의학석사 학위논문

Metabolomic analysis of follicular fluid in women with endometriosis

– A prospective study –

자궁내막증 여성에서의 난포액의 대사체 분석

– 전향적 연구 –

2023년 02월

서울대학교 대학원

의학과 산부인과전공

김 혜 경

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이 논문을 의학석사 학위논문으로 제출함

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2023년 1월

위 원 장 _____ (인)

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Abstract

Metabolomic analysis of follicular fluid in women with endometriosis

– A prospective study –

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Background: Endometriosis (EMS) is a benign gynecologic disease defined as ectopic proliferation of endometrial gland and stroma. Although the strong relationship between EMS and infertility is well known, its mechanism is still a conundrum. Recently, metabolomics has been spotlighted as a tool to elucidate the etiology, pathophysiology and mechanism of various diseases. Despite follicular fluid (FF) provides the microenvironment for follicular development and affects the quality of oocytes, there are only a limited number of metabolomic studies analyzing FF in EMS. The aim of this study is comparing the metabolomic and microbiome composition of FF of unilateral ovarian EMS with non-EMS patients.

Method: Ten women receiving oocyte retrieval were enrolled prospectively from July 2021 to July 2022 at Seoul National University Bundang Hospital. Five patients were diagnosed with unilateral EMS and the other five patients were non-EMS control group. In EMS group, FF from EMS-affected ovary was collected. Targeted quantitative metabolomics kit, which can detect 188 metabolites, and twenty bile acid (BA) quantification kit are used for metabolomic analysis. Multivariate analysis (principal component analysis) was performed to identify discriminative the differences of composition.

Result: There were six metabolites with statistical differences. In EMS group, acylcarnitine propenoylcarnitine (C3:1) was significantly increased, whereas

amino acid valine, alanine, acylcarnitine butyrylcarnitine (C4), butenylcarnitine (C4:1), and phosphatidylcholine diacyl C 38:3 (PC aa C38:3) were significantly elevated in non-EMS control group. Since antimullerian hormone level and the presence of DOR showed significant difference between EMS group and non-EMS group, the correlation with these factors and the six metabolites were performed. Valine was showed statistically significant positive correlation with AMH and C3:1 and valine had negative and positive correlation with DOR, respectively. Also, the BA kit analysis did not show any statistical difference between EMS and non-EMS patients.

Conclusion: The different levels of acylcarnitines, amino acids, and glycerophospholipids suggest that endometriosis has altered mitochondria energy metabolism in cellular level. The gut microbiome may not affect the pathophysiology of follicular development in EMS since BA kit did not show significantly different patterns.

Keyword: metabolome, endometriosis, follicular fluid, energy metabolism, local inflammation

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1. Introduction

1.1 Study Background

Endometriosis (EMS) is defined as the proliferation of endometrial glands and stroma in the outside uterus. The prevalence of EMS is reported as 10% in reproductive age women and 5−50% [1] in infertile women. It not only causes deteriorating dysmenorrhea, menorrhagia, chronic pelvic pain and dyspareunia, but also decreases ovarian reserve leading to female infertility [1]. There are a number of studies about the association between EMS and infertility. Infertility caused by EMS is also known to accompany poor in vitro fertilization (IVF) outcomes [2]. Nonetheless, the etiology and pathophysiology of endometriosis and its mechanism to infertility remain unclear.

In the aspects of etiology, traditionally, retrograde menstruation theory is most widely accepted. Besides, coelomic metaplasia, stem cell theory, vascular/lymphatic dissemination, immune dysregulation, genetic factors, environmental factors, and oxidative stress are postulated [4]. One theory cannot fully explain the origin of endometriosis and these factors are considered to interplay [7].

Meanwhile, metabolomics is a field of '−omics' elucidating chemical reactions caused by metabolites of metabolic processes. Metabolomics has a unique characteristic of capturing what is getting through in a cellular level. Therefore, metabolomics is more adequate than genetics and proteomics to reveal the disease mechanism because it gives the real information of mechanism inside the cell [6]. On the other hand, genetics and proteomics provides limited information about the possible future events. Metabolomic analysis can be made with various samples such as blood, urine, stool, and body washing fluid.

In addition, the microbiome refers any types of genetic materials from

microbes. The microbes inhabit the host and regulate the physiologic functions. It encompasses bacteria, fungi, viruses, and archaea. The microbiome is known to affect the host immunity and the progression of some of inflammatory diseases. Especially, the role of gut microbiome had been investigated in the aspect of gastrointestinal epithelium integrity, immunity, and the transport of bacteria. The composition of microbiome in female genital tract, however, is rarely investigated. Also, the effect of the microbiome on gynecologic disease including endometriosis is sparse.

