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GnRH antagonist protocol with hCG triggering ameliorates fertilization defect caused by failure of cumulus cell *pentraxin-3* expression in unilateral endometriomas

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Abstract. – OBJECTIVE: **The aim of the study was to determine the expression pattern of long *pentraxin 3* (*PTX3*) mRNA in cumulus cells (CCs) isolated from metaphase II oocytes of women with unilateral endometrioma undergoing controlled ovarian stimulation using a gonadotropin-releasing hormone antagonist (GnRHa) protocol.**

PATIENTS AND METHODS: **A total of 60 CC samples, 30 from the affected ovary and 30 from the contralateral ovary, were collected from 12 patients with unilateral endometrioma who underwent flexible GnRHa protocol with recombinant human chorionic gonadotropin (rhCG) trigger. Thirty CC samples collected from the left ovary of 12 women with male factor infertility were used as external controls. Long *PTX3* mRNA expression in each group was analyzed by real-time polymerase chain reaction (RT-PCR). Relative gene expression (fold-change) was calculated according to the $2^{-\Delta\Delta Ct}$ equation. Fertilization rates after intracytoplasmic sperm injection (ICSI) were recorded for each group.**

RESULTS: **CC-*PTX3* mRNA expression in the unilateral endometrioma group was significantly lower than the mRNA expression of the disease-free ovary (0.90±0.01 *vs.* 0.25±0.02, $p<0.01$). CC-*PTX3* mRNA expression of MII oocytes of the disease-free ovary was found to be similar to the control group (1.02±0.03 *vs.* 0.90±0.01, $p=0.107$). A significant 3.6-fold downregulation was observed in the CC-*PTX3* mRNA expression of the endometrioma group compared to the CC-*PTX3* mRNA expression in the contralateral ovary. CC-*PTX3* mRNA expression in the endometrioma group was downregulated 4.08-fold compared to the CC-*PTX3* mRNA expression of the control group (1.02±0.03 *vs.* 0.25±0.02, $p<0.001$). The cumulus morphologies of the endometrioma group with low CC-*PTX3* expression and the groups with normal CC-*PTX3* levels were similar. Fertilization rates of the endometrioma group were similar to the contralateral ovary and control groups.**

CONCLUSIONS: **Unilateral endometrioma reduces CC-*PTX3* expression but does not affect disease-free ovaries. The GnRHa protocol improved the fertilization rates, suggesting that failed CC-*PTX3* expression is an *in vivo* pathology.**

Key Words:
Unilateral endometrioma, GnRH antagonist, Long *PTX3*, qRT-PCR, Fertilization.

Introduction

One in ten women of reproductive age had endometriosis. Ovarian endometriomas are the most common presentation of endometriosis and are found in 20% of endometriosis patients[1-3]. En-

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dometrioma is a condition in which endometrial tissue, histologically and functionally similar to eutopic endometrium, settles in the ovaries and becomes a blood-filled cystic formation[2]. It is an estrogen-dependent inflammatory pathology with different clinical courses, ranging from chronic pelvic pain to infertility. Approximately 80% of endometriomas are unilateral and tend to be located in the left ovary[3,4], significantly affecting follicular morphology and function[5,6]. Due to the progressive nature of the disease, involvement of the healthy ovary also manifests with a gradual decrease in ovarian reserve and subsequent difficulty in conceiving.

The mechanism by which endometriomas negatively affect oocyte quality and quantity is not clear[5-7]. The most accepted view is that it prevents follicular development by disrupting ovarian blood flow and innervation owing to its mechanical effect[6,8]. In addition to its mechanical effect, the chronic inflammatory nature of endometrioma and its toxic iron content can cause early atresia in developing follicles[7,9,10]. The fact that iron levels in follicles close to the endometrioma are higher than those in follicles far from the cyst is evidence that free iron can cause ferroptosis in the oocyte[7]. Disruption of granulosa cell (GC) proliferation and ovarian steroidogenesis in the presence of endometrioma suggests that the negative effect of the cyst on oocytes is mediated by GCs[11]. The fact that endometriomas reduce P450 aromatase, anti-Mullerian hormone (AMH), and progesterone synthesis in GCs supports the idea that the decrease in oocyte quality and quantity occurs through GCs[11].

