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Zonulin as a potential biomarker for diminished ovarian reserve: A prospective study

Azalan yumurtalık rezervi için potansiyel bir biyobelirteç olarak zonulin: Prospektif bir çalışma

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Abstract

Objective: The purpose of this research is to investigate the relationship between zonulin levels and diminished ovarian reserve (DOR), and to evaluate the potential role of autoimmunity in the development of DOR. The study contributes to the understanding of the pathogenesis of DOR, which can be an unexpected diagnosis often associated with infertility and unpleasant physical symptoms in women.

Materials and Methods: This cross-sectional study was conducted by scanning 224 patients. The demographic characteristics of the patients were recorded. Antral follicle counts of the patients were determined by ultrasound, and Anti-Mullerian hormone (AMH) levels were examined. Follicle-stimulating hormone (FSH), luteinizing hormone, estradiol, AMH measurement, and antral follicle counts were made on the 2nd or 3rd day of menstrual bleeding. The zonulin levels of the participants were measured by the ELISA method. The patients were divided into two groups according to the presence of DOR. The patients' demographic characteristics and hormone levels were compared between these two groups, serum zonulin levels were examined, and the relationship between other hormone parameters and zonulin was investigated.

Results: When the median ages of the patients in both groups were compared, the median age of patients with DOR was 38 years, significantly higher ($p<0.001$) than the median age of those without DOR, which was 27 years. The median zonulin levels of both groups were compare; it was observed that it was 19.71 ng/mL in the group with DOR and 11.03 ng/mL without DOR, and a statistically significant difference was found between the zonulin levels of the patients in both groups ($p<0.001$). A moderate inverse correlation ($p<0.001$) between patients' zonulin and AMH levels, and a moderate correlation between FSH levels ($p<0.001$).

Conclusion: In conclusion, zonulin levels of patients with DOR were higher than women without DOR. Evaluation of zonulin levels may also be considered during the diagnosis of DOR.

Keywords: Diminished ovarian reserve, zonulin levels, autoimmunity, Anti-Mullerian hormone, AMH, infertility

Öz

Amaç: Bu araştırmanın amacı, zonulin düzeyleri ile azalmış over rezervi (DOR) arasındaki ilişkiyi ve otoimmünitenin DOR gelişimindeki potansiyel rolünü değerlendirmektir. Çalışma, kadınlar için sıkıntılı fiziksel semptomlar ve infertilite ile ilişkili beklenmedik bir teşhis olan DOR patogenezinin anlaşılmasına katkıda bulunmayı amaçlamaktadır.

Gereç ve Yöntemler: Bu kesitsel çalışma, 224 hastanın taranmasıyla gerçekleştirildi. Hastaların demografik özellikleri kaydedildi. Hastaların antral folikül sayıları ultrason ile belirlendi ve Anti-Müllerian hormon (AMH) düzeyleri incelendi. Folikül uyarıcı hormon (FSH), luteinleştirici hormon, östradiol, AMH ölçümü ve antral folikül sayıları, menstrüel kanamanın 2. veya 3. gününde yapıldı. Katılımcıların zonulin düzeyleri ELISA yöntemiyle ölçüldü. Hastalar, DOR varlığına göre iki gruba ayrıldı. Bu iki grup arasında hastaların demografik özellikleri ve hormon düzeyleri karşılaştırıldı, serum zonulin düzeyleri incelendi ve diğer hormon parametreleri ile zonulin arasındaki ilişki araştırıldı.

PRECIS: Serum zonulin may be the potential to be used as a valuable biomarker in predicting the diminished ovarian reserve with women.

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Bulgular: Her iki grup hastaların ortanca yaşları karşılaştırıldığında, DOR olan hastaların ortanca yaşı 38 yıl iken, DOR olmayan hastaların ortanca yaşı olan 27 yıldan önemli ölçüde yüksek (p<0,001) olduğu görüldü. Her iki grup hastaların ortanca zonulin düzeyleri karşılaştırıldığında, DOR olan grupta 19,71 ng/mL ve DOR olmayan grupta 11,03 ng/mL olarak bulundu ve her iki grup hastaların zonulin düzeyleri arasında istatistiksel olarak anlamlı bir fark bulundu (p<0,001). Hastaların zonulin düzeyleri ile AMH düzeyleri arasında orta derecede ters bir korelasyon (p<0,001) ve FSH düzeyleri ile orta derecede bir korelasyon bulundu (p<0,001).

