

Endothelial cell-specific molecule 1 is regulated separately in follicular and serum compartments of PCOS patients: a pilot study

N. TURKTEKIN¹, R. OZYURT²

¹Operating Room Services Department, Nisantasi University, Vocational School, Istanbul, Turkey

²Department of Obstetrics and Gynecology, Bakirköy Sadi Konuk Training and Research Hospital, Istanbul, Turkey

Abstract. – OBJECTIVE: The aim of our study is to detect endothelial cell-specific molecule 1 (ESM-1) levels in the serum and follicular fluids (FF) of patients undergoing IVF/ICSI for PCOS. The presence of ESM-1 traffic between the serum and follicular compartment was analyzed.

PATIENTS AND METHODS: A total of 50 patients, including 25 infertile patients diagnosed with PCOS according to the Revised Rotterdam criteria, and 25 patients in infertility follow-up for reasons other than PCOS, were included in the pilot study. Patients in the control group were required to have no clinical and laboratory findings of PCOS. Non-PCOS controls were selected from patients diagnosed with a male factor or unexplained infertility, and a homogeneous group was formed. Patients in PCOS and control groups were matched in terms of age and BMI. IVF/ICSI was started with antagonist protocol in both groups. Follicular fluids obtained on the day of egg collection were centrifuged, put into RNA-later, frozen, and stored until the day of analysis. Endothelial cell-specific Molecule 1 levels were measured in follicular fluid and serum samples of PCOS patients by enzyme-linked immunosorbent assay (ELISA) using the Human ESM-1 kit.

RESULTS: Serum ESM-1 levels of the PCOS group were significantly lower than FF-ESM1 levels (668.6 ± 189.2 vs. 979.0 ± 233.9 ng/L, $p < 0.02$). FF-ESM1 levels of the control group were significantly higher than serum ESM1 levels (639.3 ± 206.4 ng/L vs. 503.2 ± 102.4 ng/L, $p < 0.01$). Serum ESM1 levels of the PCOS group were significantly higher than the control group (668.6 ± 189.2 ng/L vs. 503.2 ± 102.4 ng/L, $p < 0.01$). Similarly, FF-ESM1 levels of the PCOS group were significantly higher than the control group (979.0 ± 233.9 ng/L vs. 639.3 ± 206.4 ng/L, $p < 0.01$). There was no significant correlation between serum and FF-ESM1 levels. A positive and significant correlation was found between FF-ESM1 and serum LH levels in PCOS ($r = 0.655$, $p < 0.02$). Similarly, a positive and significant correlation was found between FF-ESM1 and serum testosterone levels in PCOS ($r = 0.470$, $p < 0.03$). Moreover, a positive and significant correlation was detected between FF-ESM-1, AFC, MII, and total oocyte counts in PCOS.

CONCLUSIONS: Serum and FF-ESM-1 are regulated independently of each other in PCOS patients. ESM-1 may play a role in ovulatory dysfunction due to PCOS.

Key Words:

PCOS, Follicular fluid, ESM-1, Angiogenesis.

Introduction

Endothelial cell-specific molecule 1 (ESM-1) is a proteoglycan dermatan sulfate. This molecule, also called endocan, is secreted in the vascular endothelial cells and passes into the systemic circulation and other biological fluids^{1,2}. It can switch between compartments thanks to its dermatan sulfate and soluble properties. The glycosaminoglycan property of ESM-1 causes it to take part in many biological processes. Proliferation and neoangiogenesis in vascular endothelial cells are the most important functions of ESM-1. For this reason, it is seen as a biomarker of angiogenesis. ESM-1 also plays a role in the regulation of cell migration, adhesion, and invasive properties³. ESM-1 exhibits an increased expression in cancer cases characterized by many vascular diseases, inflammation, and neoangiogenesis^{4,5}.

Ovulation is a process characterized by expansion, neoangiogenesis, and inflammation in cumulus-oocyte complex cells. The LH peak is the main stimulus that initiates the events in this process. The effect of LH is mainly by stimulating the release of proinflammatory cytokines, increasing steroid synthesis, and the production of prostaglandins. Granulosa and theca cells are the main target areas where all these reactions take place. With the help of these mediators, the activation of immune and non-immune cells is provided, and the invasion of vascular endothelial

cells into the follicle wall is opened. As increased vascularization activates proteolytic pathways, the follicle breaks, and the oocyte is released in each cycle through other functional and structural changes⁶.

