyses and the MarkerView software (AB SCIEX, CA, USA) for multi-variate analysis (MVA). In large-scale non-targeted LC-MS metabolomic measurements, the reproducibility of the analysis may be influenced by source contamination or the maintenance and cleaning of the mass-spectrometer. Normalization is a common preprocessing method to decrease systematic change. However, normalization of the data may cause nonsystematic, compound-dependent variability (Gika et al. 2007). In this study, internal standards were used to calibrate the response of metabolite ions. Gemfibrozil was used to calibrate the metabolites in positive ion mode. Isotope-labeled d3-palmitic acid was used only for negative ion mode. By mixing equal volumes of follicular fluid from different subjects, 6 QC samples in replicates were prepared to evaluate the reproducibility of the metabolite analysis. All ion features were extracted and aligned using the MarkerView software (Applied Biosystems/MDS Sciex, Canada), to generate a data matrix consisting of peak areas corresponding to a unique m/z and retention time. After aligning peaks from the EMT and control groups, the zero-values were removed using the modified 80% rule. The score plot and loading scatter plot were obtained via principal component analysis (PCA) in the MarkerView software. The differences between groups can be seen from the score plot. Loading plots were used to identify metabolites that exerted a major influence on the group membership. Each point represented an ion that contributed to the sample separation between groups. These ions were listed according to their correlation and their abundance rank (peak area) following the primary screening. Precursor ions of metabolites were quantified by their peak areas. The Student's t-test was used for statistical comparisons. The data were presented as the mean±standard deviation. The contributing list of metabolites was determined by p-values below 0.05. The contributory list presents candidate biomarkers in the EMT group compared with the control group. The predictability of the model was determined by internal validation with 7-fold cross-validation and response permutation testing.

Metabolites with high contribution score were identified by accurate mass, isotope patterns and mass spectrometric fragmentation patterns, which were used to search databases, including KEGG, PubChem compound, METLIN, the Madison Metabolomics Consortium Database and the Human Database.

Acknowledgments: This work was supported by the National Natural Science Fund project (No. 81373676; No.81674018) and the Science and Technology Development Project of Shandong Province (2014GSF119021).

Conflicts of interest: None declared.

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