Sonuç: DOR olan hastaların zonulin düzeyleri, DOR olmayan kadınlardan daha yüksek bulundu. DOR tanısı sırasında zonulin düzeylerinin değerlendirilmesi de dikkate alınabilir.

Anahtar Kelimeler: Azalmış over rezervi, zonulin, otoimmünite, Anti-Müllerian hormon, AMH, infertilite

Introduction

There is a significant variation in the number of oocytes in each woman, and biologically, the total amount is at its maximum before the woman giving birth. There is limited scientific knowledge about the factors that control the oocyte pool and how to measure them. The decrease in the quantity and quality of oocytes in old age (usually in the mid-40s) is a normal physiological phenomenon called a decrease in ovarian reserve (DOR)[1,2]. Some women suffer from DOR a lot earlier and become infertile early (pathological DOR). According to recent estimates by the Society for Assisted Reproductive Technology's national system in the United States, 32% of in vitro fertilization (IVF) cycles (about 66,000 cycles) are diagnosed with DOR[3]. The definition of DOR is a state of low fertility due to decreased ovarian function based on clinical judgment and is usually denoted by follicular stimulation hormone (FSH) >10 mIU/mL or Anti-Mullerian hormone (AMH) <1.1 ng/mL[3-5].

For most women, the diagnosis of DOR is unexpected, stressful, often overlaps with an infertility diagnosis and is associated with unpleasant physical symptoms[6]. Many environmental and lifestyle considerations have been suggested, such as the natural age of menopause, the use of oral contraceptives, gender parity, and smoking. However, these factors do not consistently explain the changes in the age of menopause. Ovarian aging correlates strongly with the number and quality of the remaining oocytes[5,6]. Although the etiology of DOR is not yet known, it is thought that most genetic disorders may be related to autoimmunity, iatrogenic, or idiopathic reasons.

Recently, many studies have been done on the structure of the intercellular Tight Junctions (TJ) and its functionalization. The discovery of the zonula occludens toxin (Zot), an enterotoxin that affects TJ competence and developed by Vibrio cholerae, has shed light on the complex mechanisms involved in the modulation of the intestinal paracellular pathway[7,8]. The combination of affinity-purified anti-Zot antibodies and the Ussing chamber technique identified the intestinal Zot homolog called zonulin[8,9].

Zonulin is a protein that plays a role in the pathogenesis of autoimmune diseases, opening the intercellular TJs in the intestines and recycling intestinal permeability[10,11]. Regarding the relationship between zonulin and metabolic disorders, it has been shown in a recent studies[11]. A study investigating the relationship between zonulin and obesity and insulin resistance found that zonulin levels increased significantly in obese and glucose-tolerant individuals[12]. Likewise, it has been positively correlated with inflammatory markers, insulin resistance, and interleukin 6 (IL-6).

Elevated levels of zonulin have been associated with increased intestinal permeability and several inflammatory and autoimmune conditions, including celiac disease, rheumatoid arthritis, and type 1 diabetes[13]. DOR, or diminished ovarian reserve, is a condition characterized by a decreased number of eggs in the ovaries, which can lead to infertility[2,3].

There is currently no research investigating the relationship between zonulin levels and diminished ovarian reserve (DOR). However, some evidence suggested that increased intestinal permeability and inflammation may play a role in the pathogenesis of DOR. Studies have shown that women with DOR have higher levels of inflammatory markers than women with normal ovarian reserve[3,6].

This study aimed to evaluate the relationship between zonulin levels and DOR. This study will evaluate the relationship between autoimmunity, which is a factor in the pathogenesis of DOR.

Materials and Methods

This cross-sectional study was conducted by scanning 224 patients who applied to the Obstetrics and Gynecology outpatient clinic of a university hospital. Ethics committee approval was obtained for the study (Ondokuz Mayıs University/KAEK 2021/95). Written informed consent was obtained from each patient participating in the study. This study was conducted in accordance with the Declaration of Helsinki. Female patients under the age of forty who came to the obstetrics and gynecology outpatient clinic were included in the study. Forty-five patients above forty years of age and with a body mass index ≥30 were excluded from the study. Patients were selected from patients who had regular menstruation and did not have clinical and laboratory findings of PCOS. Additionally, patients with endometriosis, known autoimmune diseases, and thyroid patients, patients with previous ovarian surgery, and chronic diseases (such as diabetes mellitus and hypertension) were excluded from the study. The remaining patients were divided into two groups according to the presence or absence of ovarian insufficiency (Figure 1). The definition of DOR is a state of low fertility due to decreased ovarian function based on clinical judgment and is usually denoted by FSH >10 mIU/mL or AMH <1.1 ng/mL[3].

