RESEARCH ARTICLE

Metabolomic Analysis Of Human Follicular Fluid: Potential Follicular Fluid Markers Of Reproductive Aging

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Abstract

Objective: To assess the difference in the metabolomics profiles of follicular fluid between older and younger reproductive-aged women.

Methods: The retrospective study was conducted at the Centre of Reproduction and Genetics, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, China and comprised patient data related to the period between July and October 2015. Follicular fluid was obtained from male-factor infertility women aged 28-35 years as the younger group A, and those aged 35-42 years as the older group B. The subjects were undergoing in vitro fertilization / intracytoplasmic sperm injection and were retrospectively analysed by ultra-performance liquid chromatography-high-resolution mass spectrometry. The fragments were structurally identified using debris' information obtained from fragmented ion scans to identify the different compounds.

Results: Of the 55 cases studied, 28(51%) were in group A with a mean age of 29.57 ± 2.92 years, and 27(49%) were in group B with a mean age of 39.19±2.95 years. Compared with the group A, four types of compounds, hormones, licithin, lysophospholipids, and protein degradation fragments, were expressed significantly differentially in group B ($p<0.05$ each). Nicotine glucuronide and phosphatidylcholine were found only in Group B follicular fluid.

Conclusion: The components of follicular fluid and relative contents were found changed with ageing.

Keywords: Follicular fluid, Ageing, Biomarkers, In vitro fertilisation. (JPMA 68: 1769; 2018)

Introduction

Follicular fluid (FF) is produced by follicles and contains a large number of metabolites that play important roles in follicle development and oocyte maturation. Previous studies have shown that gonadotropins and steroids are markers of oocyte maturation in FF.[1] Other metabolites such as vascular endothelial growth factor (EGF),[2] inhibin A and B, anti-Müllerian hormone (AMH), lactoferrin, insulin-like growth factor-II (IGF-II), hyaluronan, nitric oxide,[3]leptin,[4]25-hydroxy vitamin D (25OHD), glucose, bone morphogenetic proteins (BMPs),[5] and interleukin 8 (IL-8)[6] are associated with oocyte maturation and follicular development. This microenvironment may be altered by reproductive aging.[7]Reproductive aging is marked by a decline in ovarian response, oocyte number,[1] and oocyte quality. Female age and quality of the retrieved oocytes are the major factors that affect in vitro fertilisation (IVF) outcomes. Previous studies have shown that altered FF composition is associated with reduced reproductive capacity.[5] IL-8,[6] growth differentiation factor 9 (GDF-9) and transforming growth factor beta 1 (TGF-β1),[8] inhibin, AMH,3 and bone morphogenetic protein (BMP) 155 have been found to be correlated with reproductive aging. Therefore, metabolomics analysis of FF might aid in identifying predictive markers of oocyte development to evaluate oocyte quality prior to fertilisation and predict the success of assisted reproductive technology (ART).[1,6]

Proteomic analyses have been applied to assess human FF to obtain information on the pathophysiology of conditions such as recurrent spontaneous abortion, polycystic ovary syndrome (PCOS), endometriosis, ovarian hyperstimulation syndrome (OHSS), and failure to become pregnant after IVF.[9]According to the physiological interdependence between FF and oocytes, female age-related reproductive decline may be associated with deleterious alterations in FF physiology. It is important to properly assess ovarian ageing at an early stage when counselling patients regarding their chances for pregnancy, either spontaneously or during fertility therapy. There have been few clear indicators that can be used to predict non-pregnancy and for screening tests and counselling purposes.[10]

The current study was planned to assess metabolomics profiles of FF between older and younger reproductive-aged women. It was hypothesised that the composition

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differences in FF during ovulation can be characterised using certain metabolites.

