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The role of inflammation, oxidation and Cystatin-C in the pathophysiology of polycystic ovary syndrome

Polikistik over sendromunun patofizyolojisinde enflamasyon, oksidasyon ve Sistatin-C'nin rolü

Özden Özdemir Başer[1], Ayşe Yeşim Göçmen[2], Demet Aydoğan Kırmızı[3]

[1]*Yozgat City Hospital, Clinic of Endocrinology and Metabolism, Yozgat, Turkey*

[2]*Yozgat Bozok University Faculty of Medicine, Department of Biochemistry, Yozgat, Turkey*

[3]*Yozgat Bozok University Faculty of Medicine, Department of Obstetrics and Gynecology, Yozgat, Turkey*

Abstract

Objective: The relationship between Cystatin-C levels and inflammatory, oxidant, and antioxidant markers in polycystic ovary syndrome (PCOS) was investigated.

Materials and Methods: A total of 96 participants were included in the study as PCOS (n=58) and control (n=38) groups. Tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1B), interleukin 6 (IL-6), malondialdehyde (MDA), superoxide dismutase (SOD), and Cystatin-C were evaluated by ELISA method. Relationships metabolic and endocrine parameters seen in PCOS were examined. Univariate and multivariate logistic regression analyzes were performed to identify risk factors that may affect the PCOS group. Bivariate correlations were investigated by the Spearman's correlation analysis.

Results: While Cystatin-c, TNF-α, IL-1B, IL-6, MDA were found to be higher in patients with PCOS compared with the control group, SOD was found to be lower than the control group ($p<0.05$). In the correlation analysis, increased Cystatin-C levels were found to be associated with high IL-6 ($r=0.214$, $p=0.037$) and low SOD levels ($r=-0.280$, $p=0.006$).

Conclusion: In our study, it was found that the increase in Cystatin-C levels was associated with an increase in IL-6 and a decrease in SOD. These results may bring up different treatment options to reduce cardiovascular risks for treating PCOS.

Keywords: Polycystic ovary syndrome, Cystatin-C, interleukin, oxidation, superoxide dismutase

Öz

Amaç: Polikistik over sendromunda (PKOS) Sistatin-C düzeyleri ile enflamatuvar, oksidan ve antioksidan belirteçler arasındaki ilişkinin araştırılması amaçlandı.

Gereç ve Yöntemler: Çalışmaya PKOS (n=58) ve kontrol (n=38) grubu olarak toplam 96 katılımcı dahil edildi. Tümör nekroz faktör-alfa (TNF-α), interlökin-1 beta (IL-1B), interlökin 6 (IL-6), malondialdehit (MDA), süperoksit dismutaz (SOD) ve Sistatin-C markerları ELISA yöntemi ile değerlendirildi. PKOS grubunu etkileyebilecek olası risk faktörlerini belirlemek için tek değişkenli ve çok değişkenli lojistik regresyon analizi yapıldı. İki değişkenli korelasyonlar Spearman korelasyon analizi ile araştırıldı.

Bulgular: PKOS hastalarında Sistatin-C, TNF-α, IL-1B, IL-6, MDA, kontrol grubuna göre daha anlamlı olarak yüksek bulunurken, kontrol grubunda SOD daha yüksek bulundu ($p<0,05$). Korelasyon analizinde, artan Sistatin-C seviyeleri, yüksek IL-6 ($r=0,214$, $p=0,037$) ve düşük SOD seviyeleri ($r=-0,280$, $p=0,006$) ile ilişkili bulundu.

Sonuç: Çalışmamızda kardiyovasküler hastalık belirteci olan Sistatin-C düzeylerindeki artışın yüksek IL-6 ve düşük SOD seviyeleri ile ilişkili olduğu bulundu. Bu sonuçlar PKOS tedavisinde kardiyovasküler riskleri azaltmak için farklı tedavi seçeneklerini gündeme getirebilir.

Anahtar Kelimeler: Polikistik over sendromu, Sistatin-C, interlökin, oksidasyon, süperoksit dismutaz

PRECIS: This study is a case-control study evaluating the relationship between Cystatin-C levels and inflammatory, oxidant, and antioxidant markers in polycystic ovary syndrome.