In this study, we analyzed follicular fluid (FF) of ovarian EMS patients because of its crucial role in oocyte development and infertility treatment outcome. The difference of biochemical composition in FF is known to impact the development of human oocytes in cellular and chromosomal levels [9]. Also, the composition of FF is strongly associated with clinical outcomes such as fertilization, embryo development, and even clinical pregnancy rate [10]. Moreover, FF shows higher concentration of steroid, FSH, LH, growth factors, peptides, inositol than serum samples. Therefore, we assumed that FF from ovarian EMS more strongly reflects EMS-associated metabolites and its metabolism.

1.2 Purpose of Research

This study is aimed to elucidate the etiology and mechanism of EMS and the effect of ovarian EMS to FF by using metabolomic analysis. Also, the present study is designed to investigate the relationship between EMS-related metabolites, microbiome composition and infertility.

It was hypothesized that ovarian EMS patients may have different levels of metabolites and microbiome composition with non-EMS patients and these aberrant metabolites may reflect the pathophysiology and mechanism of EMS.

2. Methods

2.1 Study Design

This is a prospective study performed between July 2021 and July 2022 at Seoul National University Bundang Hospital. A total ten individuals participated and all patients underwent standard infertility workup, treatment and gonadotropin-releasing hormone (GnRH) antagonist regimen protocols.

The inclusion criteria for both groups are women between 20 and 38 years old and who underwent oocyte retrieval for infertility treatment or fertility preservation. Infertility was defined as a failure to clinical pregnancy for more than 12 months of unprotected sexual intercourse or 6 months of unprotected sexual intercourse in women younger than 35-years-old or in women elder than 35-years-old, respectively. Infertility patients had no evidence of other causes of infertility and EMS at other times during workup. Fertility preservation included both oocyte cryopreservation and embryo cryopreservation prior to endometriosis surgery. In endometriosis group, all patients only had unilateral ovarian endometriosis and did not have evidence of other endometriosis. The endometriosis was diagnosed by transvaginal ultrasound. In ultrasound, ovarian endometriosis was defined as a cystic lesion with homogenous, low-level or 'ground glass' echogenicity. The size of a cystic lesion was at least 3-centimeter.

The exclusion criteria for both group are as following: women younger than 19-year old or elder than 39-year old, patients with congenital female reproductive tract anomaly, history of acute inflammatory diseases including pelvic inflammatory disease and acute vaginitis, the presence of malignancy, autoimmune disease or endocrine disease, use of hormone, antibiotics, or vaginal pills during the past one month, history of ovarian surgery during the past 6 months, patients with history of gonadotoxic therapy, and previous history of bowel surgery. The follow-up data of all the patients were

extracted from electronic medical records.

Meanwhile, the non-endometriosis control group consisted of patients with no evidence of any endometriosis. Patients with tubal factor infertility, male factor infertility, unexplained factor infertility, or who desire fertility preservation were recruited. As a result, four patients were receiving infertility treatment due to unexplained infertility and the other one did not have any evidence of infertility and desired fertility preservation.

2.2 Ethics

The study received institutional review board approval (IRB No. B-2108-700-303) and the informed consent was obtained from all participants before the enrollment.

2.3 Controlled Ovarian Stimulation Protocols for Oocyte Retrieval

All patients received a standard GnRH antagonist regimen starting on the early follicular phase of the menstrual cycle. Initially, recombinant follicle-stimulating hormone (rFSH, Gonal-F; Merck Serono, Geneva, Switzerland), follitropin-delta (Rekovell; Ferring, Malmo, Sweden) or FSH combined with LH, human menopausal gonadotropin (hMG, Menopur; Ferring, Malmo, Sweden) was administered to all participants. The starting dose of gonadotropin was determined by the clinicians in accordance with age, BMI and ovarian reserve. When the leading follicle reached 13-14mm in diameter, a GnRH antagonist (Cetrolix, cetrotide 0.25 mg, Merck Serono) was used to suppress premature LH surge. When two or more leading follicles reached 18mm in diameter, a recombinant human chorionic gonadotropin (rhCG) (Ovidrel 250 μg, Merck-Serono) or 0.2 mg triptorelin (Decapeptyl, Ferring), or a combination of the two (250 mg of rhCG plus 0.2 mg of triptorelin) was injected for final oocyte maturation. 36 hours after the triggering, transvaginal ultrasound-guided oocyte retrieval was performed. For oocyte

cryopreservation, metaphase II (MII) oocytes were chosen and the additional process of intracytoplasmic sperm injection (ICSI) was performed for embryo cryopreservation.

2.4 Collection and Processing of Follicular Fluid

FF was obtained from the group of follicles present in each ovary in both groups. In the endometriosis group, FF from affected side of ovary was collected. In non-EMS group, FF was collected from both sides of ovaries. All FF was centrifuged at 1500 rpm for 10 min to remove cell components. The supernatant was collected and maintained frozen at -80℃ until analysis.

