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Lipidomic Components Alterations of Human Follicular Fluid Reveal the Relevance of Improving Clinical Outcomes in Women Using Progestin-Primed Ovarian Stimulation Compared to Short-Term Protocol

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Background: Increasing the success rate of *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) is a duty of clinicians that has made many seek a variety of protocols. This study was undertaken to use a liquid chromatography-mass spectrometry (LC-MS) to define the alterations of follicular fluid (FF) lipid metabolites in patients undergoing progestin-primed ovarian stimulation (PPOS) compared with short-term protocol, revealing potential correlations between the differentially expressed lipids and ameliorative clinical outcomes.

Material/Methods: Ninety-three infertile women undergoing IVF/ICSI treatment with PPOS (n=62) or a short-term protocol (n=31) were prospectively enrolled in a randomized controlled trial. FF samples were obtained from dominant follicles at the time of oocyte retrieval. Lipid metabolism profiles were analyzed using LC-MS.

Results: Twelve lipids were found to be higher in patients treated with the PPOS protocol than in those receiving the short-term protocol, including triacylglycerols (TAG-34: 1+NH_4, TAG-58: 0+NH_4, TAG-64: 3+NH_4, and TAG-64: 8+NH_4), diacylglycerol DAG-38: 6+NH_4, phosphatidylglycerols (PG-26: 0, PG-30: 2, and PG-40: 5), phosphatidylethanolamine PE-32: 2, lysophosphatidylethanolamine LPE-14: 1, lysophosphatidylinositol LPI-12: 0, and lysophosphatidylcholine LPC-16: 0.

Conclusions: Our data demonstrate that the PPOS protocol increases the levels of 12 lipids in FF, which reveals a strong association between the differentially elevated lipids and better IVF/ICSI outcomes.

MeSH Keywords: Follicular Fluid • Lipid Metabolism • Oocytes • Progesterone

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Background

Follicular growth involves a complex pattern of hormonal signals regulated by the hypothalamus-pituitary-ovarian axis (HPOA) and by autocrine/paracrine signaling between oocytes and their adjacent somatic cells [1]. These processes have been utilized by assisted reproductive technology in the clinic for 30 years to help more infertile couples conceive. There are still many factors affecting the success rate of IVF/ICSI, especially premature LH surges, which lead to early ovulation, causing failure in up to 20% of patients undergoing IVF/ICSI [2,3]. Gonadotropin-releasing hormone (GnRH) agonists and antagonist have been used to reduce surface expression of the GnRH receptor in the pituitary or reduce GnRH sensitivity to prevent premature LH surges [4]. However, such protocols have increased the incidence of ovarian hyperstimulation syndrome and these surges range from 0.34% to 38% [5–7]. PPOS, created by Dr. Kuang, using medroxyprogesterone 17-acetate (MPA) negative feedback on the HPOA to inhibit the synthesis and secretion of pituitary LH [8–11]. This protocol is not only suitable for patients with a normal ovarian response, but has also been effectively applied in aged patients and those with low ovarian response and polycystic ovary syndrome.

The development of oocytes, fertilization, and early embryo cleavage are influenced by the composition of FF, which provides a microenvironment comprising a variety of nutrients [12]. The importance of FF in maintaining follicle development and oocyte quality has been extensively reported and discussed [13]. In particular, with the recent development in mass spectrometric technology, metabolomics has been transformed into a principal tool in biomedical research to decipher fine changes in FF metabolism in the potential of follicle and oocyte development. Nevertheless, there have been no studies published to date describing a detailed analysis of FF compositions following IVF/ICSI treatment with methods such as PPOS. As progesterone (P) is also involved in the regulation of metabolism at multiple levels, such as glucose and lipoprotein lipase activity, we hypothesized that a LC-MS analysis of lipid profiles from FF samples can identify the association between the alterations and the ameliorative clinical outcomes in the PPOS protocol.