The somatic cell-derived cells that surround the oocyte in a layer are called cumulus cells (CCs). Nuclear and cytoplasmic maturation of oocytes is achieved by bidirectional communication between CCs and oocyte[12]. Towards the end of folliculogenesis, CCs enter the expansion process, which is a critical stage for ovulation, resumption of meiosis, and fertilization[13]. Cumulus cell expansion occurs through the combined action of several genes that maintain the balance between pro- and anti-inflammatory cytokines[14-16]. Long *pentraxin 3* (*PTX3*), which is regulated by oocyte-derived GDF-9, is one of the key anti-inflammatory genes responsible for cumulus expansion[17,18]. CC-*PTX3* is required for hyaluronan-rich matrix assembly and cumulus expansion[19]. In the presence of endometrioma, deterioration of local and systemic inflammatory balance in favor of inflammation may disrupt the expression of many cumulus genes, including the long *PTX3*, negatively affecting oocyte quality may be negatively affected[16-18]. However, no study has investigated CC-*PTX3* mRNA expression in patients with endometrioma. This study was designed to determine the expression pattern of *PTX3* mRNA in CC isolated from metaphase II (MII) oocytes of patients with unilateral ovarian endometrioma undergoing controlled ovarian stimulation for assisted pregnancy.

Patients and Methods

Sample collection for this cross-sectional study was initiated after approval from the Institutional Ethics Committee (ethical approval number: 2023-10-22) and informed patient consent was obtained. Participants were selected from patients who applied to the Göztepe Medicalpark Hospital *In Vitro* Fertilization Unit. Thirty CC samples were collected from 12 patients scheduled for controlled ovarian stimulation and ICSI due to unilateral unoperated endometrioma. Thirty CC samples obtained from disease-free contralateral ovaries were used as internal controls. Thirty CC samples obtained from the left ovaries of 12 women without ovarian cysts who underwent controlled ovarian stimulation due to male factor infertility were selected as external controls.

Patients in the endometrioma and control groups were matched for age, body mass index (BMI), and infertility duration. Since endometriomas show a left-lateral predisposition, the left ovaries were used as the control group[3]. The diagnosis of endometrioma was made using transvaginal ultrasonography, and its presence was confirmed for at least two cycles. CC-*PTX3* mRNA levels obtained from endometrioma ovaries were compared with CC-*PTX3* mRNA expression in disease-free contralateral ovaries and in external control patients. Patients with a history of systemic inflammatory disease, bilateral endometrioma, ovarian surgery, and use of hormonal or anti-inflammatory drugs for the last 3 months were excluded from the study. Patients with insufficient CC were excluded from the study. Patients with insufficient CCs were excluded from the study. Patients with azoospermia were not included in the control group because it was planned to compare fertilization rates between the groups. In the baseline evaluation on the 3rd day of the cycle, luteinizing hormone (LH), follicle-stimulating hormone (FSH), anti-mullerian hormone (AMH) levels, and antral follicle count (AFC) of the par-

ticipants in both groups were recorded. The diameter of the endometrioma was measured during transvaginal examination.

A flexible GnRHa protocol for controlled ovarian stimulation was applied in both groups. Detailed information regarding the antagonist protocol can be found elsewhere[20]. Briefly, after starting recombinant follicle-stimulating hormone (rFSH) treatment on the 3rd day of the cycle, patients whose follicle diameter reached ≥14 mm were administered gonadotropin-releasing hormone antagonist (GnRHa) for pituitary suppression. Ovulation was triggered by recombinant human chorionic gonadotropin (rhCG) treatment. Cumulus oocyte complexes (COC) collected under ultrasound guidance 36 h after the ovulation trigger were subjected to cumulus isolation. We evaluated cumulus cell morphology during COC grading before denudation in all the groups. Thus, we had the opportunity to retrospectively compare the changes in CC morphology between groups with different *PTX3* expression patterns.