2.5 Targeted Metabolomic Analysis Using Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS)

A total of 188 metabolites and 15 BAs were quantified by the AbsoluteIDO® p180 kit (Biocrates Life Science AG, Innsbruck, Austria) and the Biocrates® bile acids kit (Biocrates Life Science AG, Innsbruck, Austria) with an LC-MS/MS, respectively. Since bile acid kit reflects the difference of microbiome in gut, this kit was applied to evaluate the microbiome composition of FF in both EMS and non-EMS group. This system allows high throughput capacity and quantification of metabolites in FF samples simultaneously. This system consisted of direct flow injections for acylcarnitines and glycerophospholipids and LC-MS for amino acids and biogenic amines. The analysis process was proceded by the manufacturer's instructions. To summarize, 10 μL of FF was transferred to the 96-well AbsoluteIDO® p180 kit plate and to the Biocrates® BAs kit. Once FF was vaporized under nitrogen gas, it was derivatized by phenylisothiocyanate reagent. Then, the metabolites were acquired by using 5mM ammonium acetate in methanol for standardization and direct injection analysis mass spectrometry. Also BAs were extracted by 10mM ammonium acetate and 0.015% formic acid and LC-MS/MS system was applied to

identify and quantify the BAs.

2.6 Statistical Analysis

For baseline and clinical outcomes, all statistical analyses were performed by using the Statistical Package for the Social Sciences version 25.0 (SPSS Inc., Chicago,IL, USA). Data are reported as mean ± standard deviation. The Pearson $\chi 2$ and Fisher exact test were used for categorical data. Continuous variables were demonstrated as median values as indicated and categorical variables were described as "n (%)" in the tables. This process was repeatedly performed by statistics department of Chung-Ang University for statistic validation.

Multivariate, univariate, and enrichment analyses were conducted using the Metaboanalyst 4.0 tool. Principal component analysis (PCA) was performed to evaluate the differences in overall metabolites and BAs profiles between EMS group and non-EMS group. PCA is a statistical tool used to reduce the dimensionality of datasets and to accelerate interpretability. It also enables to minimize information loss by creating uncorrelated variables. Since a great number of metabolites (188 metabolites from p180 kit and 15 BAs from BA kit) were analyzed, PCA had been adapted [18].

3. Results

The baseline characteristics of the 10 participants are present in Table 1. The level of AMH and the presence of decreased ovarian reserve were significantly higher in endometriosis group.

No statistically significant difference was found between EMS group and non-EMS group in aspects of age, BMI, gravida, duration of infertility, previous history of oocyte retrieval, total gonadotropin dose, and duration of gonadotropin use. Furthermore, clinical outcomes of EMS group and non-EMS group were analyzed. Unlike our expectation, total volume of FF, the number of oocytes retrieved, the number of mature oocytes, the number of embryos, fertilization rate and the number of good quality embryo did not show statistical differences. Among them, however, p-value of the number of mature oocytes was 0.056, which did not reach the significance level but indicates a strong relationship with EMS. This is revealed at Table 2.

Among 188 metabolites, six metabolites showed statistical difference. Acylcarnitine propenoylcarnitine (C3:1) was increased in EMS group compared with non-EMS group. On the other hand, alanine, valine, acylcarnitine butyrylcarnitine (C4), butenylcarnitine (C4:1), and phosphatidylcholine diacyl C 38:3 (PC aa C38:3) were elevated in non-EMS group. Figure 1 is a volcano plot of both p180 kit and BA kit. Figure 2 and Figure 3 present two-dimensional PCA data between EMS group and non-EMS group and relative intensity of valine, alanine, C3:1, C4:1 and C4, respectively. As figure 1 is shown, valine, alanine, C3:1, C4:1, C4, and PC aa C38:3 has statistically significant difference between two groups and their concentration has been changed more than a certain level.

Since AMH level and the presence of DOR showed significant difference between EMS group and non-EMS group, the correlation between each metabolite and AMH level and the presence of DOR was analyzed. Among the six metabolites, valine and AMH had significance relationship, while C3:1, C4:1, and PC aa C38:3 indicated a strong relationship with AMH but no

statistical significance. In the aspect of DOR, both C3:1 and valine had significance and PC aa C38:3 showed a strong relationship with p-value of 0.0797. Detailed statistic result is presented at Table 3.

Additionally, there was no difference in the analysis of BA kit comparing EMS group and non-EMS group. The result is presented as heatmaps at Figure 4.

In this study, we used bile acid kit to investigate the microbiome composition because of the association between the gut microbiome and bile acid. Bile acids are converted into secondary bile acids at the gut. This process is performed by gut microbiome. Therefore, the alterations in the gut microbiome are well reflected to the composition of bile acids. Also, it is considered that particular types of bile acids have unique effects on the inflammatory response and gut permeability [19]. As the gut microbiome is related with the alteration of microbiome of cervix, vagina, endometrial fluid, peritoneal fluid and endometriosis tissue [20, 21], we conducted bile acids analysis for follicular fluid. As the figure 4 presents, there was no difference of BAs pattern between case and control groups.