Material and Methods

Patients

Ninety-three infertile women undergoing IVF/ICSI in the Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine were prospectively recruited between March 2015 and June 2016. The patients were randomly divided into 2 protocols: PPOS protocols (4 or 10 mg) and short-term protocol. The maximum age was 35 years old, basal follicle-stimulating hormone (FSH) levels were less than 10 IU/ml, antral follicles (AFC) were greater than or equal to 5, and all had a normal body mass index (BMI) and regular menstrual cycles (MC) (27–35 days). We excluded patients with infertility induced by male factor infertility or uterine or tubal factor infertility, or unexplained infertility. Women with polycystic ovary syndrome, premature ovarian failure, cancer, a history of no used embryo, and more than 3 degrees of endometriosis were also excluded. Women were recruited according to different protocols: 31 patients receiving the short-term protocol served as the control group; 62 women were enrolled in the PPOS protocols, including 28 women with PPOS (4 mg) and 34 with PPOS (10 mg), based on the dose of MPA, undergoing IVF/ICSI treatment.

Ethics approval and consent to participate

The study was approved by the Ethics Review Committee of the Ninth People's Hospital of Shanghai and the Shanghai Jiaotong University Medical Center, China. Signed informed consent for use of FF was obtained from each patient after recommending conventional infertility treatment and the IVF/ICSI process.

Protocol

In PPOS protocol [14], MPA (Xianju Pharma, Zhejiang, China) 4 mg or 10 mg per day was taken orally from the MC3, and 150–225 IU human menopausal gonadotropin (hMG, Lizhu Pharmaceutical Trading Co., Zhuhai, China) until the day of induced ovulation. Depending on follicle development, we adjusted the dose of hMG. When the primary follicle reached 18 mm, 0.1 mg triptorelin (Huilin, Germany) and 2000–5000 IU human chorionic gonadotropin (hCG, Lizhu Pharmaceutical Trading Co., Zhuhai, China) were administrated to trigger ovulation. Transvaginal ultrasound-guided oocyte retrieval was performed 32–36 h later.

In the short-term protocol [15], triptorelin (Lizhu, Shanghai, China) 100 μg per day was injected from the MC3 until the day of hCG administration. Beginning on the day after the injection of triptorelin, 150–225 IU hMG was injected per day. Follicle development was monitored by ultrasound and we adjusted the dose of hMG based on the number and size of developing follicles. When the primary follicle reached 18 mm, or LH and P increased significantly compared with the baseline, 2000–5000 IU hCG was injected to trigger ovulation. Transvaginal ultrasound-guided oocyte retrieval was performed 32–36 h later.

Collection of follicular fluid

For each patient, the first follicle was carefully collected to avoid macroscopic blood contamination and a new needle was

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used for each patient. Each 3–5 mL of FF aspirate was centrifuged immediately at 2000 g for 20 min at 4°C, then the extracted FF supernatant was aliquoted (1.5 mL) and stored at –80°C prior to assay.

Sample preparation

Using methyl tert-butyl ether, we extracted the lipid molecules of follicular fluid twice. The organic phase was volatilized by the vacuum centrifugal concentration meter and the evaporated sample was redissolved by the mixed solvent. We transferred the redissolved samples to the sample vials for high performance liquid chromatography-electrospray time-of-flight mass spectrometry (HPLC-TOF-MS).

Metabolomic analysis of follicular fluid

The lipid molecules of follicular fluid samples were analyzed by HPLC-TOF-MS, which used a charged surface hybrid C18 chromatographic column as the chromatography/mass spectrum, 2.1×100 mm, 1.7 μm, flow velocity was 0.4 mL/min, and column temperature was held at 55°C. Gradient elution was used to elute the analyte, and we added 1-μL samples into the column conducted under electrospray ionization positive and negative ion mode. TOF/MS/MS was set to MS scanning, and total scan time cycle was 0.83 s.

Metabolic correlation analysis

Heat Map analyzed the metabolites and the similarity relation between samples to confirm which sample has a similar metabolic profile and metabolic path, and then gathered similar metabolites. The clustering software was R language (*www.r-project.org*) package Pheatmap. Correlation analysis was used for calculating coefficient of association among the metabolites, including each group of data matrix by Pearson's product–moment correlation and p-value of correcting by false discovery rate (FDR).

Statistical analysis

SPSS22.0 software was used for statistical analysis. Statistical analysis of normally distributed data was performed on 2 independent samples. The Mann-Whitney U test was used to measure non-normally distributed data. The categorical data were analyzed by chi-square test. LipidView (AB Sciex,1.2) software was utilized for lipid analysis by accurate mass and MS/MS fragment. LipidView software was used for quantitative results by comparing the target lipid peak area and their internal standard. To utilize quality control sample data for quality assurance analysis, Simca-P 13.0 (Umetrics AB, Umea, Sweden) software was used for principal component analysis (PCA). Then, partial least squares-discriminant analysis (PLS-DA) was applied to the data set, and after the construction of the PLS-DA models, a permutation test was used to prevent overfitting. Orthogonal partial least square-discriminate analysis (OPLS-DA) was applied to explore differences between the 3 groups of lipids. A 2-sample *t* test was used to assess differences between the 2 groups by R 3.3.0 (*www.r-project.org*), and $P<0.05$ and fold change >1.5 were regarded as indicating a significant difference.