Before being mechanically separated from COCs into CCs, it was subjected to COC classification: COC grade 1 indicates first polar body (+) mature oocytes (MII), COC grade 2 indicates first polar body (-) (MI) oocytes, and COC grade 3 indicates germinal vesicle (GV) oocytes. MII CCs were used for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in both groups. CCs, mechanically peeled with 290 µ diameter pipettes, were transferred to RNA-later-containing tubes and stored until RNA extraction. CCs isolated from MII oocytes were placed in individual vials. CCs of MII oocytes obtained from unilateral endometrioma ovaries were labeled CCMII-endm (+). CCs of MII oocytes collected from disease-free contralateral ovaries were labeled as CCMII-endm (-). CCs of MII oocytes collected from the left ovary of women in the control group were labeled as CCMII-con (Table I). The total number of CCs isolated from MII oocytes in all three groups and selected for Real-Time Polymerase Chain Reaction was 90. All MII oocytes were subjected to intracytoplasmic sperm injection (ICSI). The relationship between CC-*PTX3* expression and the fertilization rate was recorded.

The cumulus cell morphology of each group was evaluated before denudation. In the morphological evaluation, cytoplasmic brightness (marked bright cytoplasm was considered grade 1, moderate brightness was considered grade 2, and loss of brightness was considered grade 3), number of cumulus cell layers (if the number of layers was <5, it was considered grade 1; if it was 5-9, it was considered grade 2; and if it was >9, it was considered grade 3), cumulus cell loss (grade 1 if CC loss <50%, grade 2 if CC loss was 50-75%, grade 3 if CC >75%), and corona radiata polarization (normal corona radiata polarization was considered grade 1, and loss of polarization

Table I. Comparison of demographic laboratory characteristics between the unilateral endometrioma and control groups.

Variables	Unilateral endometrioma	Disease free ovary	Control group	*p*-values
Age (years)	27.5±2.96		26.67±3.68	0.468[a]
BMI (kg/m²)	23.19±1.64		22.88±1.40	0.586[a]
Infertility duration (yrs)	3 (2.5-4)		3 (2-4)	0.398[b]
Antral follicle count (AFC)	3 (2-4)	3 (2.5-5)	3 (3-4) (left ovary)	0.742[c]
LH (mIU/mL)	5.5 (5.4-6.6)		5.6 (4.4-6.1)	0.311[b]
FSH (mIU/mL)	5.7±1.31		5.2±0.87	0.235[a]
AMH (ng/mL)	2 (1.4-3.1)		2.3 (2.1-3.15)	0.128[b]
Endometrioma size (mm)	42.4 (31.5-50)			
Endometrial thickness (mm)	9.2 (8.8-9.5)		9.5 (9-10)	0.062[b]
Ovarian stimulation	GnRH antagonist+rhCG		GnRH antagonist+rhCG	
MII oocyte-CC	30	30	30	
Fertilization rate	66.6%	70%	66.6%	1.00[d]

Data are given as mean ± standard deviation or median (1st quartile - 3rd quartile) for continuous variables. [a]Student *t*-test, [b]Mann-Whitney U test, [c]Kruskall-Wallis test, [d]Chi-square test. BMI, body mass index; LH, luteinizing hormone; FSH, follicle-stimulating hormone; AMH, anti-mullerian hormone; MII, mature oocytes; GnRH, gonadotropin-releasing hormone; rhCG, recombinant human chorionic gonadotropin.

was considered grade 2) were considered. Thus, we had the opportunity to analyze the changes in the CC morphologies of the groups with normal and defective CC-*PTX3* mRNA expression.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Long *PTX3* mRNA expression in each group of participants was analyzed using qRT-PCR. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as a reference gene to examine mRNA expression. Total RNA isolation from cumulus cells was performed using the PureLink Total RNA Mini Kit (Invitrogen, Waltham, MA, USA), and its concentration was measured with a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA was reverse transcribed using the High-Capacity cDNA Kit (Applied Biosystems, Foster City, CA, USA). Blirt Amplyfyme SYBR Green Master Mix (Cat No.: AM-02-200; Gdańsk, Poland) was used for Real-Time PCR. The sequences of primers used for qRT-PCR were as follows: *PTX3:* Forward 5'-TGGACAACGAAATAG ACATGG-3,' reverse 5'-CTCTCATCTGCGAGTTCTCC-3,' GAPDH: forward 5'-GAAGATGGTGATGGGATTTC-3,' reverse 5'-GAAGGTGAAGGTCGGAGTC-3.'

