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Association of Arachidonic Acid, Eicosapentaenoic Acid and Serum Amyloid A Levels in Women with PCOS

Mazloomi Sahar[1], Khodadadi Iraj[1], Farimani Marzieh[2], Farimani Marzieh[2], Ghorbani Marzieh[1], Shafiee Gholamreza[3]

1. Department of Clinical Biochemistry, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran
2. Department of Obstetrics and Gynecology, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran
3. Nutrition Health Research Center, Department of Clinical Biochemistry, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

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Abstract

Background & Objective: PCOS is a multifactorial disorder characterized by inflammation caused by unknown reasons. This study aimed to investigate the Arachidonic acid (AA), eicosapentaenoic acid (EPA) levels and Serum amyloid A (SAA) protein and gene expression of follicular fluid in women with PCOS.

Materials & Methods: The study included 30 PCOS and 30 healthy women. The individuals were selected after clinical examination and the inclusion criteria. During the puncture and after removing oocytes, follicular fluids were collected from individuals. The SAA protein concentration was determined by the ELISA method. Arachidonic acid, eicosapentaenoic acid levels were determined using GC method and the statistical software SPSS 16, and the p value less than 0.05 was considered as significant.

Results: There was a significant difference in metaphase II oocytes (M2) ($p = 0.001$), number of Embryo ($p = 0.014$), Estradiol ($p = 0.025$), FSH (0.040), LH ($p = 0.035$) and Prolactin ($p = 0.023$) levels between two groups. The concentration of SAA protein level and gene expression shows a significant increase in the patient group compared to the control group ($p < 0.05$). The results showed that AA level was significantly increased in the patient group (284.12 ng/mL) compared to the control group (176.23 ng/mL) ($p = 0.001$) while, there was a significant decrease ($p = 0.001$) in EPA level in the patient group (24.56 ng/mL) compared to the control group (33.48 ng/mL).

Conclusion: It appears that higher EPA levels and lower follicular fluid AA concentrations reduce SAA levels and thus may increase follicular maturation in women with PCOS.

Keywords: PCOS, Follicular maturation, Serum amyloid A, Fatty Acids.

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Introduction

Polycystic ovary syndrome (PCOS) is the most common cause of infertility, affecting about 10-34% of women (1). PCOS is characterized by symptoms such as clinical or biochemical hyperandrogenism, chronic anovulation, and polycystic ovaries, and is widely associated with insulin resistance and hyperinsulinemia (2). Insulin resistance in women with this disease can put them at risk for type 2 diabetes, heart disease and hypertension, atherosclerosis, breast and endometrial cancers (3). Numerous pieces of evidence indicate that the initial growth of the follicle is normal in patients with PCOS, but due to problems in the selection of the dominant follicle lack of ovulation occurs (4).

Corresponding Author: Shafeei Gholamreza, Nutrition Health Research Center, Department of Clinical Biochemistry, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran
Email: g.r_shafiee@yahoo.com

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For the development of follicles, FSH injection can stimulate ovulation and probably due to a normal cycle. Other factors that can be important in the growth of follicles are inflammatory factors that are involved in regulating folliculogenesis. The role of inflamation has been specifically identified in regulating steroid secretion from the gonads and corpus luteum function and the ovulation process (5). TNF-α and IL-6 have pleiotropic effects on oocyte function. Evidence suggests that both cytokines are present in follicular fluid and in people with PCOS, their levels increase in follicular serum (6). Usually, during inflammation, leukocytes migrate to the site of destruction and cause a respiratory explosion due to excessive oxygen uptake. Thus, they increase the release and accumulation of ROS at the site of destruction (7) On the other hand, inflammatory cells can produce soluble mediators such as arachidonic acid metabolites, cytokines, and chemokines, which in turn cause more inflammatory cells to be used at the site of destruction and produce more inflammatory responses (8). These mediators can activate message transmission cascades and modify IL-1, IL-6, and IL-8 levels (9). One of the proteins in the inflammatory process is SAA protein. SAA is an acute-positive phase protein that is secreted by the liver in response to injury and inflammatory conditions and acts as an inflammatory inducer (10). An increase in SAA has been observed in obese people with insulin resistance (11). SAA affects the HDL level where it can be regulated by other proteins such as PON1, lipases as well as apoA-1. Many studies show that exogenous omega-6 fatty acids activate inflammatory cytokines, while omega-3 fatty acids have anti-inflammatory effects (12). Therefore, in this study, we decided to measure AA and EPA, SAA protein levels and gene expression in follicular fluid samples in women with PCOS.