Address for Correspondence/Yazışma Adresi: Özden Özdemir Başer MD,
Yozgat City Hospital, Clinic of Endocrinology and Metabolism, Yozgat, Turkey
Phone: +90 507 191 15 18 **E-mail:** ozdemir.oz83@hotmail.com **ORCID ID:** orcid.org/0000-0001-8368-3182

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Introduction

Polycystic ovary syndrome (PCOS) is a disease with clinical or laboratory findings of hyperandrogenism, polycystic ovary appearance, and menstrual irregularity. It is often observed in women of reproductive age[1]. The international prevalence rate ranges from 5 to 21%[2]. Although the etiology is not clearly known, disruption of oxidant mechanisms and increased inflammatory mediators are thought to be the cause[1,3,4]. Studies have shown that, depending on the increase in adipose, inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and malinaldehyde (MDA) levels increase, while superoxide dismutase (SOD) decreases[5,6]. As a result, the deterioration in oxidant-antioxidant balance and increase in inflammatory markers are observed due to increased adipose tissue and hyperandrogenemia. This situation increases diseases that increase cardiovascular risk, such as insulin resistance, obesity, dyslipidemia, and type 2 diabetes mellitus[4].

Cystatin-C is an extracellular cysteine protease inhibitor. It is a low molecular weight cationic protein[7]. It is a strong predictor of not only renal failure but also all-cause mortality, such as cardiovascular disease and diabetes mellitus[8]. It has also been significantly associated with asymptomatic coronary artery disease in patients with metabolic syndrome with normal renal function[9]. Because of these data, Cystatin-C was examined in studies due to the increased cardiovascular risk in polycystic ovarian disease, and this marker was found to be statistically significantly higher in the PCOS group than in the healthy group[10]. In previous studies, either inflammation and oxidative-antioxidative markers or markers such as Cystatin-C were studied. In this study, it was stated that high Cystatin-C levels in patients with PCOS were important in identifying patients at cardiovascular risk[11].

However, it is unclear whether the increase in Cystatin-C is due to increased inflammation or the deterioration of oxidant-antioxidant mechanisms. This study investigated the relationship between Cystatin-C elevation and inflammatory and oxidant-antioxidant mediators. If it is associated with these mechanisms, targeted therapy may come to the fore in terms of cardiovascular protection.

Materials and Methods

Study Design and Participants

Patients over the age of 18 who applied to Yozgat Bozok University Medical Faculty Hospital between 01.01.2022 and 01.04.2022 were included in the study. The Yozgat Bozok University Local Ethical Committee approved the present study (2017-KAEK-189_2021.12.29_02) and informed consent was obtained from all participants.

The diagnosis of PCOS was made according to the Rotterdam criteria. These criteria were clinical and/or biochemical hyperandrogenemia, presence of oligomenorrhea (interval between two menstrual periods more than 35 days) or amenorrhea (no vaginal bleeding for at least six months), and ultrasonographic polycystic ovary appearance (≥12 follicles measuring 2-9 mm in diameter, or ovarian volume >10 mL in at least one ovary)[12]. The presence of acne and/or hirsutism and/or alopecia were evaluated as clinical signs of hyperandrogenemia. Feriman Galway's scoring was used for hirsutism. Nine different parts of the body, upper lip, chin, chest, upper back, lower back, upper abdomen, lower abdomen, arm, and thigh, were scored between 1 and 4 and a total score of 8 and above was considered hirsutism[13]. Findings of hyperandrogenemia and menstrual patterns were recorded in the database at the first diagnosis. The demographic and laboratory data of the patients were recorded retrospectively from the hospital database. Demographic features included waist to hip ratio (WHR), body mass index (BMI), gravidity, parity, and abortion. The parameters evaluated in the study were examined from the blood samples collected for diagnostic purposes before the treatment was initiated.

Exclusion criteria from the study were the presence of chronic systemic disease, infectious and inflammatory diseases, hormone replacement therapy, use of oral contraceptives or drugs for insulin resistance, patients under 18 years of age, presence of psychiatric disorder and drug use for it, history of bariatric surgery, thyroid dysfunction. Secondary causes of clinical and/or biochemical hirsutism and oligomenorrhea, such as congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's syndrome, hyperprolactinemia, thyroid dysfunction, and adrenal disorders were excluded.