Results

Clinical characteristics of the patients

A total of 93 women were recruited for the study. Table 1 shows characteristics of the 62 patients who underwent 1 of 2 PPOS protocols (4 or 10 mg) and the 31 control participants in the short-term protocol that were finally selected for inclusion in the study. There were no significant differences among the 3 groups with respect to age, infertility time, cause of infertility, basal hormone level, antral follicle count, percentage of primary infertility, previous IVF attempts, or body mass index. Furthermore, there were no significant differences in total doses of human menopausal and chorionic gonadotropins, the numbers of oocytes retrieved, the numbers of fertilizations and embryos, or the rates of clinical pregnancy. However, significant differences were found among groups for the total doses of MPA.

Serum hormone of each groups

The levels of serum hormones detected during ovarian stimulation with the PPOS and short-term protocols are presented in Figure 1. The levels of FSH in patients receiving ovarian stimulation with the PPOS protocol were higher than in those receiving the short-term protocol, appearing as a FSH surge on the trigger day. The levels of LH decreased in the PPOS protocol but increased and were maintained at a higher level in the short-term protocol. By contrast, the levels of estrogen (E2) and P were significantly lower with the PPOS protocol than with the short-term protocol. There were no significant differences between the 4-mg and 10-mg PPOS protocols.

Overall lipid analysis and pattern recognition and functional analysis

A total of 294 lipids, including 276 positive ions and 18 negative ions, were obtained according to the quality assurance, and 12 lipid molecules were identified, including PA, PC, PE, PG, PI, PS, CE, TAGs, DAGs, Cer, SM, and free fatty acids. A PCA revealed that the metabolic profiles from FF samples differed among the 3 groups. Furthermore, an OPLS-DA model with good predictability was applied to compare the PPOS and

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Table 1. Clinical characteristics of the patients included in the study.

Parameter	Short-term n=31	PPOS (4 mg) n=28	PPOS (10 mg) n=34	P-value 1	P-value 2
Age	31.9±2.8	30.7±3.1	31.2±2.9	.298	.592
Infertility time	2.9±3.8	2.2±1.3	2.7±2.0	.109	.252
Basal FSH (IU/ml)	5.7±2.3	5.7±1.0	5.4±1.2	.961	.840
Basal LH (IU/ml)	3.3±1.4	3.5±1.2	3.8±1.0	.836	.113
Basal E2 (pg/ml)	27.3±20.5	28.8±20.0	25.5±16.7	.810	.738
Basal P (ng/ml)	0.4±1.0	0.3±0.1	0.3±0.2	.920	.487
AFC (n)	10.1±3.3	9.9±2.6	11.8±4.1	.673	.116
Primary infertility (%)	54.1	52.4	53.8	0.133	0.106
Previous IVF attempts (n)	0.9±1.4	1.0±1.1	0.9±1.5	0.929	0.966
BMI (kg/m^2)	21.7±3.7	21.7±2.4	21.4±1.9	.988	.680
Total dose of hMG (IU)	1702±138	1913±147	1894±345	.149	.372
Total dose of hCG (IU)	2067±13099	2250±1357	2150±1182	.878	.809
Total dose of MPA (mg)	0	36.3±5.0	90.0±12.9	.000*	.000*
Ocytes retrieved (n)	11.5±9.9	14.1±13.8	14.6±26.1	.143	.473
MII oocytes (n)	9.2±0.9	10.8±0.7	10.9±0.4	.980	.847
Fertilization (n)	7.7±0.6	8.7±0.7	8.8±0.5	.066	.078
Clevage (n)	7.6±0.6	8.7±0.7	8.8±0.5	.066	.078
High-quality embryos	3.2±2.6	3.9±2.6	4.0±3.4	.516	.366
All cryopreserved embryos (n)	3.2±2.5	3.9±2.5	4.0±3.3	.523	.368
Biochemical pregnancy rate per transfer (%)	48 (15/31)	57 (16/28)	58 (20/34)	.217	.164
Clinical pregnancy rate (%)	42 (13/31)	50 (14/28)	50 (17/34)	.242	.205
Birth weight (g)	2998±223	3164±634	3116±755	.245	.432