Material & Methods

This experiment is case-control study and was approved by the Ethics Committee of Hamadan University of Medical Sciences (ethics committee code: IR.UMSHA.REC.1397.386). In this study, 30 healthy individuals with regular menstrual cycles and normal ovarian morphology and 30 individuals with PCOS were selected who underwent in vitro fertilization (IVF) after the clinical examination and approval by the perinatology and gynecologist in Omid Clinical center (Hamadan-Iran). The PCOS diagnosis had been made on the basis of the Rotterdam consensus criteria. Individuals were included with two of the three features including oligo- and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries diagnosed in an ultrasonographic examination (13). Subjects were excluded if they had a disease such as Cushing's syndrome, congenital adrenal hyperplasia, androgen-secreting tumors, hyperprolactinemia, infections, inflammatory disease, oral contraceptives, insulin sensitizers or mineral supplementation at least 3 months before (14). Demographic characteristics were obtained by general information questionnaire and medical records.

Ovarian Stimulation Protocol

Before the start of the cycle, for all individulas on the second day of the menstrual cycle, a basic transvaginal ultrasound was performed in the absence of cysts or ovarian follicles above 10 mm. Then, in two groups of recombinant FSH and Cinnal-F (CinnaGen Co., Tehran-Iran), it was started at 150 to 225 units and they underwent serial ultrasonography to evaluate size of follicles. When the size of the dominant follicle reaches 14 mm, Cetrotide antagonist (Merck Serono Co., Darmstadt-Germany) was injected 0.25 micrograms daily. When at least three 18 mm (adult follicles) were found in the ovaries, the recombinant Ovidrel hCG (Merck Serono Co., Darmstadt-Germany) was injected, and after 36 hours, the follicles were punctured with a vaginal ultrasound-guided punctured. Finally, the first punctured follicle with a diameter equal to or greater than 18 mm was isolated and obtained. Blood-contaminated

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follicular fluid was not included in the study (15).

Isolation of follicular fluid and granulosa cells

Follicular fluids of each sample were poured at a sterile tube and were centrifuged at 1000×g for 4 min at 21°C, then the supernatant (follicular fluid) was separated and the pellet containing the GCs was centrifuged with 20 mL red blood cell lysing buffer (RLB) containing 2M ammonium chloride, 1M $NaHCO_3$ and EDTA in three times. Then, granulosa cells were centrifuged with DMEM F12 media at 500×g for 2 min at 21°C. Finally, the samples were centrifuged with phosphate buffered saline (PBS) at 300×g for 1 min at 21°C.

Demographic Data

After completing the questionnaire, fasting blood samples (after 12 h overnight) were obtained. The serum samples were separated and collected inside sterile tubes and were used for lipid profile parameters. The concentrations of plasma total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) and LDL-C were measured using a Hitachi 7150 automated analyzer (Hitachi, Japan).

Measurement of Arachidonic acid (n-6) and Eicosapentaenoic acid (n-3)

Follicular fluid samples were combined with 20 µL of internal standard 1µg /µL (Sigma-Aldrich, UK) and 900 µL solution of methanol: water in a ratio of 8: 1 and mixed for 10 seconds. It was then centrifuged at 4°C for 10 minutes at 4°C. About 200 µL of the supernatant was transferred to a vial for GC (CP-3800, Spectralab, Canada) and incubated overnight at room temperature. The reference standard was about 200 microliters transferred to a microtube and 1 µL was injected into the device with a column with a volume of 30 mm × 0.25 mm and an internal diameter of 0.25 mm that the flow rate should be 1mL / min. The temperature from the injection site to the end should be 270, 260, 200 and 150°C, respectively, and the delay time of the solution should be 5 minutes (16).