Statistical Analysis

IBM SPSS Statistics Version 22.0 for Windows (IBM Corp., Armonk, NY, USA) was used for data analysis. Variables with normal distribution were analyzed using an independent sample *t*-test, and non-normal distributions were analyzed using the Mann-Whitney U test or the Kruskal-Wallis test. The Chi-square test was used to compare categorical data, which were expressed as percentages. Continuous variables are given as mean±standard deviation or median (1^{st} quartile-3^{rd} quartile); two-tailed *p*-values of $p<0.05$ were considered significant. Relative gene expression (fold-change) was calculated according to the $2^{-\Delta\Delta Ct}$ equation. Statistical differences in CC-*PTX-3* expression were analyzed by one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Demographic characteristics of all three groups are presented in Table I. The mean age, BMI, infertility duration, AFC, and serum FSH, LH, and AMH levels were similar in both groups. The mean endometrial thickness on ovulation trigger day was similar in both groups. There was no difference between the two groups in terms of the total gonadotropin dose consumed, total number of oocytes collected, and serum estradiol levels on the day ovulation was triggered.

The number of MII oocyte cumulus samples subjected to qRT-PCR was 90, including 30 in the endometrioma group, 30 in the contralateral disease-free ovary, and 30 in the control group. qRT-PCR was not performed on the CCs of MI and GV oocytes. The median endometrioma diameter was recorded as 42.4 mm (31.5-50). CC-*PTX3* mRNA expression in MII oocytes of the disease-free contralateral ovary was found to be similar to that in the control group (1.02±0.03 *vs.* 0.90±0.01, $p=0.107$). CC-*PTX3* mRNA expression in MII oocytes obtained from ovaries with unilateral endometrioma was significantly lower than the mRNA expression of the disease-free contralateral ovary (0.90±0.01 *vs.* 0.25±0.02, $p<0.01$). A significant 3.6-fold downregulation was observed in the CC-*PTX3* mRNA expression of the endometrioma group when compared to the CC-*PTX3* mRNA expression in the contralateral ovary (Figure 1). CC-*PTX3* mRNA expression of MII oocytes collected from the endometrioma ovary was downregulated 4.08-fold compared to the CC-*PTX3* mRNA expression of the control group (1.02±0.03 *vs.* 0.25±0.02, $p<0.001$).

In the predenudation microscopic examination of MII oocytes with low CC-*PTX3* expression, we did not observe any significant morphological changes compared to the MII oocytes of the contralateral ovary and the control group with normal *PTX3* expression. CCs with low or normal *PTX3* levels had a bright cytoplasm, a similar number of cell layers, and polarized corona radiata. Low CC-*PTX3* levels did not lead to either loss of transparency or partial or complete loss of CCs. The two-pronuclear zygote (2PN) rates of low *PTX3* expressing endometrioma patients were similar to the 2PN zygote rates of the contralateral ovary and control groups. No difference was found between the two groups in terms of the 2PN zygote rate.

Discussion

Although endometriomas do not cause significant changes in clinical pregnancy and live birth rates compared to healthy controls, they adversely

affect both the total number of oocytes and embryo quality[6]. However, the results of studies conducted so far are unclear about the mechanisms by which endometriomas negatively affect oocyte development. In the last decade, the view that endometriomas negatively affect both oocyte and embryo development by reducing cumulus cell proliferation, progesterone synthesis, and aromatase activity has gained weight[5,11]. For all these reasons, the negative effects of endometriomas on oocyte developmental competence have been tried to be proven by studies using cumulus cell sample[5,21,22]. The number of studies investigating the changes caused by endometriomas in oocyte morphology or metabolic pathways is quite low[5,23]. If the functions of CCs are truly impaired in the presence of endometrioma, the expression of critical genes responsible for cumulus expansion should also be impaired.