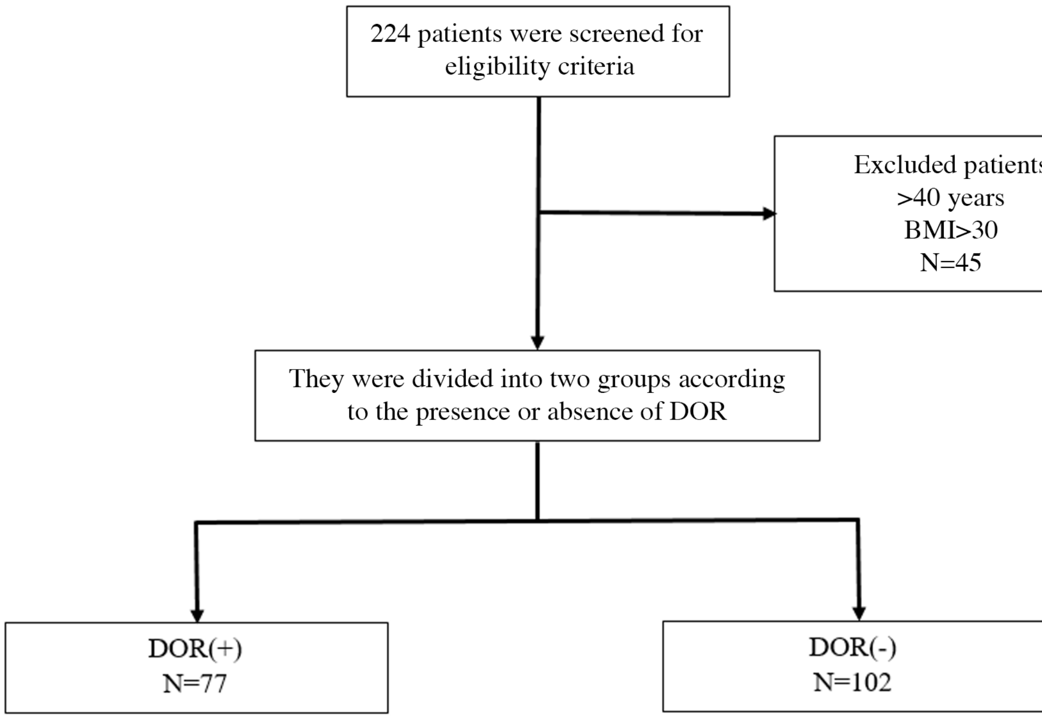

Figure 1. Patient flow diagram

Demographic and laboratory characteristics of the patients [age, body mass index (BMI), FSH, luteinizing hormone (LH), estradiol levels] were recorded. Antral follicle counts of the patients were determined by ultrasound, and AMH levels were examined. FSH, LH, estradiol, AMH measurement, and AFC counts were made on the 2nd or 3rd day of menstrual bleeding. Ultrasound scans (for AFCs) of the patients were performed by C.S.C. and S.C. at Samsun Training and Research Hospital.

Fasting blood samples of all patients were processed in a centrifuge at 3.000 rpm (rounds per minute). Serum was collected and stored at -80 °C until analysis. These stored serums were then used for measuring zonulin values.

Commercially available Enzyme-linked Immunosorbent Assay (ELISA) kits (Sun-Red Bio Company, Cat No. 201-12-5578, Shanghai, China) were used to measure human zonulin concentrations in serum. Enzymatic reactions were measured in an automated microplate photometer. The microtiter plate provided in this kit is pre-coated with a monoclonal antibody specific to human zonulin. Streptavidin-Horseradish Peroxidase is then added to each microplate well and incubated to form an immune complex. Wells containing only Human Zonulin, biotin-conjugated antibody, and enzyme-conjugated avidin will change color after adding chromogen solutions. The addition of the sulfuric acid solution terminates the enzyme-substrate reaction, and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of human zonulin in the samples is then determined by comparing the optical density of the samples with the standard curve. Human zonulin levels were expressed as ng/mL. The coefficients of intra and inter-assay variation were 4.3% (n=20) and 4.9% (n=20), respectively. The sensitivity of this test was 0.223 ng/mL, and the test range is between 0.25 ng/mL and 70 ng/mL.