Materials and Methods

The retrospective study was conducted at the Centre of Reproduction and Genetics, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, China, and comprised data between July and October 2015 of infertile women undergoing IVF/ Intracytoplasmic sperm injection (ICSI. The study was approved by the institutional ethics committee. A standard gonadotropin-releasing hormone antagonist (GnRH-ant) protocol was adopted, and 36 hours after human chorionic gonadotropin (HCG) injection, FF was collected and preserved at -80°C. FF obtained from women aged 28-35 years was called the younger group A, and from those aged 35-42 years as the older group B.

Gemfibrozil was purchased from Sigma (St. Louis, MO). ZW14 was used as an internal standard and was provided by Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences. Organic reagents acetonitrile and formic acid were of the highest grade commercially available.

FF samples (100 μL) were mixed with 300 μL of methanol containing 4 μM gemfibrozil and 4 μM ZW14. The mixture was vortexed for 5 minutes and then centrifuged at 14000 × g for 30 minutes at 4°C. The supernatant was transferred to an autosampler plate for analysis. The supernatant (2 μL) was injected into an ultra-performance liquid chromatography coupled quadrupole time-of-flight mass spectroscopy (UPLC-ESI-QTOFMS) system (ACQUITY UPLC, Waters Corp, Milford, MA). Nitrogen was used as the cone gas (50 L/h) and desolvation gas (600 L/h). The source temperature and desolvation temperature were set at 110 and 360 degrees, respectively. The capillary voltage and cone voltage were 3000 and 20 V, respectively. Injection was performed in a randomised order according to sample collection sequence before grouping to avoid complications caused by artifacts associated with 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, Milford, MA) 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 1.0 min, increased to 100% B over the next 6.0 min, and then returned to 95% A from 9.0 min to 9.2 min. The total run time was 13 min. Data was collected in both positive and negative modes on a QTOF mass spectroscopy, operated in full-scan mode at 100 – 1200 m/z. Raw data from the UPLC-ESI-QTOFMS system was processed using MarkerLynx software (Waters) to generate a data matrix consisting of peak areas corresponding to a unique m/z and retention time (RT) with normalisation. EZ info software was used to construct score plots by principal component analysis (PCA). Loading scatter S-plots were produced by supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA). The contribution list of metabolites was produced at $p>0.05$ and $p(corr)>0.6$. The contributory list was further investigated to assess the candidate biomarkers in older women compared with younger women.

To identify the metabolites with a high contribution score, metabolomics databases (Madison Metabolomics Consortium Database and METLIN)[11,12] were searched to find potential candidates.

Data was presented as the mean ± standard deviation. Student's t-test or Mann-Whitney or chi-square tests were used for statistical comparisons. Statistical significance was defined as $p<0.05$.

Results

Of the 55 cases studied, 28(51%) were in group A with a mean age of 29.57±2.92 years, and 27(49%) were in group B with a mean age of 39.19±2.95 years. Group A had 19(68%) IVF and 9(32%) ICSI cases, while group B had 13(48%) and 14(52%) cases respectively.

There were differences in follicle-stimulating hormone (FSH) ($p<0.001$) and basal antral follicle count (AFC) ($p<0.001$) between the two groups. The number of retrieved metaphase II oocytes was significantly lower in group B than group A ($p<0.05$). The mean number of oocytes from the two groups was also compared (Table-1).

Table-1: General patient information.

Index	Younger group	Older group	*P*
Age (years)	29.57±2.92	39.19±2.95	<0.001
Infertility duration (a)	3.18±1.85	93.22±2.55	0.25
Basal AFC (n)	7.69±1.28	13.26±6.80	41
FSH (IU/L)	7.69±1.28	10.87±7.40	<0.001
LH (IU/L)	4.39±1.50	5.98±8.54	<0.001
E2 (ng/L)	37.69±16.80	44.15±20.89	0.70
P (ng/L)	0.37±0.14	0.43±0.40	0.06
Fertilization IVF(%)	67.86 (19/28)	48.15 (13/27)	0.55
ICSI(%)	67.86 (19/28)	51.85 (14/27)	0.14

The fertilisation method analysed by chi-square test, 0.14 is the value of sig. Others were analysed by the Mann-Whitney test.