In the current study, we analyzed for the first time the mRNA expression of long *PTX3*, one of the main genes responsible for cumulus expansion[22], in the CC of MII oocytes of unilateral endometrioma patients and compared it with the expression levels in contralateral disease-free ovaries. We also compared CC-*PTX3* mRNA expression in the endometrioma group with the mRNA expression of healthy controls who underwent IVF/ICSI due to male factor infertility. When we compared it with CC-*PTX3* expression in the contralateral group, we found that *PTX3* expression was 3.6-fold lower in ovaries with unilateral endometriomas. Since the fold change in PTX3 mRNA was >2, we concluded that the CC-*PTX3* expression pattern in endometrioma ovaries was defective compared to that in disease-free ovaries. The 4.08-fold decrease in CC-*PTX3* mRNA expression in the endometrioma group compared to that in the healthy control group strongly supports that endometriomas may impair oocyte development quality by causing cumulus expansion defects. The fact that CC-*PTX3* mRNA expression in the disease-free contralateral ovary and the expression values of the control group were similar suggests that unilateral endometriomas did not negatively affect the cumulus cell functions of the contralateral ovary.

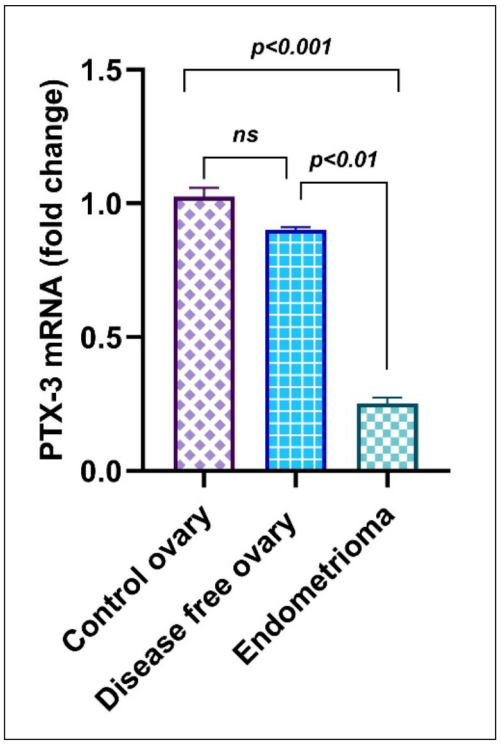

Figure 1. Graphical representation of the fold change in CC-*PTX3* mRNA expression in MII oocytes of endometrioma, disease-free contralateral ovary, and left ovary of the control group. CC-*PTX3* mRNA expression in the unilateral endometrioma group was approximately four times lower than the expression level of both the contralateral ovary and the ovary of the control group (data are expressed as the mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison test). ns., not significant.

Since *PTX3,* which is only one of the cumulus expansion genes, is not expressed sufficiently in the presence of endometrioma, it may not be correct to discuss the existence of cumulus expansion defects. Since cumulus expansion is controlled by many oocyte- and cumulus cell-derived genes, other genes may compensate for the *PTX3* expression defect[24]. If the levels of CC-*PTX3* associated with MII oocytes of patients with unilateral endometrioma are physiologically low, the degree of cumulus cell expansion in the MII oocytes of the endometrioma group will be reduced. To test this, we evaluated the images of each COC during pre-denudation grading. In the unilateral endometrioma group with *PTX3* expression deficiency, CCs did not regularly surround centrally located oocytes. Instead, CCs form a more uniform and unstable mass. In the endometrioma group, the brightness of the CCs and the number of layers formed by these cells were similar to those in the groups with normal *PTX3* expression. In addition, the absence of partial or complete loss of CCs suggests that *PTX3* deficiency does not cause significant changes in COC morphology. The decrease in the COC diameter compared to that in the control group was not large enough to be interpreted as compaction. Morphological evaluation of CCs during grading before COC denudation may provide functional

data to confirm changes in *PTX3* mRNA expression. Although *PTX3* deficiency leads to loss of polarization in the corona radiata in mice, we did not observe loss of polarization in corona radiata despite defective *PTX3* expression in the endometrioma group. This difference may be due to differences in certain functions of *PTX3* in humans and mice[25] or may depend on the severity of the decrease in *PTX3* expression in the presence of endometrioma. In mouse studies, cumulus expansion defects and loss of polarization in the corona radiata have been noted in *Ptx3-/-* mice[19]. However, in patients with endometrioma, *PTX3* expression is reduced compared to that in controls, and there is no complete absence of *PTX3* expression. CCs may expand in the presence of low *PTX3* levels, or other genes responsible for cumulus expansion may compensate for *PTX3* deficiency.