4. Discussion

In our study, five metabolites, alanine, valine, C4, C4:1, and PC aa C38:3, were decreased and one metabolite, C3:1, was elevated in endometriosis patients. There are a limited number of studies analyzing follicular fluid of endometriosis patients. Yland J et al. evaluated the level of cytokines in FF of unilateral ovarian endometriosis [3]. In the study, interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1) were higher in EMS-affected ovary than in non-EMS group. Weak differences were observed for IL-1β and IL-6. It suggests that the inflammation milieu of ovarian EMS is strongly localized rather than systemic. It has similarity with our study when compared FF of EMS-affected ovary and FF of non-EMS group. Several previous studies about peritoneal fluid and endometrial fluid of endometriosis support this argument [15-17]. One study evaluating metabolites of peritoneal fluid in endometriosis women revealed the up-regulation of lysophosphatidylcholine and derivatives of phosphoethanolamine, acylcarnitine andkynurenine in endometriosis women [15]. This study supports our hypothesis that endometriosis develops in organ level. Even though phosphatidylcholine and acylcarnitine showed significant difference, the direction of change was opposite with our study. In our study, phosphatidylcholine diacryl C38:3, butyrylcarnitine, and butenylcarnitine were down-regulated in FF of endometriosis patients.

In terms of metabolites, mitochondrial dysfunction is suspected in FF of ovarian EMS [13, 14]. A study regarding endometriosis tissue and its endometrial tissue in non-human primate (NHP) was performed [5, 11]. In this study, metabolomic analysis for 28 metabolites and mitochondrial respiratory assay for endometriosis tissue and its endometrium were performed. Mitochondrial respirometry assays presented that endometrium has decreased rates of complex-II-mediated oxygen consumption and

endometriosis tissue has lower complex I–mediated oxygen consumption rates. In metabolomics analysis of this study, EMS tissue showed significantly decreased levels of carnitine, creatine phosphate, NADH, FAD, tryptophan, and malic acid compared to normal control group. They also identified pathways of these metabolites and identified that tryptophan and nitrogen metabolism pathway in endometrium tissue of EMS in NHP has been decreased. In endometriosis tissue, riboflavin metabolism has been declined. Although this study regarded NHP, it showed similar metabolomics changes with our study. However, it analyzed endometriosis tissue and endometrium tissue. Also, our study revealed that C3:1 is elevated in EMS group. Nonetheless, similar energy pathway alterations may have occurred in human FF. At present, out study did not reveal a specific metabolomic pathway compromising multiple metabolites among the six metabolites. However, further study focusing on tryptophan, nitrogen, and riboflavin pathways can be conducted to elucidate endometriosis pathways in human. Also, acylcarnitine and phosphatidylcholine are known to participate beta–oxidation in mitochondria. Therefore, mitochondrial dysfunction is strongly suggestive. This result also correlates with many previous studies about endometriosis and mitochondria dysfunction. Recent studies found that estrogen influences the expression shape, and function of mitochondria including its adenosine triphosphate (ATP) energy generation and antioxidant defenses [13]. This process is shown in Figure 5. It can be grounds for our result since our metabolomic analysis indicates strong relationship with mitochondrial beta–oxidation dysfunction.

What is more, it is suggested that similar metabolomic alterations can exist on endometriosis of other locations. It is attributed to that pelvic, deep infiltration, bowel, and thorax endometriosis reveal the same histologic characteristics with ovarian endometriosis. According to a study analyzing peritoneal fluid of endometriosis patients revealed metabolomic change. The

levels of IL-6, IL-10, IL-13, and TNF-α were elevated in peritoneal fluid of endometriosis patients accompanying infertility than healthy control group [22]. In thoracic endometriosis, catamenial pneumothorax shows high expression of CD10 with sensitivity as high as 88.1~96.8%. This is comparable with the expression of CD10 in pelvic endometriosis. However, there is no metabolomic study about thoracic endometriosis yet because of its low prevalence and limited accessibility to pleura. Further metabolomic investigation for thoracic endometriosis is needed. Especially pleural effusion and pleura tissue can be applied to analysis. Since about 50% of thoracic endometriosis patients accompany pelvic endometriosis, retrograde menstruation theory has a limitation to explain the etiology. At present, Sampson's implantation theory, coelomic metaplasia, stem cell theory, and vascular or lymphatic dissemination are suggested as hypothesis. From this reason, additional metabolomic studies dealing with thoracic endometriosis may expand our knowledge for the disease.

The influence of gut microbiome on host immunity and several inflammatory diseases are well established. Proinflammatory environment and altered immunity recently drew attention as related factors to EMS. To be specific, EMS shows increased proinflammatory cytokines in serum, peritoneal, and FF [7]. Previous studies on the microbiome analysis show that potentially pathogenic species such as *Gardnerella*, *Streptococcus*, *Escherichia*, *Shigella* and *Ureaplasma* are increased in gut, vagina, or cervix of EMS patients. Despite FF plays a key role in the maturation and development of oocytes, the microbiome analysis of FF is not conducted yet. In this study, we used BA kit to analyze the microbiome of FF. This BA kit can analyze 20 BAs. Since gut microbiota convert primary BAs into secondary BAs, BA kits reflect host-microbiome interaction [12]. There was no statistically significant difference of BA kit of FF in EMS group and non-EMS group (Figure 4). This suggests that the gut microbiome may not affect FF. However, further microbiome

analysis of FF in ovarian EMS is needed since BA kit is an indirect method of analyzing the microbiome.