Polycystic ovary syndrome (PCOS) is the most common endocrine pathology in reproductive age and is characterized by anovulation, LH surge defects, and hyperandrogenemia⁷. The mechanism leading to ovulatory dysfunction in these patients is not fully known. In addition to inflammation and neoangiogenesis, inter-cumulus biomechanical forces may also contribute to follicle rupture⁸. Considering its role in the proliferation and angiogenesis of the vascular endothelial bed, ESM-1 may be involved in ovulatory dysfunction due to PCOS. There is no study investigating ESM-1 levels in biological fluids in PCOS patients. This study was planned to detect ESM-1 levels in the serum and follicular fluids of patients undergoing IVF/ICSI for PCOS. The presence of ESM-1 traffic between the serum and follicular compartment was analyzed.

Patients and Methods

A total of 50 patients, including 25 infertile patients diagnosed with PCOS according to the Revised Rotterdam criteria, and 25 patients in infertility follow-up for reasons other than PCOS, were included in the pilot study. The minimum number of participants required to complete our study with a 95% confidence level ($\alpha=0.05$) and 80% power was 50 (25 for each group). The participants were selected from among the patients who applied to the Istanbul IVF Center between 2020-2021 with the complaint of infertility and were decided on IVF/ICSI. After receiving approval from the same center (Date: 15.06.2023, No.: 618), the study was started. Strict compliance with the Declaration of Helsinki was demonstrated throughout the study period. An informed consent form was obtained from all participants. Patients showing at least two of the criteria for hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology were considered PCOS. In the ultrasonographic evaluation, the condition of 12 or more polycystic appearance between 2 and 9 mm in one or both ovaries was sought. Ovarian volume $<10 \text{ mm}^3$ was also considered as polycystic morphology. Patients in the control group were required to have no clinical and laboratory findings of PCOS. Non-PCOS con-

trols were selected from patients diagnosed with a male factor or unexplained infertility, and a homogeneous group was formed. Patients in PCOS and control groups were matched in terms of age and BMI. IVF/ICSI was started with antagonist protocol in both groups. Follicular fluids obtained on the day of egg collection were centrifuged, put into RNA later, frozen, and stored until the day of analysis.

Participants in both groups underwent basal hormonal examination. Antral follicle counts were recorded with TVS. Blood samples were taken from ovulatory PCOS cases on the third day of their spontaneous menstrual cycle. Venous blood samples were collected from anovulatory cases following progesterone withdrawal bleeding. In the control group, on the third day of the spontaneous menstrual cycle, venous blood was taken for chromosomal evaluation. Serum luteinizing hormone, follicle-stimulating hormone, testosterone and insulin levels of both groups were measured, and insulin resistance was calculated with HOMA-IR. Patients with Cushing's syndrome, thyroid disease, non-PCOS idiopathic hirsutism, hyperprolactinemia, type 2 diabetes, and other endocrine diseases were excluded from the study. Those who used lipid-lowering drugs, hormonal drugs, and insulin-sensitizing drugs in the last 6 months were excluded from the study.

Controlled Ovarian Stimulation

Patients in both PCOS and control groups underwent ovulation stimulation with the antagonist protocol. Details of this protocol can be found elsewhere in literature. Recombinant follicle-stimulating hormone (Gonal-F, Merck İlaç Grubu A.Ş., Turkey) was started on the 3rd day of the cycle by adjusting the dose according to the BMI, age, and previous history of the patients. Antagonist therapy (Cetrotide 250 μg , Merck Serono, Turkey) was initiated in patients with a follicle diameter of 14 mm or more. Two or more follicles $>17 \text{ mm}$ were found to induce ovulation with recombinant hCG or triptorelin acetate. Eggs were collected 36 hours after hCG or triptorelin triggering. The blood and follicle fluids taken during egg collection were centrifuged and stored by freezing. If the follicle fluids were too bloody despite centrifugation, they were not included in the study. The primary outcome measure of the study was to compare serum and FF-ECSM1 levels in PCOS and control groups.