The patients were divided into two groups according to the presence of DOR. The patients' demographic characteristics and hormone levels were compared between these two groups, and serum zonulin levels were examined. Additionally, the relationship between other hormone parameters and zonulin was investigated. Finally, univariate regression analysis was performed for the presence of DOR, and the zonulin cut-off value to predict the presence of DOR was calculated.

Statistical Analysis

The SPSS 25.0 (IBM, NY, USA) program was used for statistical analysis. Kolmogorov-Smirnov test was performed to evaluate whether the distribution in the groups was parametric. An independent sample t-test was used for comparison between groups. Correlation analysis was performed with the Spearman's test, and logistic regression analysis was applied. $P<0.05$ was determined as statistically significant.

Results

Age, BMI, the number of antral follicles, and hormone levels of the patients are shown in Table 1. When the median ages of the patients in both groups were compared, the median age was 38 (35-39) in the group of patients with DOR and 27 (24-33) in the group without DOR ($p<0.001$). When the mean BMIs of the patients were compared, it was found that they were

Table 1. Selected clinical data for the study groups

	DOR (+) (n=77)	DOR (-) (n=102)	p-value
Age (y)	38 (35-39)	27 (24-33)	**<0.001**
BMI (kg/m^2)	25.4±2.4	24.9±2.3	0.171
AMH (ng/mL)	0.3 (0.03, 0.5)	3.1 (1.9-4.8)	**<0.001**
Antral follicle count (median)	2 (1, 3)	10 (9, 2)	**<0.001**
FSH (mIU/mL)	9.9 (7.9-21.5)	6.2 (5.6-7.3)	**<0.001**
Estradiol (mIU/mL)	55.9 (30.1-80)	41. 6 (37.4-53.1)	0.088
LH (mIU/mL)	9.6 (5.3-11.4)	8.7 (4.9-11.3)	0.121
Zonulin (ng/mL)	19.7 (10.5-53.5)	11.0 (9.2-13.2)	**<0.001**

25.43±2.39 and 24.86±2.29, respectively, and no statistically significant difference was found (p=0.171). When the median antral follicle numbers of the patients were compared, it was seen that the median numbers of the patients in the groups with and without DOR were 2 (1-3) and 10 (9-23), respectively (p<0.001).

The median AMH levels of the patients with and without DOR were 0.3% and 3.1, respectively. When the median FSH hormone levels of the patients were compared, it was observed that it was 9.90 (7.9- 21.5) and 6.22 (5.6- 7.5) in the groups with and without DOR, respectively, and there was a statistically significant difference between the two groups (p<0.001). Similarly, when the median zonulin levels of both groups were compared, it was observed that it was 19.7 (10.5, 53.5) in the group with DOR and 11.03 (9.2, 13.2) without DOR, and a statistically significant difference was found between the zonulin levels of the patients in both groups (p<0.001). There was no statistically significant difference between the median estradiol levels in both groups (p=0.088).

Spearman's test was performed to show the correlation between the patients' AMH, zonulin, FSH, and estradiol levels. A moderate inverse correlation (p<0.001, rs:-0.498) between patients' zonulin and AMH levels (Figure 2a), and a moderate correlation between FSH levels (p<0.001, rs:0.313) (Figure 2b). A strong inverse correlation was also observed between patients' FSH and AMH levels (p<0.001, rs:-0.600) (Table 2). No significant correlation was observed between age and zonulin levels (r=0.225, p=0.07). There was no correlation between estradiol levels and other hormone levels (p>0.05).

Logistic regression analysis was performed to show the effect of hormone levels, age, and BMI on ovarian insufficiency. The regression model was statistically significant: x^2 (4) = 111.8, p<0.001. The model explained 62.3% (Nagelkerke R^2) of the variance in DOR and accurately predicted 82.1% of the cases. Sensitivity was 84.3%, specificity was 79.2%, positive predictive value was 79.2%, and negative predictive value was

Table 2. Correlation analysis of the patients' zonulin levels and hormones

		Zonulin	AMH	FSH
Zonulin	r		-0.498	0.313
	p		<0.001	<0.001
AMH	r			-0.600
	p			<0.001
Antral follicle count	r	-0.452	0.930	-0.368
	p	<0.001	<0.001	<0.001

