



## Expression Analysis of the Small GTP-Binding Protein Rac in Pterygium

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### Abstract

**Objectives:** To determine the roles of small GTP-binding proteins Rac1, Rac2, and Rac3 expression in pterygial tissue and to compare these expressions with normal conjunctival tissue.

**Materials and Methods:** Seventy-eight patients with primary pterygium were enrolled. Healthy conjunctival graft specimens obtained during pterygium surgery were used as control tissue. The real-time polymerase chain reaction method on the BioMark HD dynamic array system was utilized in genomic mRNA for the gene expression analysis. Protein expressions were analyzed using western blot and immunohistochemical methods.

**Results:** *RAC1*, *RAC2*, and *RAC3* gene expressions in pterygial tissues were not markedly elevated when compared to the control specimens ( $p>0.05$ ). As a very low level of *RAC1* gene expression was observed, further protein expression analysis was performed for the Rac2 and Rac3 proteins. Western blot and immunohistochemical analysis of Rac2 and Rac3 protein expression revealed no significant differences between pterygial and healthy tissues ( $p>0.05$ ).

**Conclusion:** This is the first study to identify the contribution of Rac proteins in pterygium. Our results indicate that the small GTP-binding protein Rac may not be involved in pterygium pathogenesis.

**Keywords:** Pterygium, Rac, gene expression, protein expression, immunohistochemical analysis

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### Introduction

Pterygium is a benign and common ocular surface disease characterized by abnormal conjunctival fibrovascular tissue growth on the cornea. Pathologically, pterygium is a proliferative, invasive, and highly vascularized tissue. It is generally accepted that pterygium is a conjunctival degenerative and proliferative disorder. Due to the presence of various common features between pterygium and neoplasia, pterygium is also considered as a neoplastic-like growth lesion.<sup>1</sup> Many environmental factors such as inflammation, ultraviolet irradiation, and chronic irritation have been postulated to be causative factors.<sup>2</sup> Genetic factors are also important in the etiology of pterygium.<sup>3</sup> However, the exact molecular mechanisms of pterygium development are not fully understood. Accumulating evidence indicates that many growth factors may contribute directly or indirectly to the pathogenesis of pterygium.<sup>4</sup> Some studies have reported that cyclo-oxygenase (COX), vascular endothelial growth factor (VEGF), and various proinflammatory cytokines are associated with the development and formation of pterygium.<sup>5,6,7</sup>

Low molecular weight (20-30 kDa) small GTPases are monomeric G-proteins that bind guanine nucleotides and hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate. The human Ras superfamily of small GTPases consists of 166 members, which is subdivided into five major subfamilies (Rho, Ras, Arf/Sar, Rab, Ran) and "unclassified" proteins based on their functional and sequence similarities.<sup>8</sup> The Rho family of small GTPases contains 20 members including Rac1, Rac2, and Rac3. The three distinct mammalian Rac isoforms, mapped by different genes, share between 89% and 92% identity in their respective amino acid sequences.<sup>9</sup> It has been reported that Rho GTPase activates proteins involved in VEGF-induced cell migration and that VEGF signaling



requires Rac activation during chemotaxis.<sup>10,11</sup> Rac activation also induces an increase in endothelial cell focal adhesion and stress fiber formation.<sup>11</sup> Rac activity is crucial for efficient cell movement and migration.<sup>12,13</sup> These effects may contribute to the development of the wing-like or triangular-shaped tissue growth of the conjunctiva tissue in pterygium. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme present in non-phagocytic and phagocytic cells is a Rac-regulated complex that generates reactive oxygen species (ROS) for the purposes of intracellular signaling and innate immunity.<sup>14</sup> Since Rac proteins can regulate the oxidative burst in phagocytic cells and are involved in ROS production, Rac may contribute to the inflammatory processes of pterygium development.<sup>15,16</sup> Although Rac family members may have several roles in certain cellular functions, their roles in pterygium pathophysiology remain uncharacterized. Therefore, the goal of this research was to assess the expression of the small GTP-binding proteins Rac1, Rac2, and Rac3 in pterygial tissue.

## Materials and Methods

### Participants

This prospective study was performed in the Ophthalmology Department of Gaziantep University Hospital and the Ophthalmology Clinic of the Gaziantep Dr. Ersin Arslan Training and Research Hospital. The study was approved by the Gaziantep University Local Clinical Ethics Committee (decision no: 2017/312, date: 11.09.2017), and the tenets of the Declaration of Helsinki were adhered to throughout this study. Pterygium specimens were collected during surgery from 78 consecutive patients with primary pterygium (40 male and 38 female). Normal conjunctival tissue samples from the superior temporal bulbar conjunctiva were taken during pterygium excision surgery with conjunctival autograft transfer.<sup>17</sup> Each patient underwent routine eye examinations. Inclusion criteria were as follows: (1) age 18 years or above; (2) presence of primary grade 2 or 3 pterygium; and (3) enrolling in the study voluntarily and providing informed written consent.