Measurement of Serum and Follicular Fluid ECSM1 with ELISA

Endothelial cell-specific Molecule 1 (ESM1) levels were measured in follicular fluid and serum samples of PCOS patients by enzyme-linked immunosorbent assay (ELISA) using the Human ESM1 kit (Sunred Biotechnology Company, Shanghai, China). The measurement was performed in accordance with the manufacturer's instructions specified in the catalog. Absorbances were measured with a microplate reader at a wavelength of 450 nm. The concentrations corresponding to all absorbances were calculated in ng/L with the formula obtained using the standard curve graph. The measurement range of the Human ESM1 kit was 8 ng/L – 2,000 ng/L, and the sensitivity was 7.506 ng/L. The intra-assay CV value of the Human ESM1 kit was <10%, while the inter-assay CV value was <12%.

Statistical Analysis

The data of both groups were analyzed by applying the Statistical Package for Social Sciences software for Windows (IBM Corp., Armonk, NY, USA). Kolmogorov-Smirnov test was used to analyze whether the data showed normal distribution. Demographic and laboratory data of the groups were evaluated with the Mann-Whitney U test. Serum and FF-ESM1 levels, demographic, and labo-

ratory parameters were analyzed by the Spearman correlation method. The results obtained were presented as mean±SD or percentage. A $p<0.05$ was considered significant in all comparisons.

Results

Demographic, laboratory, and ESM1 levels of PCOS and the control group are presented in Tables I and II in detail. In Figure 1, serum and FF-ESM1 levels are shown in the bar graph. Age and BMI values of the two groups were similar. Although there was a tendency for an increase in BMI in the PCOS group, the difference was not significant. Serum testosterone and LH levels of PCOS patients were significantly higher than the ones of the control group. The LH/FSH ratio was found to be higher in the PCOS group compared to the controls. Serum FSH values were found to be similar among the groups. All three groups were similar in terms of serum FSH levels. The number of antral follicles was significantly higher in the PCOS group than in the controls. The HOMA-IR value of PCOS patients was significantly higher than the one of the controls. The number of collected oocytes and 2 PN zygotes was higher in the PCOS group than in the controls. MII oocyte count was significantly high in PCOS.

Table I. Demographic findings of each group of participant.

	PCOS (n=25)	Control (n=25)	p-values
Age (yrs)	25.8±3.09	26.2±2.11	0.33
BMI (kg/m ²)	24.3±3.67	23.7±4.30	0.08
LH (mIU/mL)	8.77±3.07	5.11±2.09	0.03
FSH (mIU/mL)	5.11±2.37	4.90±1.20	0.46
LH/FSH	1.74	1.04	0.02
Testosterone (ng/dL)	43.2±9.21	34.3±6.44	0.01
HOMA-IR	2.64±1.55	1.33±0.33	0.02
AFC	18.6±2.33	9.22±3.29	0.01
Total oocyte	13.2±4.09	7.44±3.20	0.01
MII oocyte	9.15±4.05	5.13±2.22	0.01

Table II. Serum and FF-ECSM1 levels of both groups.

	PCOS	Control	p-values
N (%)	25 (50%)	25 (50%)	0.26
Serum ESM1 (ng/L)	668.6±189.2	503.2±102.4	0.01
FF-ESM1	979.0±233.9	639.3±206.4	0.01
p-values	0.02	0.01	