AMH: Anti-Mullerian hormone, FSH: Follicular stimulation hormone

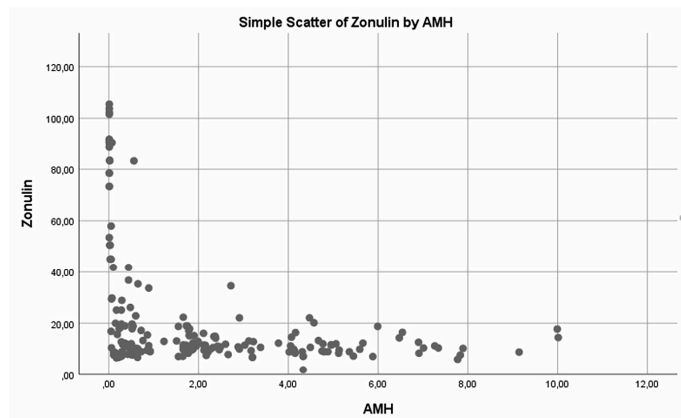

Figure 2a. Correlation analysis of Anti-Mullerian hormone (AMH) and zonulin levels

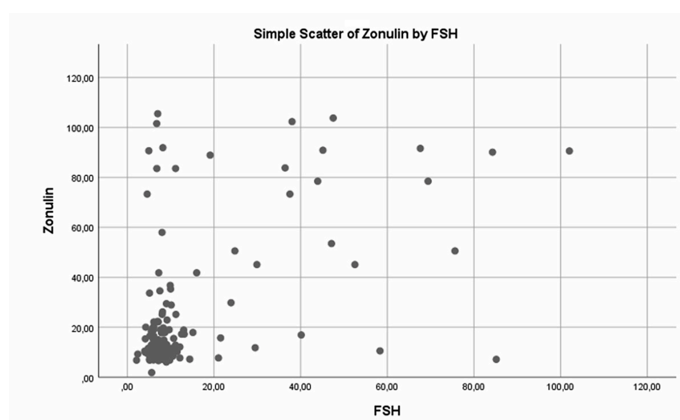

Figure 2b. Correlation analysis of follicular stimulation hormone (FSH) and zonulin levels

84.3%. Age and zonulin levels were statistically significant predictors (Table 3).

Receiver operating characteristic (ROC) curve analysis was performed to predict the distinction of zonulin level from DOR. It was observed that the area under the ROC curve was between 0.734 (95% confidence interval 0.652 and 0.815) and was at an acceptable level of distinction. The sensitivity and specificity of the zonulin value of 22.6 were 46.8% and 99.9% (Figure 3).

Table 3. Logistic regression analysis

	B	S.E.	p-value	HR	%95 CI	
					Lower	Upper
FSH	-0.041	0.031	0.191	0.960	0.903	1.021
Zonulin	-0.105	0.029	<0.001	0.900	0.851	0.953
Age	-0.212	0.039	<0.001	0.809	0.749	0.874
BMI	-0.020	0.145	0.892	0.980	0.737	1.303

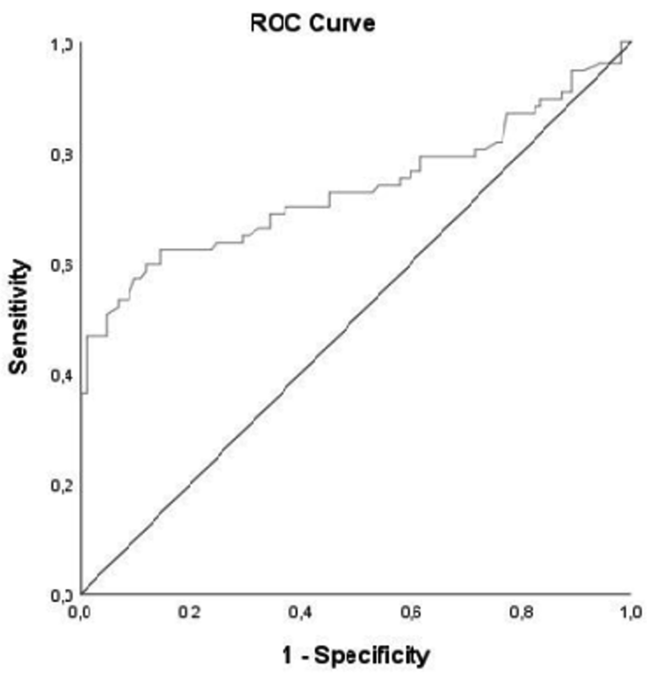

Figure 3. ROC curve analysis of zonulin

ROC: Receiver operating characteristic

Discussion

At the end of the study, it was determined that zonulin levels were significantly higher in the group with ovarian insufficiency and that zonulin levels were moderately negatively correlated with FSH and AMH levels. Additionally, age and zonulin levels were found to be significant as parameters that could predict ovarian insufficiency, and the zonulin cut-off value determined to indicate ovarian insufficiency was recorded as 22.6.