Plus–minus values represent the mean ±SD. The categorical datas were analysed by Chi-square. The baseline hormonal profile was typically assayed in patients treated with on menstrual days 2–4. P-value 1 for the comparison between short-term *vs.* PPOS (4 mg), P-value 2 for the comparison between short-term *vs.* PPOS (10 mg). AFC – antral follicles; BMI – body mass index; E2 – estrogen; FSH – follicle-stimulating hormone; hCG – human chorionic gonadotropin; hMG – human menopausal gonadotropin; LH – luteinizing hormone; MC – mentrual cycle day 3; MPA – medroxyprogesterone 17-acetate; P – progesterone; PPOS – progestin primed ovarian stimulation; * represent a statistically significant difference (P<0.05).

short-term groups. These analyses revealed significant differences among the 3 groups (Figure 2).

Differences in metabolites with ovarian stimulation

Based on the OPLS-DA model, together with the p-value of the *t* test (p<0.05) and a greater that 1.5-fold difference, significant augmentation in the levels of 12 lipids metabolites were observed in the PPOS groups compared with the short-term group. As detailed in Figure 3, those lipids included triacylglycerols (TAG-34: 1+NH_4, TAG-58: 0+NH_4, TAG-64: 3+NH_4, and TAG-64: 8+NH_4), diacylglycerol DAG-38: 6+NH_4, phosphatidylglycerols (PG-26: 0, PG-30: 2, and PG-40: 5), phosphatidylethanolamine PE-32: 2, lysophosphatidylethanolamine LPE-14: 1, lysophosphatidylinositol LPI-12: 0, and lysophosphatidylcholine LPC-16: 0.

Two-way hierarchical clustering of these metabolites revealed small differences within groups, with good sample repeatability. Overall, patterns of lipid expression in the PPOS groups differed significantly from that of the short-term group, with most

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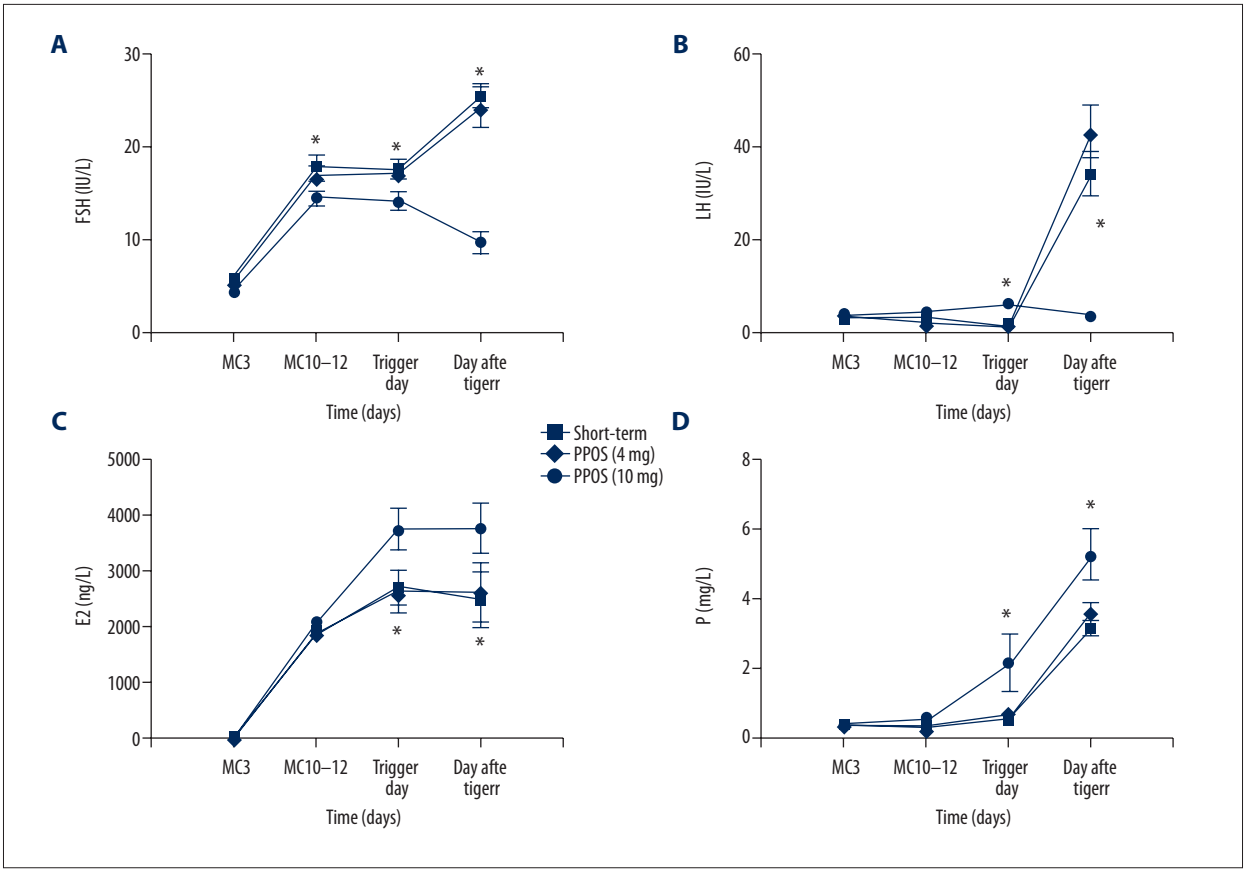