Although it is known that CC-*PTX3* is involved in the mechanism associated with cumulus expansion in humans and is also a potential biomarker for oocyte quality, there are not enough studies investigating CC-*PTX3* levels in infertile populations. Ouandaogo et al[26] reported that *PTX3* expression was downregulated in CCs obtained from the oocytes of PCOS patients at different stages of nuclear maturation. It is thought that low-grade chronic inflammation in PCOS disrupts the redox balance of the follicle, causing defects in CC-*PTX3* expression and expansion[18,26]. *PTX3* expression increases in response to local inflammation and exerts a protective effect against inflammation by regulating apoptotic cell loss[27]. An increase in oocyte apoptosis, follicle atresia, and inflammatory cytokine production in the affected ovary compared to the unaffected ovary is critical evidence that endometriomas can disrupt cumulus granulosa cell function[8,9,11]. Increased local and systemic inflammation in the presence of endometrioma may restrict the role of *PTX3* in cumulus matrix stability[28]. However, despite the CC-*PTX3* expression defect in MII oocytes of patients with endometrioma, *PTX3* expression in the disease-free contralateral ovary was normal, suggesting that local inflammation rather than systemic inflammation is the determinant of *PTX3* expression.

CCs are an important indicator of oocyte development, as they participate in the production and transfer of factors that ensure nuclear and cytoplasmic maturation of the oocyte[29]. The fertilization and implantation potential of oocytes with inadequate or defective cumulus expansion is limited[14,22]. Thus, the fertilization rates of patients with unilateral endometrioma expressing low *PTX3* levels would be expected to be lower than that of the control group. However, the fact that the ICSI outcomes of patients with endometriomas were similar to those of the contralateral ovary and the control group led us to question the role of CC-*PTX3* in fertilization. The answer to this question was clarified in an experimental study conducted by Salustri et al[19]. The authors reported that although oocytes obtained from *Ptx3-/-* mice were fertilized *in vitro*, *in vivo* fertilization did not occur. Supporting these experimental results, the ICSI results of patients in the endometrioma group were similar to those of the healthy controls, suggesting that low CC-*PTX3* expression prevents fertilization *in vivo*, whereas ICSI overcomes this problem. In agreement with this, in a recent study[30], our team showed that CC-*PTX3* expression in PCOS patients varies according to the nuclear maturation stage and that ICSI overcomes CC-*PTX3* expression defects. Salustri et al[19] and our previous study[30] are critical in showing that oocyte development is normal in humans and mice, despite the CC-*PTX3* expression defect. Our study is unique as it shows that despite low CC-*PTX3* expression, MII oocytes from endometrioma patients are fertilized by ICSI at a rate similar to that in healthy individuals. However, the grading we used to evaluate CC morphology is an important limitation, as it is a system developed by us and has not been agreed upon by everyone.

Conclusions

Long *PTX3 i*s just one of the essential genes responsible for cumulus expansion. Low CC-*PTX3 mRNA* expression in women with unilateral endometrioma may lead to unexplained infertility without ovulatory dysfunction. Therefore, ICSI should be the first treatment option in women with endometrioma because of the risk of insufficient CC-*PTX3* expression, as it provides fertilization rates similar to those of healthy controls.

Conflict of Interest

The authors declare no competing financial or non-financial interests.

Authors' Contributions

NDG, OC, AE, and MK conceptualized, administered the project, and wrote the manuscript; SM performed cumulus cell isolation; NC and MY biochemical, hormonal analysis, statistical analysis, and graphic design; SD performed RT-PCR anal-

ysis, review, and editing paper; KC, SC, and RFA, entry and storage of the obtained data, and collection of relevant literature. All authors have read and approved the final manuscript.

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Availability of Data and Materials
All datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval
This study was approved by the Ethics Committee of Fırat University (ethics approval number: 2023-10-22, date: 27-07-2023). Strict compliance with the standards of the Declaration of Helsinki was ensured throughout the study.

Informed Consent
All participants included in the study provided informed consent prior to their involvement in the study.

AI Disclosure
No artificial intelligence or assisted technologies were used in this study.

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