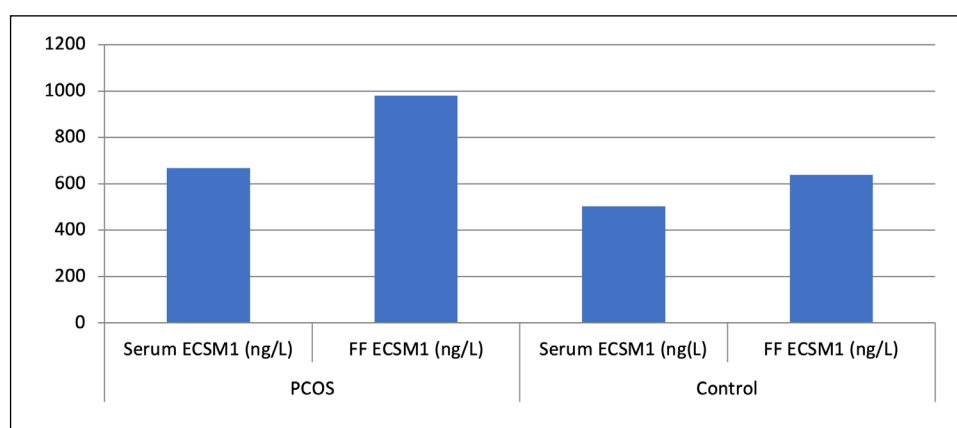


Figure 1. Graphic representation of both groups in terms of serum and FF-ESM1 levels.

We compared the groups in terms of ESM1 levels both within and among themselves (Table I and Figure 1). Serum ESM1 levels of the PCOS group were significantly lower than FF-ECSM1 levels (668.6±189.2 vs. 979.0±233.9 ng/L, $p<0.02$). FF-ESM1 levels of the control group were significantly higher than serum ECSM1 levels (639.3±206.4 ng/L vs. 503.2±102.4 ng/L, $p<0.01$). Serum ESM1 levels of the PCOS group were significantly higher than the ones of the control group (668.6±189.2 ng/L vs. 503.2±102.4 ng/L, $p<0.01$). Similarly, FF-ESM1 levels of the PCOS group were significantly higher than the levels of the control group (979.0±233.9 ng/L vs. 639.3±206.4 ng/L, $p<0.01$).

There was no significant correlation between serum and FF-ESM1 levels. These data are evidence that ESM1 levels in the two compartments are regulated differently. A positive and significant correlation was found between FF-ESM1 and serum LH levels in PCOS ($r=0.655$, $p<0.02$). Similarly, a positive and significant correlation was found between FF-ECSM1 and serum testosterone levels in PCOS ($r=0.470$, $p<0.03$). Moreover, a positive and significant correlation was detected between FF-ESM-1, AFC, MII, and total oocyte counts in PCOS. There was no significant correlation between age, BMI, HOMA-IR, serum, and FF-ESM-1 levels in PCOS and control groups. In the control group, a positive and significant correlation was found between FF-ESM-1, MII oocyte, AFC, and total oocyte counts.

Discussion

Polycystic ovary syndrome is a disease with subfertility and ovarian and endometrial components. Hyperandrogenemia, insulin resistance,

endometrial receptivity defect, and obesity are some of the causes of subfertility. However, the most common cause of subfertility due to PCOS is considered to be ovulatory dysfunction. LH surge defect, polycystic ovarian development, androgen elevation, and insulin resistance may be the cause or result of ovulatory dysfunction⁷⁻⁹. As the common result of all these pathogenetic mechanisms, monthly follicle development cannot be achieved, most of the patients are anovulatory. In physiological conditions, follicle development and rupture are regulated by inflammatory reactions and angiogenic mechanisms⁷. ESM-1 is an important biomarker involved in endothelial proliferation and angiogenesis¹⁰. We studied serum and follicular fluid ESM-1 levels for the first time in PCOS. We observed that FF-ESM-1 levels increased significantly when compared with serum levels. This finding suggests that ESM-1 is regulated differently in serum and in the follicular compartment. If there was a transition of ESM-1 from the follicular area to the serum, the serum and follicle fluid ESM-1 levels should have been similar. The fact that we did not detect any correlation between serum ESM-1 and FF-ESM-1 suggested that the two compartments had different internal dynamics in terms of ESM-1. Under these conditions, it is not possible to talk about the existence of a saturable transport system between the serum and the follicular compartment. However, high intrafollicular ESM-1 levels were also detected in the control group without PCOS. Therefore, ESM-1 differences between serum and follicular compartments are not specific to PCOS. Since both serum and follicular fluid ESM-1 levels of the PCOS group were higher than the levels of the healthy controls, we can think that ESM-1 exhibits a more intense expression pattern in PCOS.