Figure 1. The trends of serum hormones during ovarian stimulation in the PPOS and short-term protocols. E2 – estrogen; FSH – follicle-stimulating hormone; LH – luteinizing hormone; P – progesterone; PPOS – progestin-primed ovarian stimulation; MC3 – menstrual cycle day 3; MC10–12 – menstrual cycle day 10–12; (**A**) FSH (IU/L); (**B**) LH (IU/L); (**C**) E2 (ngl/L); (**D**). P (ng/L); n=6; * represent short-term protocol and PPOS protocols, and P<0.05.

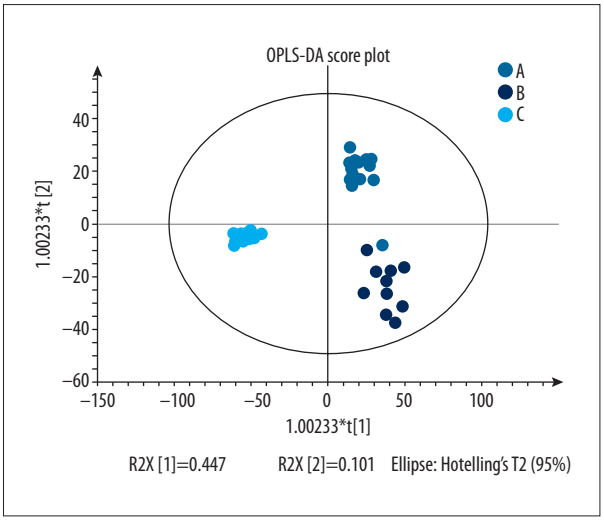

Figure 2. Score plot of OPLS-DA analysis. OPLS-DA – orthogonal partial least square-discriminate analysis; PPOS – progestin-primed ovarian stimulation; A – Short-term protocol, B – PPOS (4 mg) protocol, C – PPOS (10 mg) protocol.

of the lipids showing upregulation with 10 mg PPOS (Figure 4A). Correlational analyses showed that there was a correlation between the selected variables and a high correlation between TAG-34: 1+NH4, TAG 58: 0+NH4, TAG 64: 3+NH4, TAG 64: 8+NH4 and DAG 38: 6+NH4 (Figure 4B).

Discussion

In this study, we performed LC-MS analysis of FF lipid composition from patients undergoing IVF/ICSI treatment with 4 or 10 mg PPOS or a short-term protocol. We identified 12 differentially expressed lipids that were associated with the dose of MPA in the PPOS treatment that achieved high-quality oocytes and satisfactory pregnancy outcomes. Future studies will reveal the mechanism(s) for the alteration in the lipid profile.