Measurement of SAA level

Follicular fluid SAA protein concentration was measured using an ELISA commercial kit (ZellBio GmbH, Germany) by ELISA reader (synergy HTX, Biotech, USA). The basis of the measurement in the kit was the non-competitive sandwich method. The standard PCSK9 range is 2.5-20 ng/mL.

SAA gene expression

The gene expression level of SAA gene as indicators of inflammatory factor was measured by real-time. First, Total RNA was extracted from the granulosa cells using SinaPureTM RNA kit (SinaClon Bioscience, Tehran, Iran), according to the manufacturer's instruction. The quality and integrity of total RNA was confirmed by agarose gel electrophoresis whereas its concentration was determined by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). One microgram of total RNA samples was used for cDNA synthesis according to the kit instruction (BioFact, Seoul, South Korea) and specific primer sets (Table 1) were designed using Allele ID6.0 software, considering β-actin as housekeeping gene. Finally, quantitative PCR was carried out using the SYBR Green master mix (Amplicon, Denmark) in the LightCycler®96 instruments (Roche Life Science Deutschland GmbH, Sandhofer, Germany). The observed signals from the RT-PCR were validated by verifying the bands on 2% agarose gel electrophoresis, compared with the 100 bp DNA ladder marker. The expression level of SAA gene was calculated relative to the expression of β-actin using $2^{-\Delta\Delta Ct}$ formula.

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Table 1. Target genes and their relative primer sequences

Target Gene		Primers	PCR Product (bp)
SAA	Forward	5'- CAGACAAATACTTCCATGCT -3'	303
	Reverse	3'- ATTGTGTACCCTCTCCCC -5'	
β-actin	Forward	5'- CAAGAGATGGCCACGGCTGCT -3'	275
	Reverse	3'- TCCTTCTGCATCCTGTCGGCA -5'	

Statistical Analysis

Data were analyzed using the statistical software SPSS 16 (SPSS Inc., Chicago, USA) and one-way ANOVA followed by Tukey multiple comparisons test to analyse differences between groups. For real-time PCR, the changes in gene expressions were calculated as $2^{-\Delta\Delta Ct}$. Results were presented as mean ± SD and the p value less than 0.05 was considered as significant.

Results

Baseline clinical characteristics findings

The baseline clinical and biochemical characteristics are shown in Table 2. The minimum and maximum maternal ages of participants were 28.98 and 34.32 years, respectively with no statistical difference between groups in age (p = 0.422). There was no statistical difference in GV (p =0.241), duration of infertility (p =0.850), β-HCG (p =0.790) and AMH (p =0.310) factors between two groups. However, there was a significant difference in BMI (0.023), number of oocyte M1 (p =0.040), number of oocyte M2 (p =0.001), number of Embryo (p =0.014), Estradiol (p =0.025), FSH (p =0.040), LH (p =0.035) and Prolactin (p =0.023) levels between the two groups.

Arachidonic acid and Eicosapentaenoic acid

The results showed that Arachidonic acid (AA) level was significantly increased in the patient group (284.12 ng/mL) compared to the control group (176.23 ng/mL) (p =0.001). But, the finding showed that there was a significant decrease (p =0.001) in EPA level in the patient group (24.56 ng/mL) compared to the control group (33.48 ng/mL) (Table 2).