Exclusion criteria were as follows: (1) history of any previous ocular surgery such as pterygium excision, vitrectomy, trabeculectomy, cataract extraction, and squint surgery; (2) history of trauma such as chemical injury or conjunctival laceration within the last three months; (3) presence of other conjunctival pathology or corneal pathology; (4) presence of other ocular surface disease such as Sjogren syndrome; (5) presence of infection such as conjunctivitis; (6) history of topical medication use such as immunosuppressants, mitomycin C, or corticosteroids; and (7) current or previous contact lens use.

### Gene Expression Analysis

Total RNA was isolated from the tissues using the miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's instructions. The purity and concentration of RNA were measured by spectrophotometrically (Epoch,

BioTek, Winooski, VT, USA). cDNA synthesis from RNA was performed using the Ipsogen RT Kit (Qiagen, Hilden, Germany) and the protocol recommended by the manufacturer. Polymerase chain reaction measurements with RAC primers were done using the BioMark HD system (Fluidigm, South San Francisco, CA, USA). Expression of each gene was measured and messenger RNA (mRNA) expression was determined.  $\beta$ -actin (*ACTB*) was used as a housekeeping gene for internal control. Relative mRNA levels were quantified using the  $2^{-\Delta\Delta C_t}$  method, according to the formula:  $\Delta\Delta C_t = \Delta C_{tRAC} - \Delta C_{tACTB}$ , where  $C_t$  = threshold cycle.<sup>17</sup>

### Western Blot Analysis

Frozen tissue samples were homogenized in HEPES buffer using a tissue homogenizer (Tissue Lyser LT, Qiagen, Hilden, Germany), and stored at  $-80^\circ\text{C}$  until analysis. Protein concentrations were determined using the Bradford method (Thermo Fisher Scientific, IL, USA). The protein samples were incubated with a sample buffer (5  $\mu\text{L}$ ) and HEPES buffer and heated for 5 min at  $95^\circ\text{C}$ . Then, 20  $\mu\text{g}$  of proteins from the tissue specimens were run in 10% sodium dodecyl sulfate polyacrylamide gels and electroblotted onto polyvinylidene difluoride (PVDF) membranes at  $4^\circ\text{C}$  overnight. After proper blocking with non-fat dry milk and washing, the membranes were treated overnight with primary antibodies to Rac2 (sc-517424, SantaCruz Biotechnology, Dallas, TX, USA, 1/300) or Rac3 (ab124943, Abcam, Cambridge, UK, 1/1000) at  $4^\circ\text{C}$ .  $\beta$ -actin (sc-47778, SantaCruz Biotechnology, Dallas, TX, USA, 1/1000) antibody was utilized as a loading control. Then secondary antibodies were incubated with the PVDF membranes for 90 minutes at room temperature (anti-mouse immunoglobulin G [IgG] for anti-Rac2, sc-516102, SantaCruz Biotechnology, Dallas, TX, USA, 1/1000, or goat anti-rabbit horseradish peroxidase for anti-Rac3, ab6721, Abcam, UK, 1/3000). The antibody-reactive bands were visualized using enhanced chemiluminescence signals (Super Signal West Pico, cat. no. 34080, Thermo Fisher Scientific, IL, USA). The densities of the bands were recorded using a gel image analysis system (ChemIDoc XRS+ Imager, Bio-Rad, Hercules, CA, USA), then normalized according to  $\beta$ -actin levels.<sup>17</sup>

### Immunohistochemical Analysis

To perform immunohistochemical studies, formalin-fixed and paraffin-embedded tissue blocks were sectioned into 5- $\mu\text{m}$  slides using a microtome. Rac2 (PA5-29281, polyclonal rabbit IgG, Thermo Fisher Scientific, Rockford, USA, 1/100) and Rac3 (EPR6679B, rabbit monoclonal anti-Rac3 antibody, Abcam, Cambridge, USA, 1/100) antibodies were applied using an automated immunohistochemistry-staining device (Ventana, Bench Mark Ultra Auto-Stainer, Roche Diagnostics, IN, USA). Intensity of Rac2 and Rac3 immunoreactivities were scored on a 0-3 rating scale. A single researcher (Ö.E.) scored all samples for consistency. Intensity of staining was graded as follows: 0, <10% and accepted as negative; 1+, 11 to 20%; 2+, 21 to 75%; 3+, >75%.<sup>17</sup>

### Statistical Analysis

Data were presented as mean  $\pm$  standard error of the mean or percentage. The Kolmogorov-Smirnov test was applied to assess for data normality. The unpaired Student's t-test or Mann-Whitney U test was utilized to compare the means of two groups as appropriate. QIAGEN GeneGlobe online program (<http://www.qiagen.com/geneglobe>) was used for gene expression analysis. All results were presented as fold changes, with values between 0.001 and 0.5 considered significant downregulation and values above 2.0 considered significant upregulation.<sup>18</sup> The Mann-Whitney U test was utilized to identify marked differences between immunohistochemical scores. Correlations were determined using the Spearman rank correlation test. Statistics were performed using GraphPad InStat (version 3.05, GraphPad Software Inc., San Diego, CA, USA). Differences with a p value less than 0.05 were considered statistically significant.