Anthropometric Measurements

A weight measurement of the patients was made with a digital scale, with at least clothes and no shoes. Height measurements were made while standing without shoes. BMI was obtained by dividing weight in kilograms (kg) by height (m^2) (kg/m^2). Determined according to BMI World Health Organization's criteria. WHR; was obtained by dividing the waist circumference measured at the thinnest point between the rib and the iliac crest with the hip circumference measured from the widest part of the hips.

Ultrasonography Assessment

Gynecological ultrasound was performed on the second or third day of menstruation with a 7.5 MHz transvaginal transducer or a 5 MHz transabdominal transducer. Antral follicles were measured in three dimensions, and those with an average diameter of 2-9 mm were counted.

Biochemical Measurements

All blood samples used in the study were taken between 08.00 and 09.00 in the morning in the early follicular phase on the second or third day of the menstrual cycle. Pituitary, adrenal and gonadal axis hormones were checked in all patients due to amenorrhea and hirsutism complaints. Liver and kidney

function tests, hemogram, serum lipid levels, fasting plasma glucose, and fasting insulin levels were measured. Serum follicle-stimulating hormone, luteinizing hormone (LH), prolactin, insulin, and thyroid-stimulating hormone (TSH) levels were determined by chemiluminescent immunometric assays using a Cobas 6000 analyzer (Roche, Swiss) method. Fasting glucose, total cholesterol, high-density lipoprotein cholesterol and triglyceride levels (TG) were measured spectrophotometrically using an enzymatic colorimetric assay (Roche Integrated system, Mannheim, Germany). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula. Insulin resistance was calculated using the homeostatic model assessment for the insulin resistance index (HOMA-IR). The HOMA-IR formula is fasting plasma glucose (mg/dL) x fasting serum insulin (mU/mL)/405[14].

Blood samples were collected from each patient after a 12-hour fasting period for TNF-α, interleukin- 1 beta (IL-1β), and IL-6. Whole blood samples were centrifuged for 10 min at 4000 rpm, and the supernatants were kept at -80 °C until the assays were performed by an investigator who was blind to each patient's status. Commercial enzyme-linked immunosorbent assay (ELISA) kits were used for measuring Cystatin-C, TNF-α, IL-1β, and IL-6 (Bioassay technologies, China) levels using appropriate wavelengths on a microplate reader (BioTek Instruments, EL x 800 TM, USA) following the assay instructions. Concentrations were calculated over the standard curves. Serum MDA level was determined according to Göçmen et al.[15] Total SOD activity was examined using the SOD Activity Assay kit (Rel Assay Diagnostics kit; Mega Tıp, Gaziantep, Turkey), according to the manufacturer's instructions.

Statistical Analysis

The statistical package program SPSS 20 (IBM Corp. released 2011. IBM SPSS Statistics for Windows, version 20.0, Armonk, NY: IBM Corp.) was used to evaluate the data. Data were expressed as mean ± standard deviation and in percentages. Continuous variables were investigated using analytical methods (Kolmogorov-Simirnov/Shapiro-Wilk's test) to determine whether they were normally distributed. The Mann-Whitney U test was used for the non-parametric numerical data, while the Student's t-test was adopted for the parametric numerical data. Relationships between categorical variables were analyzed by the chi-square test. Bivariate correlations were investigated by the Spearman's correlation analysis. Univariate and multivariate logistic regression analyzes were performed to identify risk factors that may affect the PCOS group. P<0.05 were accepted as statistically significant.

Results

A total of 96 patients were included in the study, 60.4% of whom were PCOS (n=58) and 39.6% were from the control group (n=38). When the demographic data of both groups were analyzed, gravida and parity were found to be significantly lower in the PCOS group (p<0.05) (Table 1). When the laboratory data of the patients were evaluated, it was observed that the TSH level was statistically significantly lower in the PCOS group (p<0.05). There was no significant difference between the two groups in fasting glucose, fasting insulin, and cholesterol levels, which are cardiovascular risk markers, but Cystatin-C level was found to be high in the PCOS group (p<0.05) (Table 2). When the inflammatory, oxidant, and antioxidant markers of both groups were compared, it was seen that IL-1β, IL-6, TNF-α, and MDA were statistically significantly higher and SOD was low in patients with PCOS (p<0.05) (Table 2).