Table 2. Clinical and fatty acids features of women with PCOS and controls

Variable	Control (n=30)	PCOS (n=30)	p-value
age (Year)	30.12±1.07	31.65±2.67	0.422
BMI (kg/m^2)	±4.71 24.62	30.61±3.15	0.023*

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No. oocyte MII (%)	78.3 ± 5.6	45 ± 4.1	0.001*
MI (%)	15 ± 1.1	9 ± 2.9	0.040*
GV (%)	4 ± 0.99	3.2 ± 1.8	0.241
Duration of infertility (year)	5.03±0.75	±0.85 5.86	0.850
No. Embryo	13.89±3.28	8.40±0.94	0.014*
β-HCG (IU/L)	10.65±3.43	10.68±4.52	0.790
Estradiol (pmol/L)	36.5±4.43	57.64±7.82	0.025*
LH (IU/L)	4.4±0.78	8.2±0.12	0.035*
FSH (IU/L)	8.22±0.97	5.27±0.72	0.040*
Prolactin (mU/L)	11.92±2.42	19.21±3.41	0.023*
AMH (ng/mL)	4.4±0.23	8.5±0.48	0.010*
AA (ng/mL)	176.23 ± 4.23	284.12 ± 8.51	0.001*
EPA (ng/mL)	33.48±0.72	±0.36 24.56	0.001*

BMI: body mass index; MII: metaphase II, MI: metaphase I, GV: Germinal vesicle, AA: Arachidonic acid, EPA: Eicosapentaenoic acid, AMH: anti-Müllerian hormone. The groups were also matched for age and BMI, and there was a significant difference in gestational age at birth, neonatal weight, diastolic blood pressure, and proteinuria between the two groups. Values are given as mean ± SD. Thesymbol (*) represents significant differences

SAA concentration

The concentration of SAA protein in the patient group compared to the control group shows a significant increase ($p < 0.05$) (Chart 1A). The mean SAA levels in the serum of women with PCOS and control were 2.46 and 1.63 ng/mL, respectively.

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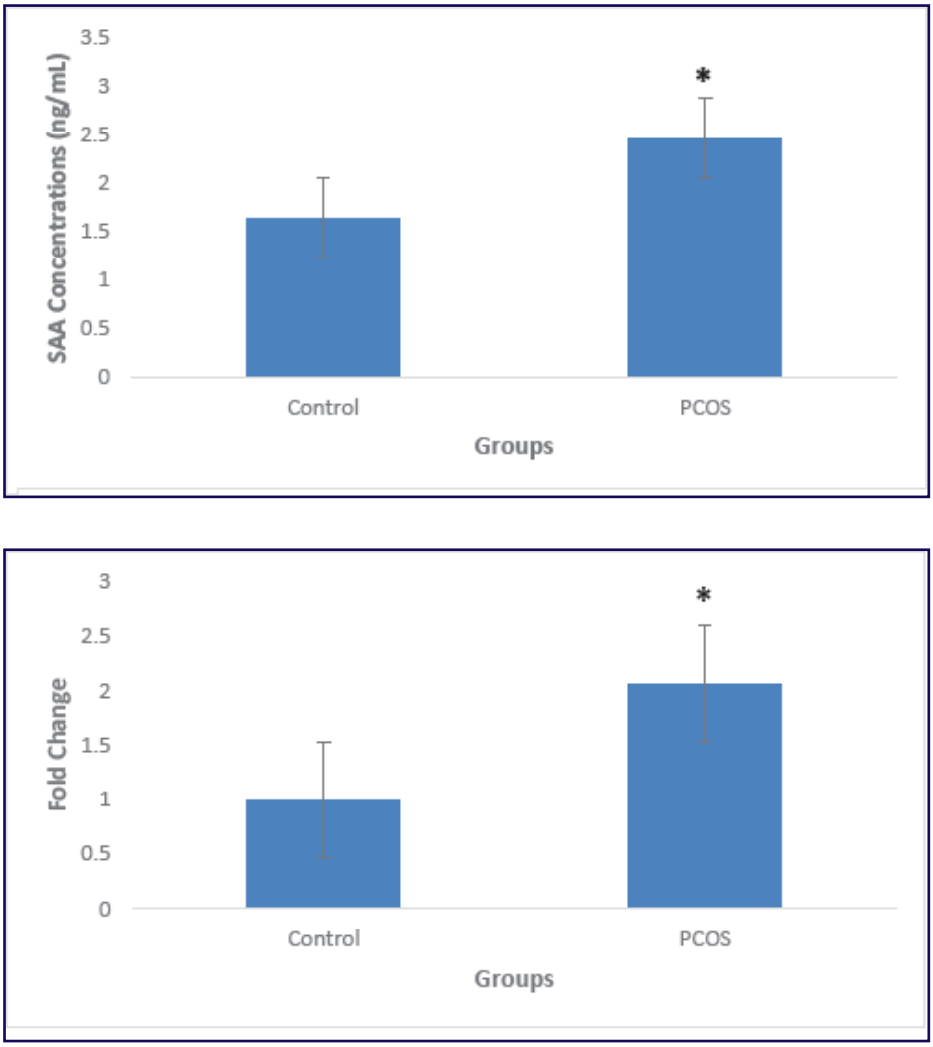