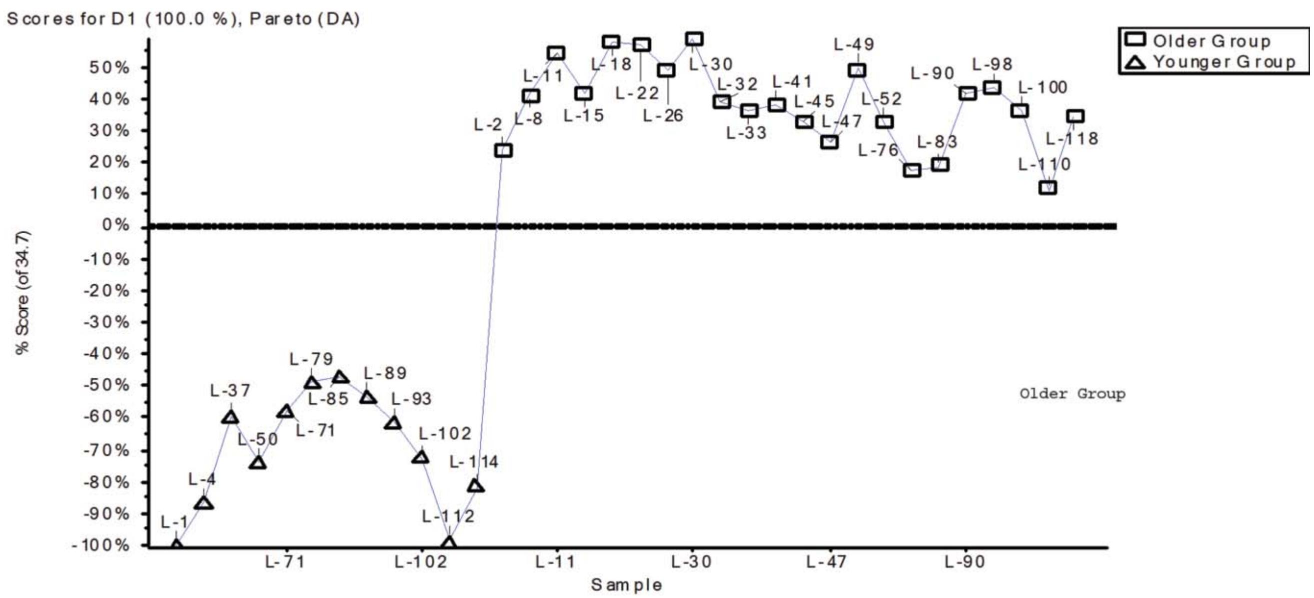

Figure-1: Comparison of PLS-DA arrangement of follicular fluid samples between different groups.