In the multivariate regression analysis, TNF-α [odds ratio (OR)=1.2, 95% confidence interval (CI)=1.1-1.3], IL-1β (OR=1.1, 95% CI=1.1-1.3), IL-6 (OR=3.9, 95% CI=1.1-13.5) and Cystatin-C (OR=11.7, 95% CI=2.8-98.1) levels were found to be independently high in the PCOS group (Table 3).

When the relationship between Cystatin-C elevation and these markers was evaluated (in the bivariate correlation), it was observed that the increase in Cystatin-C was associated with an increase in IL-6 levels (r=0.214, p=0.037) and a decrease in SOD levels (r=-0.280, p=0.006) (Table 4).

Discussion

This study showed that IL-1β, IL-6, TNF-α, and MDA were significantly higher and SOD was low in patients with PCOS. Again in the study, Cystatin-C, which is a risk factor for cardiovascular diseases, was found to be high in the PCOS group. When the relationship between the elevation

Table 1. Demographic data of PCOS and control group

	Control	PCOS	OR	95% CI		p
Age (years)	30.2±5.2	28.2±4.0	1.998	0.090	3.906	0.075
BMI (kg/m²)	5.9±0.9	6.1±1.3	-0.178	-0.715	0.359	0.851
WHR	0.8±0.1	0.8±0.1	-0.030	-0.061	0.001	0.075
Gravidity	1.8±1.3	0.6±0.9	1.281	0.698	1.865	<0.001
Parity	1.4±1	0.4±0.7	0.988	0.556	1.420	<0.001
Abortion	0.3±0.9	0.1±0.4	0.192	-0.149	0.532	0.391

Data are shown as mean ± SD. BMI: Body mass index, WHR: Waist circumference hip circumference ratio, PCOS: Polycystic ovary syndrome, OR: Odds ratio, SD: Standard deviation, CI: Confidence interval

Table 2. Comparison of biochemical characteristics between the two groups

	Control	PCOS	OR	95% CI		p
Fasting glucose (mg/dL)	93.7±43	88.9±8.1	4.862	-7.186	16.909	0.301
Fasting insulin (µIU/mL)	10.3±5.2	13.7±20.9	-3.395	-11.136	4.347	0.314
FSH (IU/L)	5.7±1.6	5±1.5	0.772	-0.483	2.027	0.180
LH (IU/L)	5±2.4	8.8±15.7	-3.795	-15.819	8.228	0.278
E2 (IU/L)	36.3±19.5	45.1±22.6	-8.869	-27.015	9.278	0.233
TSH (mIU/L)	2.7±1.5	2.0±0.7	0.765	0.272	1.257	0.015
LDL (mg/dL)	94.3±35.1	101.3±25	-6.966	-20.303	6.372	0.366
HDL (mg/dL)	52.9±11.8	56.4±14.5	-3.521	-9.722	2.680	0.242
Cholesterol (mg/dL)	169.8±36.4	175.3±27.6	-5.498	-19.669	8.672	0.416
Triglyceride (mg/dL)	93.4±49.4	97.7±52.4	-4.325	-27.839	19.189	0.758
TNF-α (pg/mL)	27±7.2	36.4±10.0	-9.366	-13.103	-5.629	<0.001
IL-1β (pg/mL)	35.1±8.6	47.6±9.9	-12.431	-16.334	-8.528	<0.001
IL-6 (pg/mL)	1.4±0.8	2.0±0.8	-0.593	-0.922	-0.264	<0.001
SOD (IU/mL)	13.5±1.8	12.6±2	0.942	0.134	1.751	0.006
MDA (µmol/L)	0.9±0.2	1±0.2	-0.121	-0.202	-0.040	0.001
Cystatin-C (mg/L)	0.8±0.1	0.9±0.1	-0.056	-0.103	-0.009	0.016

FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, E2: Estradiol, TSH: Thyroid stimulating hormone, LDL: Low density lipoprotein, HDL: High density lipoprotein, TNF-α: Tumor necrosis factor-alfa, IL-1β: Interleukin-1 beta, IL-6: Interleukin-6, SOD: Superoxide dismutase, MDA: Malondialdehyde, PCOS: Polycystic ovary syndrome, OR: Odds ratio, CI: Confidence interval