Chart 1. (A) The concentration of SAA protein in the patient group compared to the control group shows a significant increase. (B) The results of the SAA gene expression shows a significant increase in the patient group compared to the control group. The symbol (*) represents significant differences ($p<0.05$)

SAA Gene expression

The finding showed that there was a significant increase (2.06 Fold) in SAA gene expression in the patient group compared to the control group ($p <0.05$) (Chart 1B).

Correlational analysis of SAA, AA, EPA and M2

According to correlational analysis, the SAA variable in the control group had a significant negative association with EPA (p-value = 0.023, $\beta = -0.766$) and M2 (p-value = 0.028, $\beta = -0.729$). But in this group, there was a significant positive association between SAA level and parameters of AA (p-value = 0.031, $\beta = 0.660$) and between EPA level and parameter of M2 (p-value = 0.014, $\beta = 0.863$) as shown in Chart 2.

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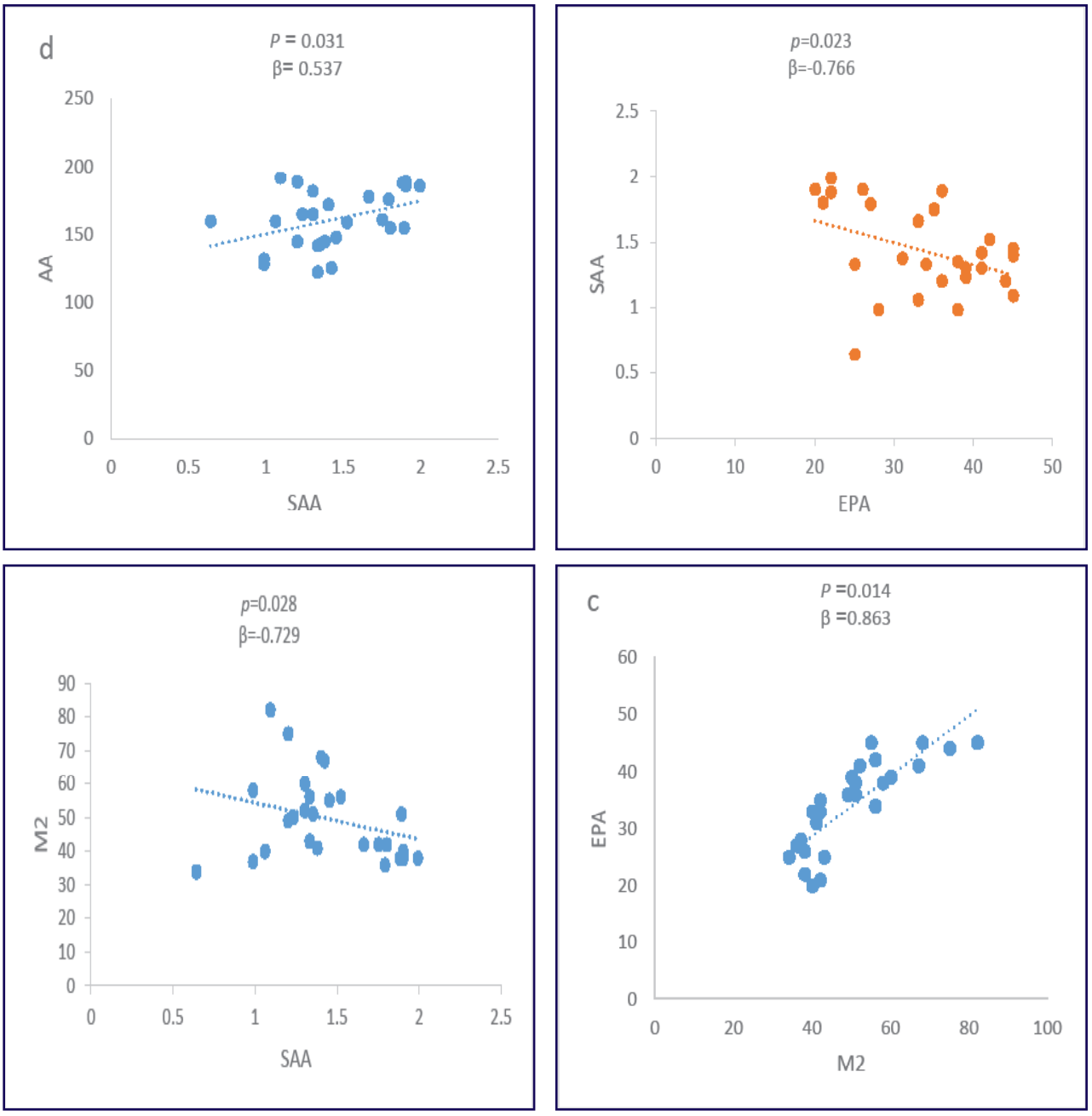