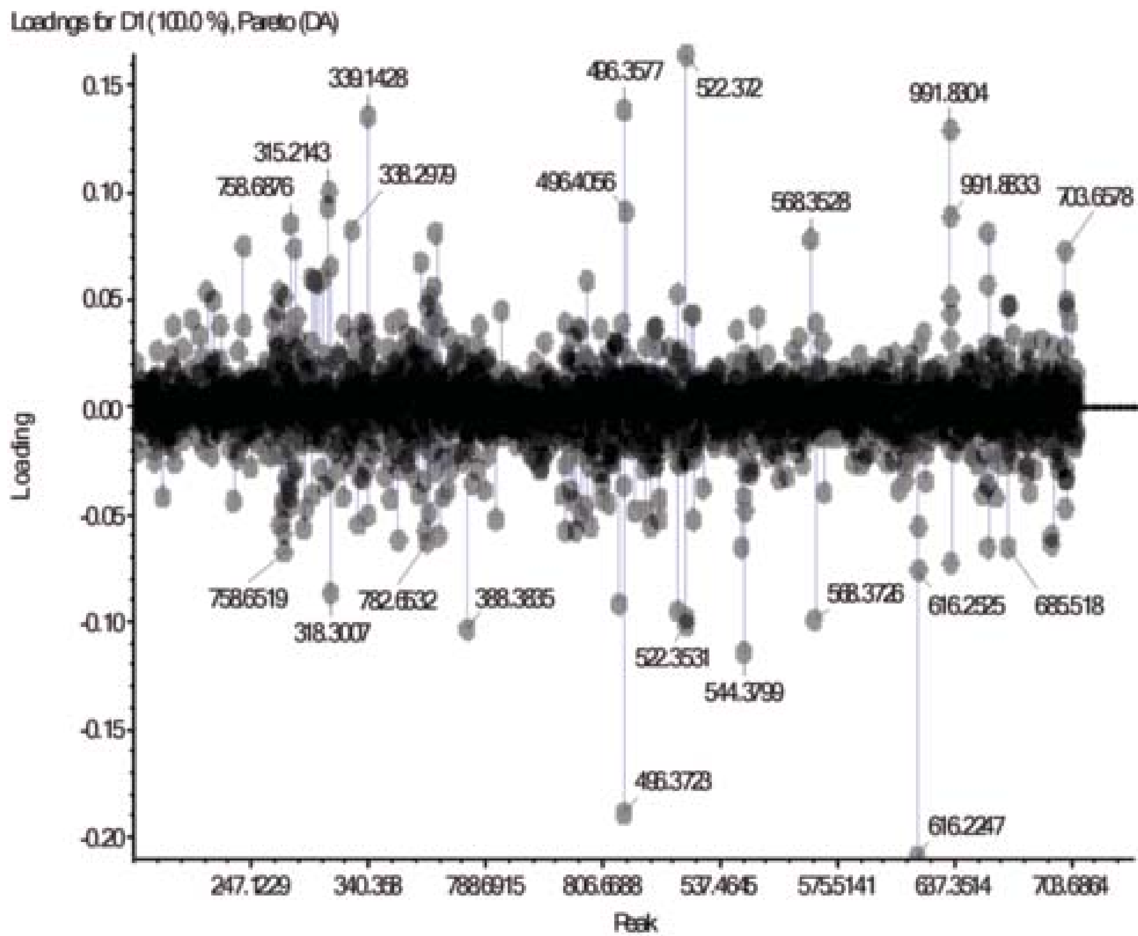

Figure-2: Comparison of load graphs between the two groups.

PCA modelling of FF from younger women revealed that the baseline and control samples grouped together. FF samples from the older women deviated from the baseline and control samples, indicating significant metabolic changes between the two groups (Figure-1). Notably, they varied along the first component in the scores plot, indicating that some metabolites in FF from the older women had significantly changed. After pattern recognition of FF samples from the younger group, OPLS-DA was used to produce loading scatter S-plots (Figure-2). Mass spectroscopy (MS) values that were thought to be endogenous metabolites were used for mass-based searches in the metabolomics databases. Metabolites that matched for characteristics and accurate masses were diacylglycerol (DAG) (14:1(9Z)/22:2(13Z,16Z)/0:0), triacylglycerol (TAG) (18:1(11Z)/24:0/20:5(5Z,8Z,11Z,14Z,17Z)), Lyso-phosphatidylcholine LPC (14:0), LPC (16:0), LPC (18:0), phosphatidylcholine (PC) (16:0/22:0), 4-oxo-retinoic acid, nicotine glucuronide, and 3-hydroxynonanoyl carnitine (Table-2).

Table-2: Differential compounds between two groups.