There is no definitive test to assess ovarian reserve, and contradictory results between ovarian reserve tests are commonplace. Therefore, various tests are used to assesment the ovarian reserve. In a worldwide study, 51% of infertility centers chose AMH as the best test to measure the ovarian reserve, 40% chose the ASH as the best test, and only 6% chose the basal FSH[14]. A common clinical challenge is to provide advice to patients with contradictory ovarian reserve test results. With regard to direct diagnoses of DOR, 20% of samples from a large national testing center have inconsistent FSH and AMH[15]. Our study determined that zonulin level could be a significant indicator of ovarian insufficiency and correlated inversely with AMH values and positively with FSH values.

Inflammation of the small intestine leads to changes in bowel patency. It has also been shown that there is a correlation between serum zonulin level and fasting insulin, fasting triglycerides, and IL-6. The zonulin gene overlaps with the haptoglobin 2 gene under the control of IL-6[13,16]. Moreover, in patients with normal glucose tolerance, zonulin is associated with high levels of uric acid, HbA1c, serum IL-6, and low HDL. Ohlsson et al.[16] studied the relationship between zonulin and ovarian function and found that rates of zonulin were associated with ovarian function and were higher in patients with severe menstrual irregularities.

The ovaries are not immunologically protected, so the autoantibodies suggest that the ovaries are detected and that an etiology of autoimmune can produce DOR. The most common autoimmune diseases associated with primary ovarian failure are hypothyroidism, type I diyabetes mellitus, hiperparatiroidizm, sistemik lupus erytematosus, and tipik lupus erythematosus[15-17]. Both early follicular exhaustion and dysfunction may be present in all these conditions.

There are few studies on the relationship between zonulin and metabolic disorders[18,14,19]. One study investigated the relationship between zonulin and obesity and insulin resistance. It has been found that levels of zonulin appear to increase considerably in people who are obese and glucose intolerant and are positively correlated with insulin resistance and inflammatory markers such as IL-6. There are few studies have investigating serum zonulin levels in PCOS patients[4,20]. Zhang et al.[21] reported that zonulin levels have increased significantly in women with disease compared to controls and a strong correlation between insulin resistance, obesity, dyslipidemia, and menstrual disorders. The high levels of zonulin in patients with ovarian insufficiency patients in our study confirm that it has an auto-inflammatory role and causes the development of DOR. Further studies must confirm the role of changes in intestinal permeability and zonulin in insulin resistance and autoimmunity.

Studies have shown that zonulin can be a marker for autoimmune diseases. The pathogenesis of DOR is unclear, and many factors are under investigation. In response to the problem that autoimmunity, which may cause a loss of reserve at an early age, may also be one of these possible factors, we planned to study with the thought that zonulin could be an indicator.

Study Limitations

Our study is the first to evaluate a markers such as zonulin in women with ovarian insufficiency. The information obtained from this study will shed light on future studies. This study had some limitations. The first limitation of the study is that the sample size is small, and it is a single-center study. Due to the study's cross-sectional nature, evaluation as a factor in developing ovarian insufficiency is limited. Another limitation of the study is that cytokine levels such as IL-1, TNF-alpha, and IL-6 were not evaluated.

Conclusion

As a result, the zonulin levels of patients with DOR were higher than women without DOR. Modeling that can be done with other parameters will provide very convenient data for diagnosing DOR.

Ethics

Ethics Committee Approval: Ethics committee approval was obtained for the study (Ondokuz Mayıs University/KAEK 2021/95).

Informed Consent: Written informed consent was obtained from each patient participating in the study.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Concept: Se.Ç., N.Y., C.S.C., S.Ç., Design: Se.Ç., N.Y., C.S.C., S.Ç., Data Collection or Processing: Se.Ç., C.S.C., Analysis or Interpretation: Se.Ç., N.Y., Literature Search: Se.Ç., N.Y., C.S.C., S.Ç., Writing: N.Y., Se.Ç.

Conflict of Interest: No conflict of interest was declared by the authors.

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