Chart 2. Association analysis of between SAA, AA, SOD, EPA and M2 serum levels in the control group. According to association analysis, the SAA variable had a negative association with EPA (a), M2 (b), but in this group, there was a significant positive association between SAA level and parameter of AA (c) and EPA with M2 (d). Data were given as mean ± SD and significant difference was p <0.05

Also, the SAA variable in the PCOS group had a significant negative relationship with EPA (p-value = 0.013, β = -0.891) and M2 (p-value = 0.044, β = -0.648). But in this group, there was a significant positive relationship between SAA level and parameters of AA (p-value = 0.036, β = 0.685) and between EPA level and parameter of M2 (p-value = 0.025, β = 0.823) as shown in Chart 3.

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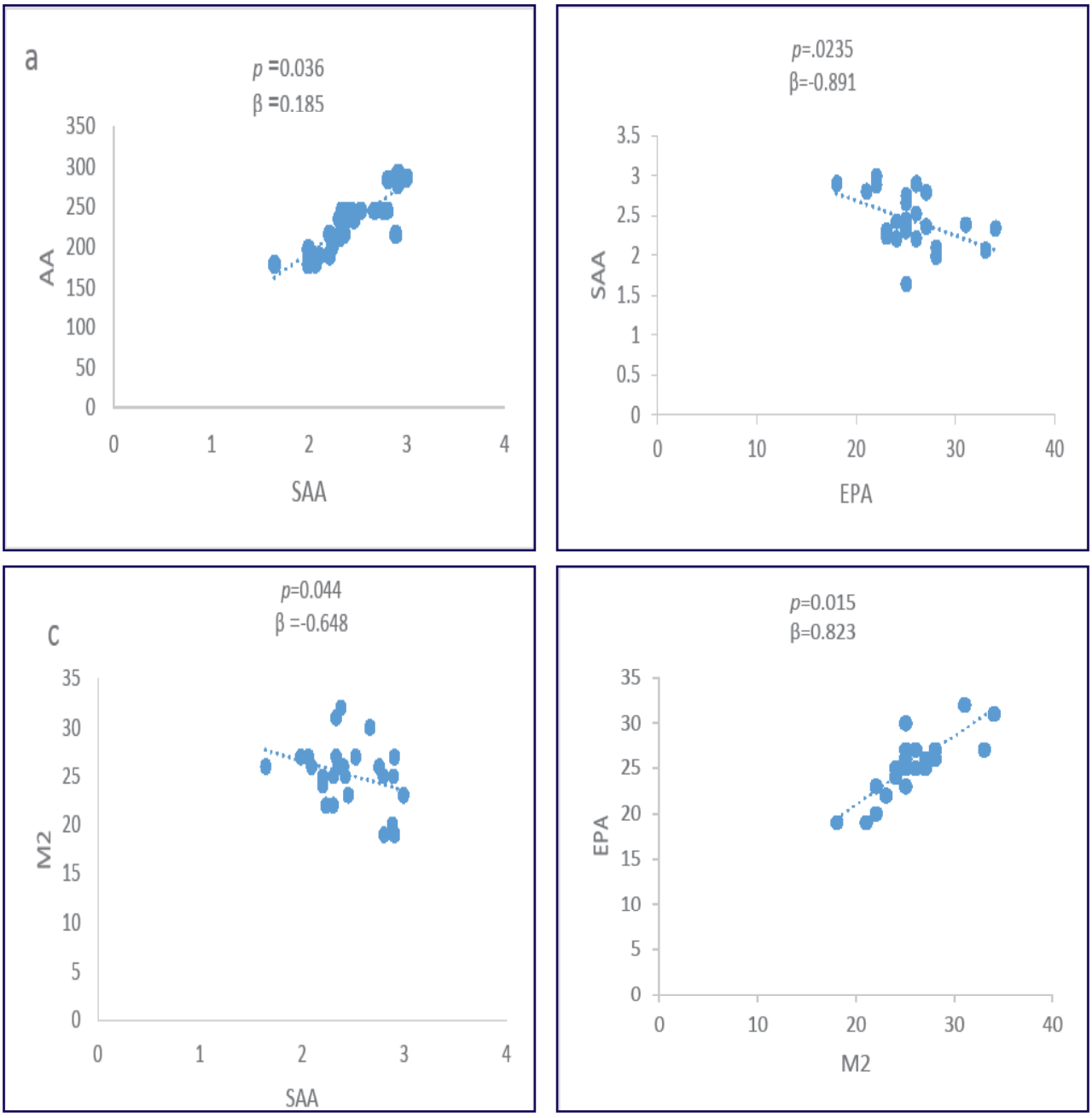

Chart 3. Association analysis of between SAA, AA, SOD, EPA and M2 serum levels in the preeclampsia group. According to association analysis, the SAA variable had a negative association with EPA (a), M2 (b), but in this group, there was a significant positive association between SAA level and parameter of AA (c) and EPA with M2 (d). Data were given as mean ± SD and significant difference was p <0.05

Discussion

SAA is an acute phase protein that is produced locally in the ovary, the expansion and maturation of the follicle is inversely related to its amount in the follicular fluid (17). One of the factors that can be important in the growth of follicles are cytokines that are involved in regulating folliculogenesis. Their role has been specifically identified in regulating steroid secretion from the gonads and corpus luteum function and regulating the ovulation process (18). Inflammatory cells can produce soluble mediators such as arachidonic acid metabolites, cytokines, and chemokines, which in turn cause