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요약 (국문초록)

자궁내막증 여성에서의 난포액의 대사체 분석

– 전향적 연구 –

자궁내막증은 자궁 내막의 분비선 및 기질이 자궁 외의 장소에서 증식하는 양성 부인과 질환이다. 자궁내막증과 난임의 높은 연관성은 많은 연구에서 입증되었으나, 자궁내막증이 난임에 작용하는 기전은 아직도 완전히 밝혀지지 않았다. 최근, 대사체학이 다양한 질환의 병인, 병태생리, 기전을 밝히는 도구로 각광받고 있다. 난포액은 난포의 발달에 필요한 미세 환경을 제공하고 난자의 질에 영향을 주는 물질이다. 그러나 자궁내막증 환자에서 그 난포액을 분석한 대사체학 연구는 제한적이다. 본 연구의 목적은 단측성 난소 자궁내막증에서 난포액의 대사체 구성과 마이크로바이옴 조성을 비자궁내막증 군과 비교하는 것이다. 단측성 난소 자궁내막증 환자의 검체는 모두 자궁내막증에 이환된 쪽의 난소에서 채취한 난포액을 이용하여 분석하였다.

2021년 7월부터 2022년 07월에 걸쳐 분당서울대학교병원에서 10명의 난자 채취 예정 환자가 전향적으로 연구에 참여하였다. 5명은 단측성 난소 자궁내막증으로 진단된 환자였고, 나머지 5명은 비자궁내막증 대조군이었다. 이후 키트를 사용한 표적 대사체 분석이 진행되었다. 188개의 대사체를 분석할 수 있는 키트와 20개의 담즙산을 분석할 수 있는 정량 키트가 사용되었다. 다변량 분석과 주 성분 분석을 통해 난포액 구성 성분의 유의한 차이를 확인하였다.

6개의 대사체에서 통계적 유의성이 확인되었다. 자궁내막증 군에서는 아실카르티닌 C3:1 (propenoylcarnitine)이 통계적으로 유의하게 증가하였고, 비 자궁내막증 군에서는 발린, 알라닌, 아실카르티닌 C4:1 (butenylcarnitine), C4, PC aa C38:3이 유의하게 증가하였다. 한편, 자궁내막증 군과 비자궁내막증 군의 임상적 지표에서 유의한 차이를 보인 항뮬러관호르몬 수치와 난소기능저하 여부에 대해 6개의 대사체와의 연관성 분석을 시행하였고, 발린과 항뮬러관호르몬 사이에서 양의 상관관계를 보였다. 또한 C3:1과 발린은 난소기능저하와 각각 음의 상관 관계와 양의 상관 관계를 보였다. 또한 담즙산 분석 키트에서는 유의한 차이가 확인되지 않았다.

자궁내막증 환자에서 아실카르니틴, 아미노산, 글리세로인지질의 수치 차이는 이 질병으로 인한 세포 수준에서의 미토콘드리아의 에너지 대사 변화를 암시한다. 담즙산 분석 키트에서 유의한 패턴의 차이가 없었기 때문에, 자궁내막증 환자에서 장 마이크로바이옴이 난포 발달에 미치는 영향은 없을 것으로 생각된다.

Table 1. Baseline characteristics of the study population

Characteristics	EMS (n=5)	Non-EMS (n=5)	*p* value
Age (years)	35.6 ± 2.6	36.2 ± 2.2	0.703
BMI (kg/㎡)	18.8 ± 1.7	23.1 ± 4.3	0.071
Primigravida	5 (100%)	5 (100%)	
Oocyte/Embryo cryopreservation			
Yes	2 (40.0%)	1 (20.0%)	
No	3 (60.0%)	4 (80.0%)	
Decreased ovarian reserve			<0.05
Yes	5 (100.0%)	0 (0.0%)	
No	0 (0.0%)	5 (100%)	
AMH	0.9 ± 0.3	4.0 ± 0.9	<0.05
Duration of infertility (month)	28.7 ± 28.3	64.5 ± 48.3	0.31
Previous history of oocyte retrieval (number)	0.6 ± 0.9	2.4 ± 4.3	0.405
Total gonadotropin dose (IU)	2760.0 ± 536.7	1664.4 ± 1234.9	0.106
Duration of gonadotropin use (day)	9.2 ± 1.8	7.2 ± 2.4	0.172
Clinical pregnancy rate (%)	2/3 (66.7%)	2/4 (50%)	

Abbreviation: EMS, endometriosis; BMI, body mass index; AMH, anti-mullerian hormone