Molecular Formula	Identity (Older group vs Younger group)	Fold change* (O/Y)	T-test (p)
$C_{20}H_{26}O_3$	4-oxo-retinoic acid↑	2.4	0.03
$C_{22}H_{46}NO_7P$	LysoPC (14:0) ↓	0.3	0.02
$C_{24}H_{50}NO_7P$	LysoPC (16:0) ↓	0.5	0.03
$C_{26}H_{54}NO_7P$	LysoPC (18:0) ↓	0.4	0.04
$C_{16}H_{31}NO_5$	3-hydroxynonanoyl carnitine↑	2.5	0.04
$C_{39}H_{70}O_5$	DG(14:1(9Z)/22:2(13Z,16Z)/0:0) ↓	0.6	0.04
$C_{65}H_{114}O_6$	TG(18:1(11Z)/24:0/20:5(5Z,8Z,11Z,14Z,17Z)) ↓	0.3	0.03
$C_{16}H_{22}N_2O_6$	Nicotine glucuronide↑	9.6	0.001
$C_{46}H_{94}NO_7P$	Phosphatidylcholine (16:0/22:0) ↑	7.5	0.001

*Fold change is the area ratio of peaks between the two groups, the higher the ratio is, the greater the difference is.

Discussion

Differences in lipid compounds were found between the older group and the younger group. DAG (14:1(9Z)/22:2(13Z, 16Z)/0:0) and TAG (18:1(11Z)/24:0/20:5(5Z,8Z,11Z,14Z,17Z)) levels were significantly lower in the older group compared to the younger group, and therefore, because DAG and TAG are components of oocyte lipid metabolism, the energy metabolism of oocytes in the older group differed from that of oocytes in the younger group. DAG, which is actually glycerol fat, is a fat metabolic intermediate, and it was the first lipid second messenger to be discovered. DAG is converted into phosphatidyl inositol (PI), phosphatidyl inositol-4-phosphate (PI-4-P), and phosphatidyl inositol-4, 5-diphosphate (PI-4, 5-[P2]) by phospholipase C (PLC) pyrolysis, and protein kinase C (PKC) is the main affector. DAG binds to PKC at a conservative C1 domain structure, leading to activation of PKC, which regulates the cell cycle, cell survival and apoptosis in malignant transformation.[13] During oocyte ageing, irregular follicles become more prominent, and lipid properties change during oocyte maturation. TAG is stored in oocytes, and it can be used for beta oxidation in the mitochondria to produce energy during oocyte maturation. TAG is one of the most abundant lipids in oocytes, constituting more than 50% of oocyte lipids[14] and representing a huge potential energy reserve for oocytes. DAG and TAG levels were reduced in the older age group, which would affect the energy metabolism and development of the oocytes. Thus, it is likely that in kidney deficiency and infertility, oocyte energy reserves decrease, resulting in a decrease in their quality. However, the specific mechanism of this decrease requires additional studies.

Phospholipids play an important role during oocyte maturation and embryo development. In addition, during oocyte maturation, the concentrations of phospholipids and cholesterol are key factors following fertilisation of the oocytes for membrane formation during rapid cell division. In oocytes, PI accounts for 6% of total phosphatide and is rich in arachidonic acid (20:4n-6), stearic acid (18:0), and palmitic acid (16:0). The membrane phospholipids are hydrolysed to produce two second messengers, inositol 1, 4, 5-triphosphate (IP3) and DAG, which are particularly important in oocyte maturation and fertilisation. The most well-studied lysophospholipid related to female reproductive function is lysophosphatidic acid (LPA). Of the phospholipids, it is the smallest but has the most simple structure, and it has been shown as an important signalling molecule in various biological processes, such as cell proliferation, differentiation, activation, morphogenesis, and cytokine secretion.[15] LPA is produced by membrane phospholipids through two major pathways/enzymes: autocrine motility factor (ATX) and phospholipase A2 (PLA2).[16] It has been shown that LPA supplementation could improve oocyte function by greatly increasing the messenger ribonucleic acid (mRNA) levels of the oocyte maturation mediators follistatin (FST) and GDF-9 while reducing co-transcriptional splicing (CTS) mRNA levels in oocytes .[17] In addition, stimulation of oocytes, which have high B-cell lymphoma 2 (BCL2) transcription levels and low BCL2-Associated X (BAX) protein transcription levels, with LPA leads to a significant decrease in the BAX/BCL2 ratio, and, in granulosa cells, LPA has several roles, including autocrine and paracrine functions, and increases the