Table 3. Univariate and multivariate logistic regression analysis to identify possible risk factors that may affect the PCOS group

	Multivariate					Univariate				
	B	p	OR	95% CI		B	p	OR	95% CI	
Age (years)	-0.060	0.515	0.9	0.8	1.1	-0.099	0.044	0.9	0.8	1.0
TSH (mIU/L)	-0.843	0.070	0.4	0.2	1.1	-0.678	0.009	0.5	0.3	0.8
TNF-α (pg/mL)	0.152	0.004	1.2	1.1	1.3	0.122	0.000	1.1	1.1	1.2
IL-1β (pg/mL)	16.549	0.017	1.1	1.0	1.3	5.765	0.000	0.0	1.1	1.2
IL-6 (pg/mL)	1.354	0.033	3.9	1.1	13.5	1.377	0.018	0.3	1.5	5.9
SOD (IU/mL)	-0.194	0.364	0.8	0.5	1.3	-0.247	0.027	0.8	0.6	1.0
MDA (µmol/L)	1.364	0.528	3.9	0.1	268.8	3.229	0.006	25.2	2.6	247.9
Cystatin-C (mg/L)	11.630	0.012	11.7	2.8	98.1	4.764	0.025	117.2	1.8	7518.4

TSH: Thyroid stimulating hormone, TNF-α: Tumor necrosis factor-alfa, IL-1β: Interleukin-1 beta, IL-6: Interleukin-6, SOD: Superoxide dismutase, MDA: Malondialdehyde, PCOS: Polycystic ovary syndrome, OR: Odds ratio, CI: Confidence interval

of Cystatin-C and inflammatory, oxidant, and antioxidant mediators was evaluated, it was observed that there was a significant correlation with the increase in IL-6 and decrease in SOD.

Studies have shown that Cystatin-C is a good predictor of cardiovascular events[16,17]. It has been reported that it may be an indicator of future cardiovascular risk in women with PCOS[11]. Çınar et al.[18] stated that increased Cystatin-C levels in patients with PCOS are an early indicator of negative clinical outcomes. Statistically significant negative outcomes in the PCOS group in this study were BMI, WHR, FS, triglyceride, LDL, total cholesterol, estradiol, dehydroepiandrosteno-sulphate, free testosterone, LH, high sensitive C-reactive protein. Gozashti et al. [10] also found high Cystatin-C levels in patients with PCOS. In our study, there was no significant difference between the two groups in terms of fasting glucose, fasting insulin and lipid levels, which are cardiovascular risk factors, while Cystatin-C was found to be high regardless of these risk factors.

TNF-α, IL-1β, IL-6 are markers that show inflammation. Studies have shown that TNF-α is higher in women with PCOS

Table 4. Correlation analysis between Cystatin-C and inflammatory and oxidative markers

	Cystatin-C (mg/L)	
	r	p
TNF-α (pg/mL)	0.056	0.590
sIL-1β (pg/mL)	-0.030	0.775
IL-6 (pg/mL)	0.214	0.037
SOD (IU/mL)	-0.280	0.006
MDA (µmol/L)	0.132	0.199

TNF-α: Tumor necrosis factor-alfa, IL-1β: Interleukin-1 beta, IL-6: Interleukin-6, SOD: Superoxide dismutase, MDA: Malondialdehyde

than in the healthy population[19]. TNF-α has been particularly associated with insulin resistance and hyperandrogenemia and is higher in follicular fluid than in serum[20-22]. It has also been stated that high TNF-α levels in patients with PCOS cause the development of type 2 diabetes mellitus (type 2 DM), infertility, atherosclerosis and some cancers[23].