The main characteristic of the PPOS protocol is the use of a high dose of MPA to reduce LH levels to produce more follicles during the follicular phase. Although a study found that P can inhibit follicular activity in llamas by suppressing LH

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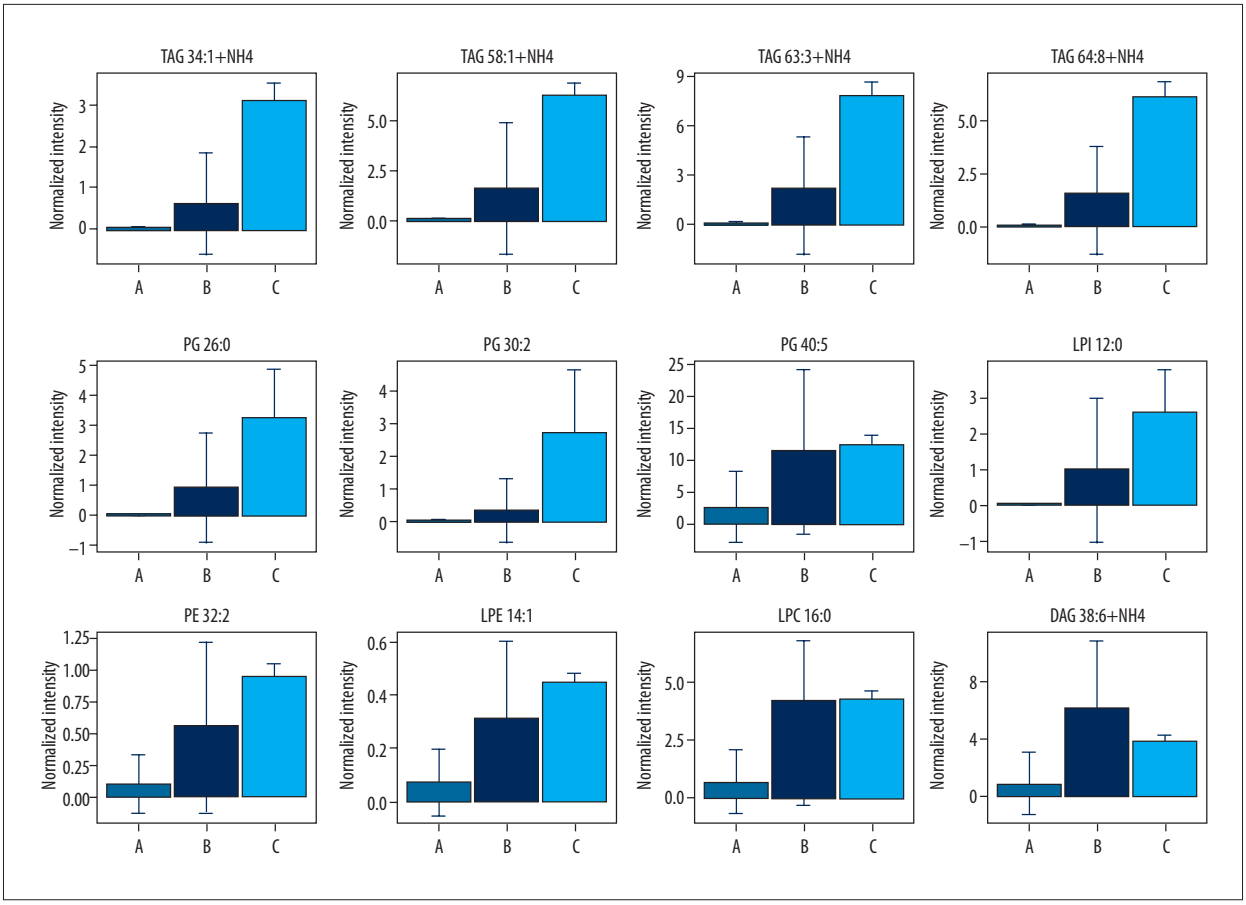

Figure 3. Differences in metabolite levels among 3 groups. DAG – diacylglycerol; LPC – lysophosphatidylcholine; LPE – lysophosphatidylethanolamine; LPI – lysophosphatidylinositol; PE – phosphatidylethanolamine; PG – phosphatidylglycerol; TAG – triacylglycerol; PPOS – progestin-primed ovarian stimulation; A – Short-term protocol, B – PPOS (4 mg) protocol, C – PPOS (10 mg) protocol.

pulsatility [16], another study showed that P treatment correlates with the emergence of young follicles [17]. Moreover, the cumulus complex secretes P to promote follicle maturation, and this high P environment had no effect on the process of follicular development in humans [18]. Indeed, it is widely accepted that P can slow the frequency of LH secretion in the early stages of follicle development, effectively suppressing premature LH surges, but does not damage the follicular development at later stages [19–21]. Interestingly, PPOS also induced an increase in basal serum FSH by using hMG, which surged on the trigger day. Studies suggest that a surge in FSH before ovulation also promotes the development of the follicles, and the appearance of the FSH surge before ovulation effectively improves the developmental potential of follicles [22–24]. However, the underlying molecular mechanisms remain elusive.