Table 2. Clinical outcome of the study population

Characteristics	EMS (n=5)	Non-EMS (n=5)	*p* value
Total volume of FF (ml)	3.1 ± 2.0	3.3 ± 2.8	0.809
Number of oocytes retrieved	1.6 ± 0.9	3.92 ± 2.6	0.1
Number of mature oocytes	1.8 ± 1.5	5.4 ± 3.3	0.056
Number of embryos	1.5 ± 0.7	5.0 ± 4.1	0.319
Fertilization rate (%)	53.4 ± 66.0	80.8 ± 24.1	0.462
Number of good quality embryo	0.5 ± 0.7	1.8 ± 1.0	0.185

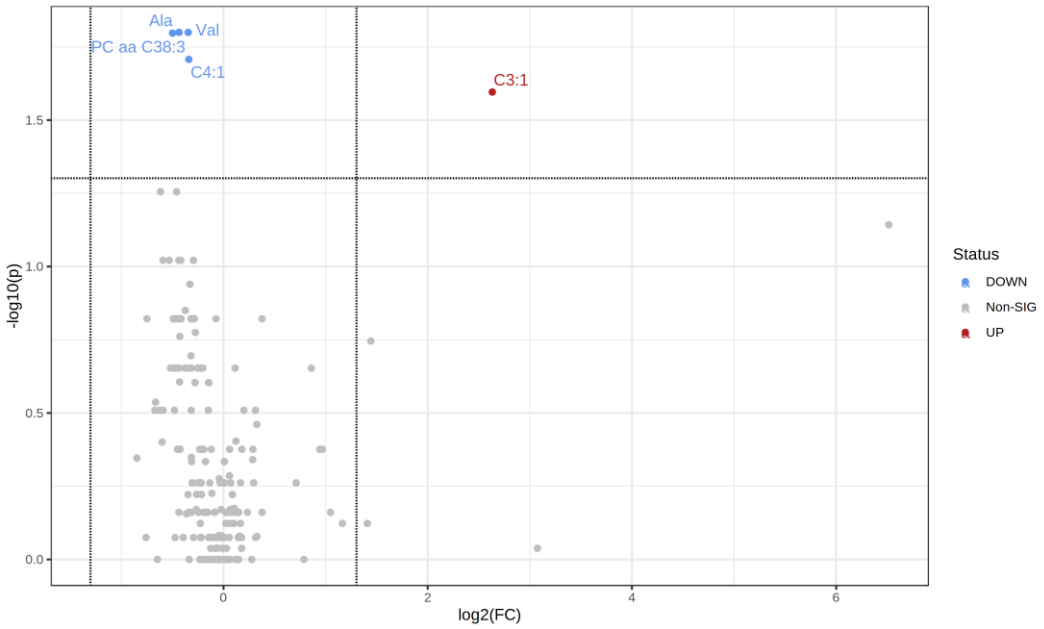
Abbreviation: EMS, endometriosis; FF, follicular fluid

Table 3. Correlation between each metabolite and AMH level and DOR

Metabolites	AMH		DOR	
	Correlation	*p* value	Correlation	*p* value
Alanine	0.5339	0.1119	−0.4336	0.2106
Valine	0.7676	<0.05	−0.6635	<0.05
C3:1	−0.7579	0.0111	0.9559	<0.05
C4:1	0.5807	0.0783	−0.4657	0.175
PC aa C38:3	0.5815	0.0779	−0.5785	0.0797

Abbreviation: Ala, alanine; Val, valine; C3:1, propenoylcarnitine; C4:1, butenylcarnitine; PC aa C38:3, phosphatidylcholine diacyl C 38:3; AMH, anti−mullerian hormone; DOR, decreased ovarian reserve