Other cytokines known to increase in PCOS are IL-1β and IL-6. It is thought that *IL-1β* gene activation, which plays a key role in the inflammatory response, may affect steroidogenesis in granulosa cells[24]. It has also been reported that increased IL-1β causes follicular atresia and inhibits oocyte maturation[25]. In the study of Alkhuriji et al.,[26] it was observed that IL-1β levels were high in patients with PCOS with obesity. The increase in IL-1β in these patients is thought to be due to anovulation[27]. It has been shown that IL-6 levels, one of the inflammatory cytokines, are increased especially in patients with PCOS with insulin resistance[28]. IL-6 is thought to have proinflammatory properties that cause insulin resistance[29]. Additionally, it has been observed that insulin resistance and obesity stimulate *TNF-α* and *IL-6* gene expression in adipose tissue in patients with PCOS[30]. Although there was no statistical difference in BMI and WHR between the PCOS and control groups in our study, TNF-α, IL-1β, and IL-6 were found to be statistically significantly higher in the PCOS group. These mediators were independently elevated in patients PCOS when performed in a multivariate analysis. This indicates that inflammation plays an important role in the pathophysiology of PCOS. As it is known, proinflammatory mediators increase the risk of cardiovascular diseases[31].

MDA is an indicator of intracellular and cell membrane damage, and lipid peroxidation[32]. SOD is one of the major antioxidant enzymes that neutralizes free oxygen radicals[33]. They are mediators that show oxidative stress in patients with PCOS[5]. It is stated that insulin resistance, obesity, dyslipidemia, and hyperandrogenism seen in patients with PCOS increase MDA levels and decrease SOD levels[34]. Increased MDA levels are an indicator of lipid oxidation and this is a risk factor for cardiovascular diseases[5]. Studies on SOD levels are conflicting. While studies have shown that it decreases in patients with PCOS, there are also studies indicating that SOD levels increase in response to increased oxidant levels in the circulation[5,35]. Polat and Şimşek[36] reported in their study that Turkish women with PCOS had mutations in the *SOD-1* and *SOD-2* genes and did not have sufficient antioxidant capacity. In our study, MDA was found to be high and SOD to be low in patients with PCOS.

In our study, there was no difference between the two groups in terms of BMI, WHR, fasting glucose, fasting insulin, and lipid levels, while a significant difference was found between Cystatin-C, inflammatory, oxidant, and antioxidant markers. This shows that inflammation and oxidant-antioxidant pathway are affected independently by obesity, metabolic syndrome, and insulin resistance. Additionally, although routine cardiovascular risk factors seem to be normal, high Cystatin-C levels made us think that these mediators may be related. In the correlation analysis performed for this purpose, the increase in Cystatin-C was correlated with the increase in IL-6 and the decrease in the SOD level. Gozashti et al.[10], in their study, no relationship was found between elevated Cystatin-C and inflammatory mechanisms in patients with PCOS. There is no other study in the literature examining this relationship. Clarification of this relationship is also important in terms of treatment. Polat and Şimşek[36] who detected *SOD-1* and *SOD-2* gene mutations in patients with PCOS, suggested adding antioxidant supplementation to the treatment due to decreased antioxidant capacity. When the relationship between IL-6 and SOD and Cystatin-C is evaluated, it may be necessary to add antioxidant supplements and anti-inflammatory agents to the treatment for cardiovascular protection. However, more studies are needed to include them in routine treatment.

Study Limitations

Our study has some limitations. A limitation is that the patient sample is too small and the PCOS cannot be divided into subgroups. Not looking for oxidant-antioxidant markers other than MDA and SOD may be another limitation. In addition, we did not apply antioxidant supplements and anti-inflammatory treatments to these patients. Therefore, we do not have post-treatment results. However, even if BMI, WHR, fasting glucose, fasting insulin, and lipid values are not different from the control group, it is important to show the elevation of Cystatin-C in these patients and to correlate this elevation with IL-6 and SOD.

Conclusion

Our study showed that Cystatin-C levels were high in patients with PCOS, even though there was no difference between the control group and the PCOS groups in terms of other cardiovascular risk factors. It is also the only study showing the relationship between increased Cystatin-C levels and IL-6 and SOD. This result may be effective in the treatment plan of the patients. However, our results should be confirmed with studies conducted with more patients.

Ethics

Ethics Committee Approval: The Yozgat Bozok University Local Ethical Committee approved the present study (2017-KAEK-189_2021.12.29_02).
Informed Consent: Informed consent was obtained from all participants.
Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.Y.G., Concept: Ö.Ö.B., A.Y.G., Design: Ö.Ö.B., Data Collection or Processing: Ö.Ö.B., Analysis or Interpretation: A.Y.G., D.A.K., Literature Search: Ö.Ö.B., A.Y.G., D.A.K., Writing: Ö.Ö.B., D.A.K.
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