Our analyses revealed that PGs, including PG-26: 0, PG-30: 2, and PG-40: 5, were higher in FF from patients treated with the PPOS protocol. PG is a lipid mediator synthesized by arachidonic acid in the cyclooxygenase pathway, which plays important roles in LH secretion, follicular development, fertilization, and blastocyst implantation [25,26]. Also, PGs are involved in mammalian ovulation by stimulating the follicular wall smooth muscle contraction, leading to follicular rupture and ovulation, which are mediated by the cAMP signaling system. Furthermore, a reduction of PGs results in many spermatozoa that cannot penetrate cumulus cells when levels of PGs are low [27–29]. Thus, the finding from our study of higher PG levels with the PPOS protocol is consistent with these results. PGs receptors located in many tissue cell membranes play a different function [30]. They mediate the physiological functions of PGs through their second messenger pathways, stimulating or inhibiting the activity of adenylatecyclopase (AC) in the cell membrane to increase or decrease the level of cAMP. This suggests that PGs not only promote the ovulation of mature follicles, but also act as a second messenger of GnRH-mediated granule cells on cAMP response to promote the proliferation of follicular granulosa cells by mediating the PKA signaling pathway of the FSH in cells [31], which in turn plays an important role in the selection of dominant follicles.

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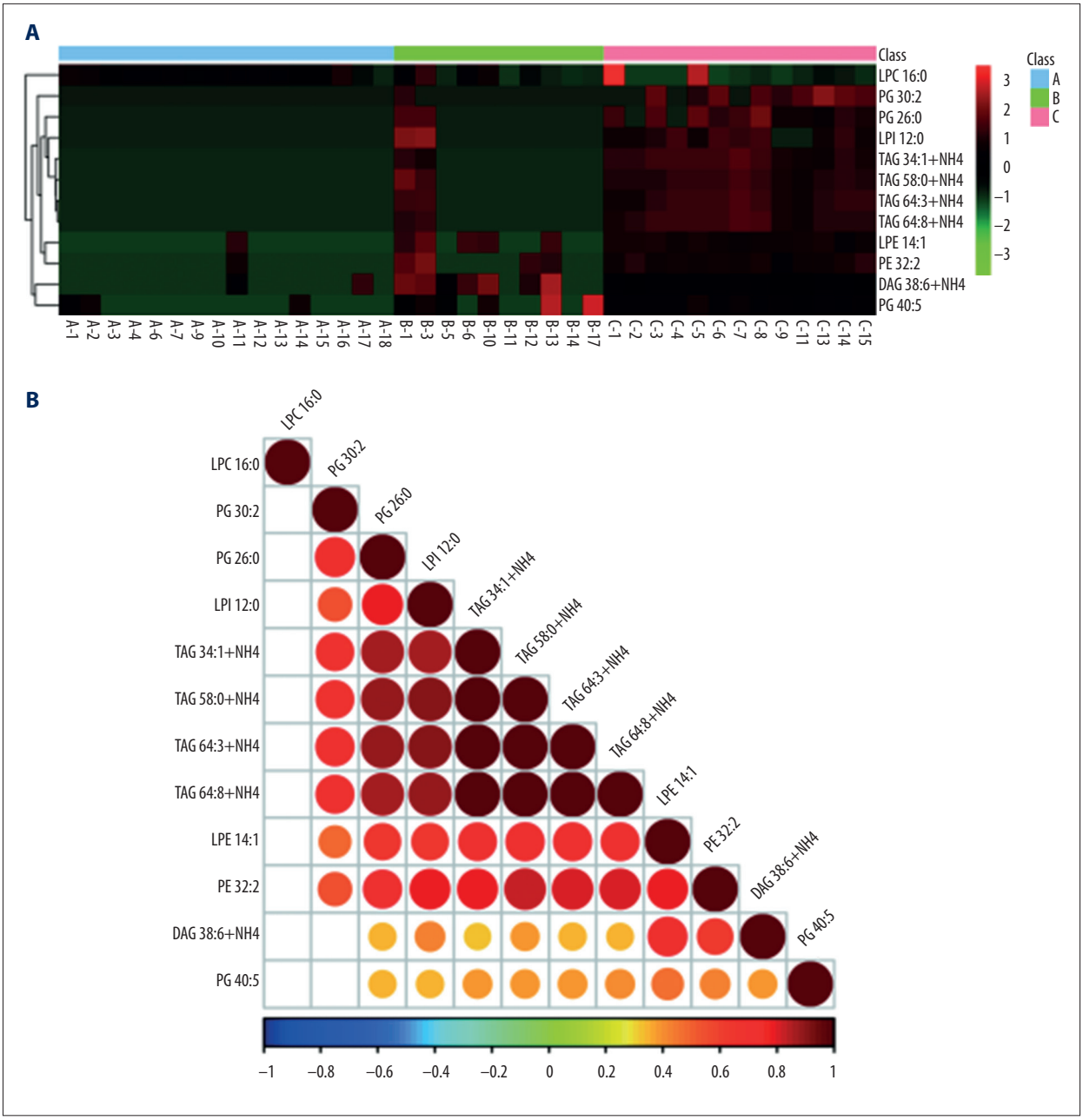