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more inflammatory cells to be used at the site of destruction and produce more types of responses (19).The chronic inflammation and oxidative stress have been identified in PCOS pathogenesis. The clinical observations indicate that early follicle growth is not normal in patients with PCOS and non-ovulation occurs for unknown reasons (20, 21).

In this study, we showed that the SAA protein levels significantly increase in the PCOS group compared to control group women. Also, there was a significant difference in SAA gene expression in two groups. In line with our findings, a study by Tan BK et al (2011) found that obese people with higher insulin resistance had higher SAA levels and in women with PCOS, serum SAA levels were higher than in the control group (22). Another study (2014) showed that serum SAA levels in women with polycystic ovary syndrome were higher than in control groups (23). It seems that one of the causes of increase in SAA in this study and previous studies is that the pro-inflammatory cytokines, such as IL-6, IL-1, and TNF-α, appear to regulate the expression of SAA1 and SAA2 genes in hepatocytes. As seen in both serum amyloid A in acute inflammatory conditions in mice shows an increase of up to 1000 times (24). Also, in this study association analysis showed that there was a negative relationship between SAA level and number of oocytes M2 (as follicular maturation). The similar results of our studies (2013) showed that SAA levels were associated with follicular maturation so that increasing the amount of SAA reduces the probability of fertility in women (25). Maybe the reason for this is that follicular fluid provides a favorable environment for oocyte growth and development and has a direct impact on oocyte quality. The increased inflammatory factors in the follicular fluid can cause adverse effects on the growth and development of oocytes and reduce the success of IVF (26).