expression of the FSH receptor (FSHR) and 17 beta hydroxy steroid dehydrogenase (17 beta HSD) to stimulate the synthesis of estradiol (E2) .[18]When used in the vagina, LPA receptor (LPAR) antagonists can reduce the pregnancy rate, increase the longevity of the corpus luteum (CL), and stimulate oocytes to promote lactobacillus prostaglandin (PG) and E2 synthesis;[19] thus, LPA plays an important role in the development and maturation of oocytes.

It has also been reported that addition of LPA to the maturation medium can promote the connection between oocytes and surrounding cells, embryonic development, oocyte and cumulus cell apoptosis, and oocyte gene expression. LPC is a kind of phospholipid, and under normal physiological conditions, most cellular LPC is quickly metabolised or acetylated. PC and sphingomyelin (SM) are functional structural units of the plasma membrane, and their composition determines most of the physical and chemical properties of the membrane, including liquidity, permeability, and thermal behaviour.[20] PC can regulate the activity of various ion channels during cell preparation. PC has also been shown to lead to continuous increases in intracellular calcium ions (Ca2+) or be associated with the activation of selective cation channels. Through metabolomics analysis, the current study found lower expression in the levels of LPC (14:0), LPC (16:0), LPC (18:0), and PC (16:0/22:0) between the younger and older groups. These four compounds are types of phosphatide, but the phospholipid PC (16:0/22:0) was only found in the older group. Whether these four phospholipids would affect embryonic development, oocyte and egg high apoptosis gene expression, ability of oocytes, and oocyte development and maturation as LPA is not known and requires further research.

Further, 4-Oxo-retinoic acid (4-oxo-RA) is an active geometric isomer of retinoic acid (RA). The biological activity of geometric isomers of RA is affected by CYP26A1, CYP26B1, and CYP26C1, which are three types of cytochrome P450 enzymes (CYP26) that metabolise polar metabolites,[21] and this is involved in many physiological processes, including energy metabolism, mainly by affecting the metabolic activity of RA in cells to regulate gene expression. During embryonic development and in adult epithelial tissues, RA is a key regulator of gene expression, mainly by activating two receptors within the nuclear receptor superfamily, retinoic acid receptor (RAR) and class retinol X receptor (RXR). RAR/RXR was found to affect Epo expression during embryogenesis, affecting the growth of the embryo.[22] RA is an active metabolite of vitamin A (VA, retinol), which is a lipophilic micronutrient. Moreover, its derivatives with similar or equivalent active compounds are often referred to as retinol, and it has been found to play a role in a wide range of mammalian physiological processes, such as spermatogenesis, fertilisation, pregnancy, morphogenesis and organogenesis, and perinatal foetus growth. In adults, retinoids regulate reproduction, immunity, vision, and metabolism, and maintain the function of the skin, lungs, bone marrow, liver, and nervous system. RA also has genomic and non-genomic regulatory functions.[23] A recent study reported that the RA biosynthesis inhibitor WIN 18446 may be used in the treatment of obesity and as a potential new male contraceptive. In the body, WIN 18446 can significantly reduce RA concentration, leading to increased serum retinol levels and reduced visceral obesity to reduce spermatogenesis. These findings shed new light on basic retinol physiology and indicate that WIN 18446 has potential for the treatment of obesity and as a male contraceptive drug,[24] but the role of RA inhibitors in the female reproductive system has not been studied. The findings of this study indicated that in older women, 4-oxo-RA affects reproductive function, but the exact mechanism, specifically related to pregnancy and foetal development requires further study.