Figure 4. Differentially expressed lipids in PPOS protocol. DAG – diacylglycerol; LPC – lysophosphatidylcholine; LPE – lysophosphatidylethanolamine; LPI – lysophosphatidylinositol; PE – phosphatidylethanolamine; PG – phosphatidylglycerol; TAG – triacylglycerol; PPOS – progestin-primed ovarian stimulation; (**A**) Heatmap of expression profiles for the 12 lipids that showed significant expression changes, green through red color indicates low to high expression level; A – short-term protocol, B – PPOS (4 mg) protocol, C – PPOS (10 mg) protocol; (**B**) Metabolite-associated correlation analysis, the highest correlation is 1 for the complete positive correlation, the correlation is a minimum of –1 for a complete negative correlation, and the blanks were statistically correlated with a P>0.05 and a color-coded portion of P<0.05.

Another important finding of our study was that levels of TAGs and DAGs in FF samples were increased by PPOS. As second messengers, these metabolites play important roles in cell signal transduction, proliferation, differentiation, and survival [32–34]. However, little is known about the effects of high TAG levels on human oocyte development. Animal studies have shown that TAGs can be used as the key energy source in bovine oocyte maturation and embryo development *in vitro* [35]. TAGs account for 60% of the neutral lipids in oocytes and accumulate with oocyte growth in the insect *Rhodnius prolixus* [36].

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Future studies evaluating the relationship between TAG levels and human oocyte development are needed. With regard to DAGs, a previous study has shown that P stimulates *Rana* sp. oocytes to release large amounts of DAG [37]. Consistent with this, we found the IVF/ICSI treatment that induces high levels of P increases DAGs. Eckberg and Szuts similarly found that DAGs initiate the meiotic maturation of oocytes [38]. Furthermore, Albessard et al. found that levels of TAG, DAG, and PA were higher in ready-to-spawn ovaries than in spent females [39]. These findings demonstrate the crucial relationship between TAG and DAG, and the developmental potential of oocytes in a high P environment.

A remarkable finding of this study is the elevation of LPC and LPI. LPC can activate several second messengers, including extracellular-signal-regulated kinases and protein kinase C, which have been shown to be involved in the regulation of follicular development and oocyte maturation [40–42]. Likewise, LPI (specifically, LPI-12: 0), acts as a second messenger in the regulation of P and activates phosphoinositide 3-kinase signaling, which also modulates follicle development via the mammalian pre-implantation nutrition ligand, and is needed for cell survival and the first embryo division [43,44]. Therefore, we propose that the P-induced elevation of LPC and LPI levels in FF of the PPOS protocol regulates follicular development by activating several second messengers.

Our findings also showed that levels of PE and LPE were altered in FF by the PPOS protocol. However, further studies are needed to investigate the significance of these changes.

Conclusions

In conclusion, the results of these analyses demonstrate that use of the PPOS protocol alters the lipid profiles in FF. Moreover, these changes reveal the strong relevance of the differentially elevated lipids and better IVF/ICSI outcomes. The mechanisms by which these changes influence IVF/ICSI outcomes remain to be elucidated.

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Cooflicts of interests

None.

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