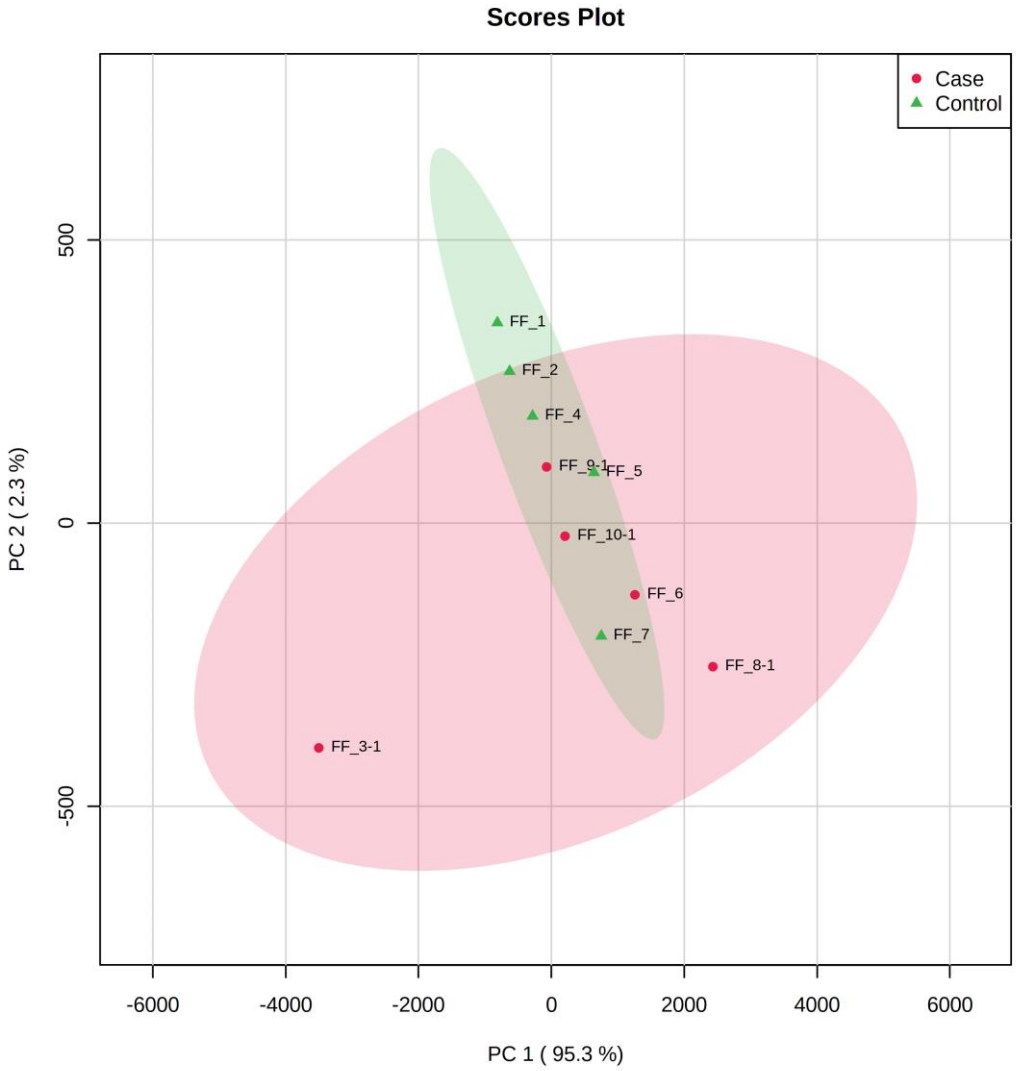
Figure 1. Volcano plot for p180 kit and BA kit



For volcano plot, x-axis present log scaled fold-change and y-axis is log scaled p-value. The cut-off value is 1.1 which means that certain metabolites from follicular fluid of endometriosis-affected ovary have been altered more than 1.1-fold. To sort the statistically different metabolites between case and control groups, the cut-off value of p-value was given as 0.05.

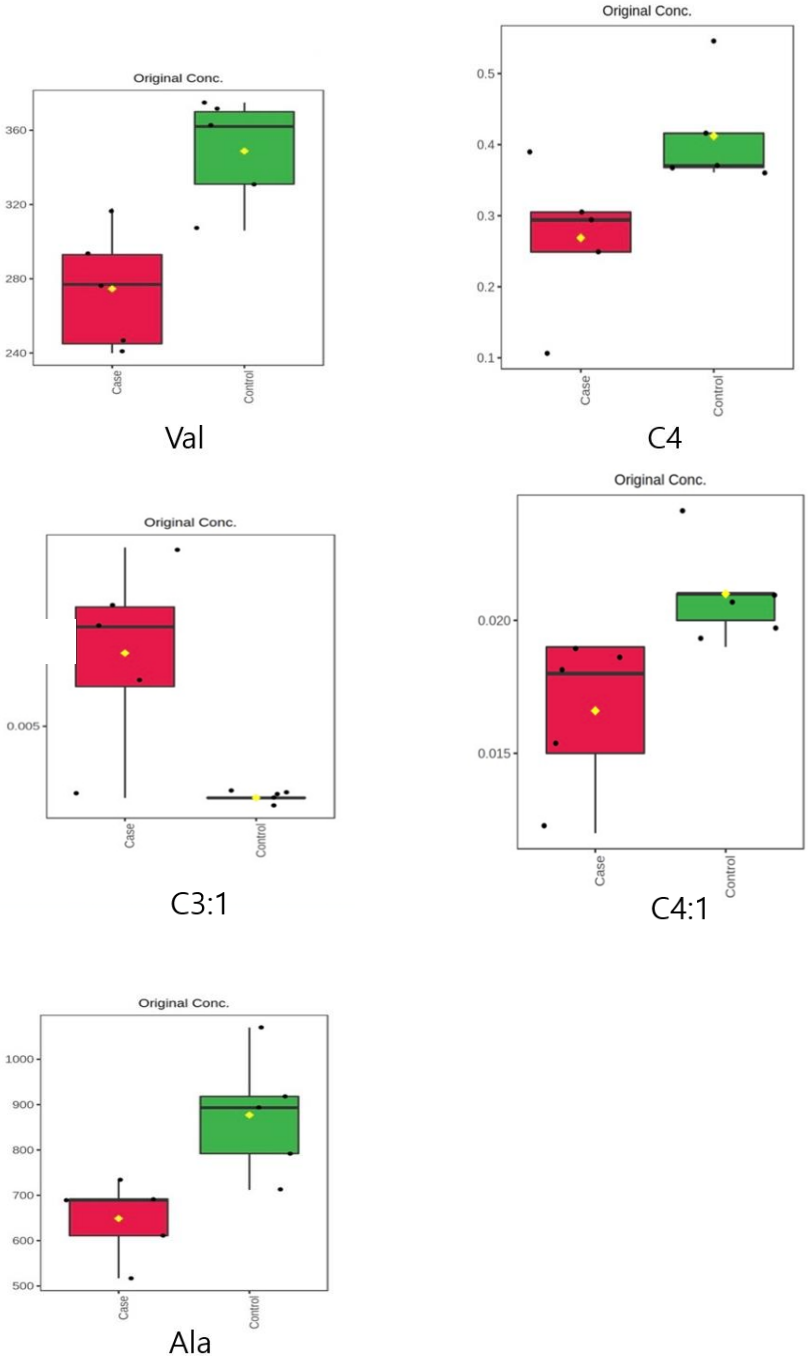
Abbreviation: BA, bile acid; Ala, alanine; Val, valine; C3:1, propenoylcarnitine; C4:1, butenylcarnitine; PC aa C38:3, phosphatidylcholine diacyl C 38:3