It is well established in literature that increased ovarian mass, supported by new blood vessel proliferation in stroma and theca, is a key feature of PCOS. Recent studies¹¹ suggest a role for angiogenic factors in this phenomenon. High serum and follicular fluid vascular endothelial growth factor levels in PCOS patients who underwent IVF/ICSI support the role of neoangiogenesis in ovulatory dysfunction due to PCOS. ESM-1 may contribute to both VEGF production and new vessel formation because of its role in endothelial migration and adhesion³. Since ESM-1 expression is stimulated by inflammation, chronic inflammation due to PCOS may be responsible for the increase in ESM-1 in follicular fluid^{4,5}. Reporting^{12,13} of increased microRNA levels in the cumulus granulosa cells and follicular fluids of PCOS patients may explain the increased expression of ESM-1. In addition, disruption of VEGF-mediated angiogenesis in mature follicles in C57BL/6 mice on high fat diet are important indicators that PCOS phenotypes and BMI values contribute to the deterioration of angiogenesis in the follicular microenvironment^{14,15}.

The positive correlation between FF-ESM-1 and serum LH and testosterone levels suggests that the LH peak stimulates the synthesis of this molecule. Increasing androgens in the presence of PCOS also positively affect FF-ESM-1 production. However, we could not detect a relationship between insulin resistance and ESM-1. The significant relationship between ESM-1 and AFC and total oocyte count is evidence supporting the role of this molecule in healthy follicular development. However, there is a critical question that we must answer here. If FF-ESM-1 levels are so high in PCOS, why does anovulation occur? It is very difficult to answer this question with this study design. In this study, we applied antagonist and hCG in addition to rFSH treatment. All these drugs contribute to the development and rupture of follicles at different rates. FF-ESM-1 levels may be increased because the drugs given in the presence of high AFC allow the development of a large number of oocytes. In particular, triptorelin acetate and recombinant hCG, which we use for ovulation triggers, may have increased inflammation and angiogenesis and caused an increase in ESM-1 levels. The main reason for the increased FF-ESM-1 levels compared to serum may be the exogenous drugs we applied. The increase in FF-ESM-1 levels in the control group also supports this idea. The more pronounced increase in ESM-1 in PCOS may be due to high AFC and increased endogenous LH from the beginning. Looking at

ESM-1 levels in PCOS patients without ovarian stimulation is a necessary condition for us to have a clearer discussion.

Our study is important in terms of presenting a new biomarker for endothelial dysfunction and neoangiogenesis, which are possible etiological factors of ovulatory dysfunction in infertile PCOS. Demonstrating that ESM-1 levels are regulated independently in serum and follicular fluid is of additional importance as it shows that neoangiogenesis is not systemic but specific to the follicular microenvironment. However, it is not possible to make a clear interpretation of neoangiogenesis and ovulatory dysfunction by looking at only one marker. Investigation of multiple angiogenic markers at both mRNA and protein levels will allow us to have a clearer discussion. It is clear that there is a need for case-controlled studies in which multiple markers with a high number of participants are analyzed.

Conclusions

Despite the small number of cases, this study is the first clinical study investigating FF-ESM-1 levels. ESM-1 levels are regulated independently in the serum and follicular compartment. A significant increase was found in FF-ESM-1 levels in both PCOS and healthy controls. This increase may be due to the antagonist protocol and ovulation triggering. It may be possible to reach a clearer conclusion with new studies to be conducted in spontaneous cycles and without any ovulation stimulation drug administration.

Conflict of Interest

The authors declare no competing interests.

Ethics Approval

Study protocols and procedures were approved by the Istanbul IVF Center (Date: 15.06.20223, No.: 618).

Authors' Contributions

N.T: Conceptualization, validation, writing, review and editing, R.O: Conceptualization, validation, writing, review and editing.

Data Availability

Data can be accessed when deemed appropriate by the authors and the IVF Center.

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ORCID ID

Nurettin Turktekin: 0000-0001-8167-3124

Ramazan Özyurt: 0000-0001-6822-2222

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