The finding of our study showed that AA concentration was higher in the PCOS group compared to the control group. Conversely, it was shown that there was a significant decrease in EPA level compared to the control group. Inflammatory cytokines and oxidant molecules have been shown to release arachidonic acid from membrane phospholipids. IL-1 activates arachidonic acid-dependent phospholipase A2 and activates the JNK / SAPK message pathway and increases cyclooxygenase 2 synthesis (27, 28). Regarding the association of fatty acids with PCOS, a study (2017) showed that serum and follicular levels of decanoic acid, stearic acid in PCOS and control groups were not different, but serum and follicular levels of palmitoleic acid, oleic acid were higher in obese women with PCOS individuals compared with the control group (29). Metabolic status has a direct effect on the microscopic environment of the ovarian follicle and can be associated with inflammation and increased oxidative stress so that it can be seen with a potential reduction in oocyte development (30). Correlational analysis showed that there was a positive relationship between AA and SAA levels but between SAA and EPA levels there was a negative relationship. In an interventional study, the results showed that omega-3 and 6 intakes were inversely related to inflammatory biomarkers (IL-6, IL-8, IL-1, IL-10, IL-12, CRP, TNF-α) and there is a positive relationship with adiponectin in heart patients (31). In a study, the use of myoinositol supplements increased follicles and their quality in PCOS, and myoinositol also increased insulin sensitivity in these women (32).

The results of another study by Sato (2014) have shown that oral intake of eicosapentaenoic acid is associated with a decrease in epicardial adipose tissue and visceral abdominal fat. The treatment with eicosapentaenoic acid improves some metabolic problems such as insulin resistance, fatty liver and hypertension (33). It has been made clear that eicosapentaenoic acid and docosahexaenoic acid and their metabolites have anti-inflammatory effects while Arachidonic acid activates inflammatory cytokines and is substrate for cyclooxygenase and lipoxygenase enzymes to form prostaglandins and leukotrienes (34).

There are lipid biomarkers associated with female fertility, for example phospholipids can induce cell mass to transform into different cells, and some studies have reported an association between fatty acid profiles with oocyte maturation and embryonic proliferation (35) A study by Shaker et al (2012) found that the ratio of saturated to unsaturated fatty acids was negatively correlated with follicular maturation. Linoleic acid showed a positive relationship while arachidonic acid showed a negative relationship with follicle maturation (36). Even in an animal study (2015), the results showed that long-term administration of arachidonic acid reduced the levels of eicosapentaenoic acid and docosapentaenoic acid (37). These results show that according to the pro-inflammatory properties of arachidonic acid, in contrast to the anti-inflammatory effects of eicosapentaenoic acid, due to the increase of oxidative stress and inflammation factors such as SAA and so, it has a negative effect on the function of the ovaries in follicular maturation and causes the occurrence or progression of PCOS disease. Finally, the results of this study can be considered in line with nutrition studies, because it showed that a high level of EPA could reduce SAA and may increase follicular maturity.

Conclusions

Due to the mechanism of arachidonic acid (w6) and eicosapentaenoic acid (w3) in the inflammatory process, it seems that the measurement of SAA can be considered in polycystic ovary syndrome. Also, it can be suggested that consuming sufficient amounts of EPA and lower amounts of AA in the diet can be effective in preventing the PCOS disease.

Acknowledgments

This experiment is a case-control study and was approved by the Ethics Committee of Hamadan University of Medical Sciences (ethics committee code: IR.UMSHA.REC.1397.386).

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Conflict of Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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