Figure 2. Two-dimensional PCA data between EMS group and non-EMS group



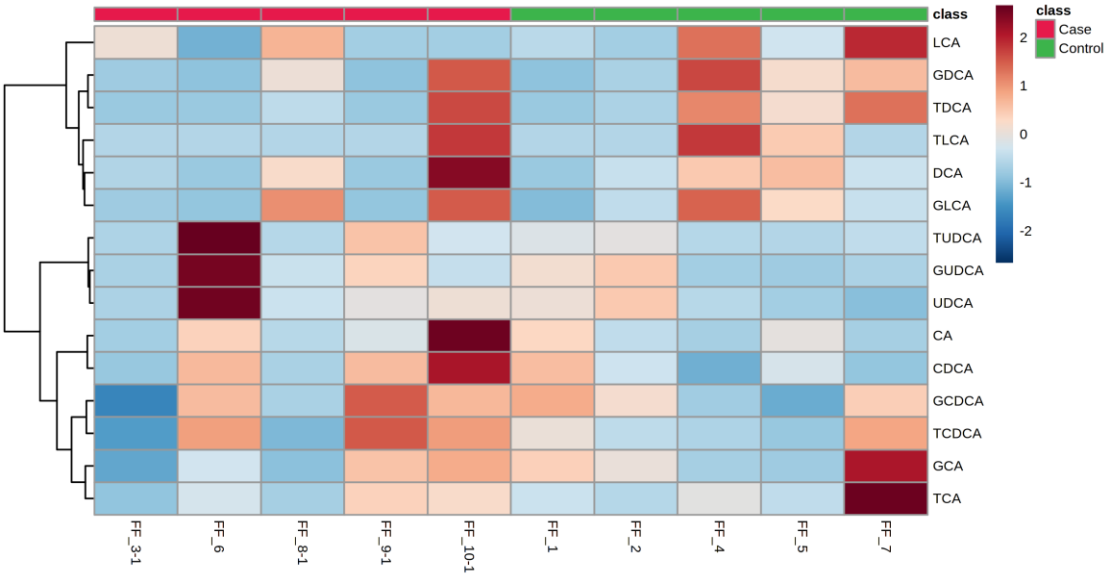
Abbreviation: PCA, Principal component analysis; EMS, endometriosis; FF, follicular fluid

Figure 3. Relative intensity of identified metabolites



Abbreviation: Ala, alanine; C3:1, propenoylcarnitine; C4, butyrylcarnitine; C4:1, butenylcarnitine; Val, valine

Figure 4. Heatmaps and boxplots of bile acids of FF in EMS and non-EMS patients



For heatmaps, the concentration of each bile acid was represented as a log scale. Normalization was performed by the mean value of each bile acid. The red and green column indicate EMS and non-EMS patients, respectively.

Abbreviation: FF, follicular fluid; EMS, endometriosis; TUDCA, tauroursodeoxycholic acid; GUDCA, glycoursodeoxycholic acid; UDCA, ursodeoxycholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; GCDCA, chenodeoxycholic acid glycine conjugate; TCDCA, taurochenodesoxycholic acid; TDCA, taurodeoxycholic acid; GDCA, deoxycholic acid glycine conjugate; TMCA(a+b), tauro-b-muricholic acid; TLCA, lithocholyltaurine; DCA, deoxycholic acid; GLCA, lithocholic acid glycine conjugate; LCA, lithocholic acid; GCA, glycocholic acid; HDCA, hyodeoxycholic acid; TCA, taurocholic acid

■ Figure 5. Metabolic pathway of major substrates in mitochondria and energy generation