Nicotine glucuronide (nicotine) accounts for 3-5% of the converted nicotine, and in the human body, before it is excreted in the urine, nicotine is modified by the catalyst uridine diphosphate-glucuronic acid glycosyl transferase (UGT). Nicotine induces oxidative stress and increases apoptosis in neurons, deoxyribonucleic acid (DNA) damage, active oxygen and lipid peroxidation. It affects cell proliferation and apoptosis and induces insulin resistance and metabolic syndrome. Nicotine can also cause a hypo-oestrogenic state by inhibiting 21-hydroxylase, and it can increase androgen levels and lead to chronic irregular ovulation and menstruation cycles. Nicotine can also induce inappropriate cytokine production and irregular bleeding in the endometrium. Another study found that nicotine can affect the ovaries and induce changes in oocytes. Nicotine-treated oocytes have a non-spherical shape and rough surface, and tearing and irregular transparent tape, and is an oocyte maturation disorder.[25]

In this study, we compared the FF compounds found in the older group and younger group, and only the samples from the older group contained nicotine glucuronide, which, when present in the FF during oocyte development, has an adverse effect on the reproductive system, decreasing female reproductive function.

The 3 hydroxynonanoyl carnitine (3-hydroxy pelargonic

acid carnitine) belongs to the family of acyl carnitines and contains O-acylating carnitine. Acyl carnitine is necessary for fatty acid metabolism, and, during growth and ageing, it is the key enzyme involved in lipid metabolic changes; thus, in older women, fatty acid production increases in the oocytes, and oocytes with lipid particles are more likely to exhibit cytoplasmic damage, leading to reduced in vivo and in vitro fertilisation ability. The metabolism of fatty acids by fatty acid oxidation (FAO) produces adenosine triphosphate (ATP), which is an important process of the meiotic maturation of oocytes and early embryonic development. FAO occurs through the peroxidase proliferator-activated receptor (PPAR) signalling pathway in many organisms. The PPAR nuclear receptor acts as a transcription factor to regulate lipid metabolism, and there are three main types: PPAR alpha, PPAR gamma, and PPAR delta; each is activated by endogenous prostaglandins and fatty acids.[26] Moreover, 3-hydroxy pelargonic acid carnitine is necessary for fatty acid metabolism, and it can promote the oxidation of fatty acids and the production of ATP. Other fatty acids, such as steroid hormones, are important participants in transport and are important regulators in cellular signalling pathways. Excess abdominal adipose tissue is often seen in metabolic syndrome, and abnormal lipid metabolism may result in an increase in saturated fatty acids (SFAs), which can enhance the expansion of fat tissue and then activate inflammatory signals and insulin-induced insulin resistance. In another study, fatty acids were shown to participate in oocyte maturation, fertilisation, and embryo development.[27] Polyunsaturated fatty acid (PUFA) supplementation has been shown to delay pregnancy[28] and to affect the function of the ovaries and uterus.

The current study has its limitations. It is only a primary screen of biomarker compounds in FF of ageing patients undergoing ART. To make the findings meaningful for clinical diagnosis and treatment, further studies on the mechanism and regulation of specific factors is necessary.

To our knowledge, however, no study has been published on the association between the metabolites detected by MS in our study and ovarian ageing; thus, these findings require additional investigation. Because the exact functions of these metabolites in the ovary are not fully understood, further studies are required to clarify the roles of these markers in female fertility. This information could be useful before the initiation of an IVF cycle. Also, we found differences in certain metabolites in FF between younger and older women. Further studies are needed to clarify the exact mechanism of action of these metabolites.

Conclusion

Novel candidate markers were found for oocyte number and, importantly, reproductive ageing during the IVF cycle, indicating that deoxycorticosterone and LysoPC may play important roles in folliculogenesis.

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Conflict of Interest: None.

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