### Results

Among the 78 pterygium patients, there were 40 men (51.3%) and 38 women (48.7%), demonstrating approximately equal gender distribution. The mean age of the patients was  $52.4 \pm 1.4$  years (range, 29-72 years). There were no marked differences in *RAC1*, *RAC2*, and *RAC3* gene expressions in pterygial tissues when compared to the controls (n=30, [Figure 1](#), p values were 0.819, 0.326, and 0.112 for *RAC1*, *RAC2*, and *RAC3*, respectively). Since a very low level of *RAC1* expression was observed, further analyses were performed with the Rac2 and Rac3 proteins. In the western blot analysis, there were no marked differences in Rac2 and Rac3 protein expression in pterygial tissues when compared to controls (n=30, p values were 0.330 and 0.309 for Rac2 and Rac3, respectively, [Figure 2](#)).

The immunohistochemical data revealed weak staining for Rac2 ([Figure 3A, B](#)) and Rac3 ([Figure 3C, D](#)) mainly localized to the pterygium epithelial and capillary endothelial cells. Almost no stromal cell staining was observed in pterygial tissue. However, these cells were also stained with the Rac2 or Rac3

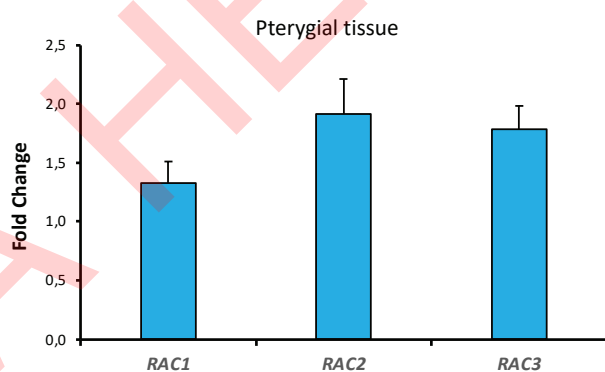
antibodies in the control samples ([Figure 3](#)). Although there was a trend for Rac2 or Rac3 upregulation in pterygial tissue, these increases did not show statistical significance (p values were 0.2113 and 0.2524 for Rac2 and Rac3, respectively, [Figure 4](#)).

Correlation analysis revealed positive correlation between *RAC1* and *RAC2* gene expressions ( $r=0.606$ ,  $r^2=0.367$ ,  $p<0.001$ ) and between *RAC2* and *RAC3* gene expressions ( $r=0.367$ ,  $r^2=0.135$ ,  $p=0.046$ ). No significant correlations were detected between gene and protein expressions ( $p=0.239$  for Rac2,  $p=0.609$  for Rac3). There was also no marked correlation between Rac2 and Rac3 protein expressions ( $r^2=0.012$ ,  $p=0.531$ ).

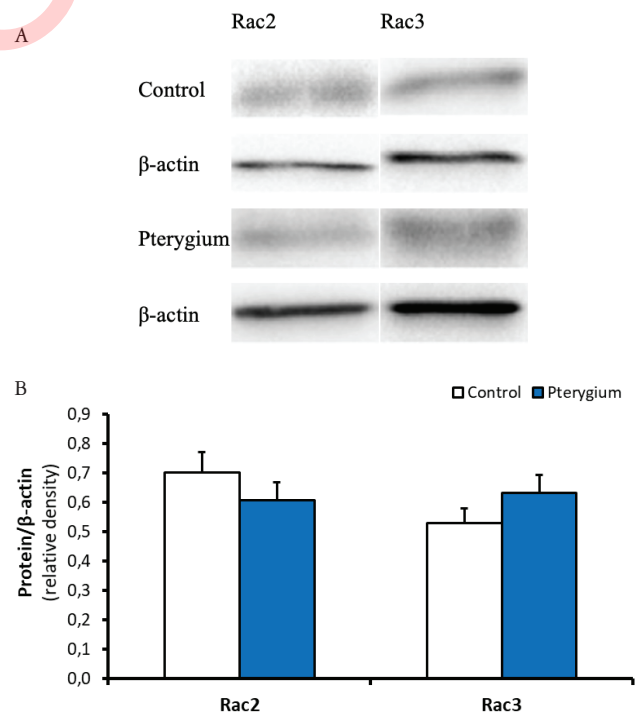
### Discussion

In the present study, we observed that *RAC1*, *RAC2*, and *RAC3* gene expression was not markedly modified in primary pterygium specimens when compared to normal conjunctival tissues. Additionally, no elevation in protein expression was observed. These findings suggests that gene expression and posttranslational Rac protein formation are not involved in pterygium development or the regulation of pterygium growth across the ocular surface. Although several studies have reported that cellular immunity and inflammatory response play a crucial role in pterygium formation,<sup>19,20</sup> our results do not support the idea that Rac proteins participate in the inflammatory process of pterygium development.

Rac activity is essential for cell movement.<sup>12</sup> Rac is generally accepted to be a regulator of initial cell-cell contact,

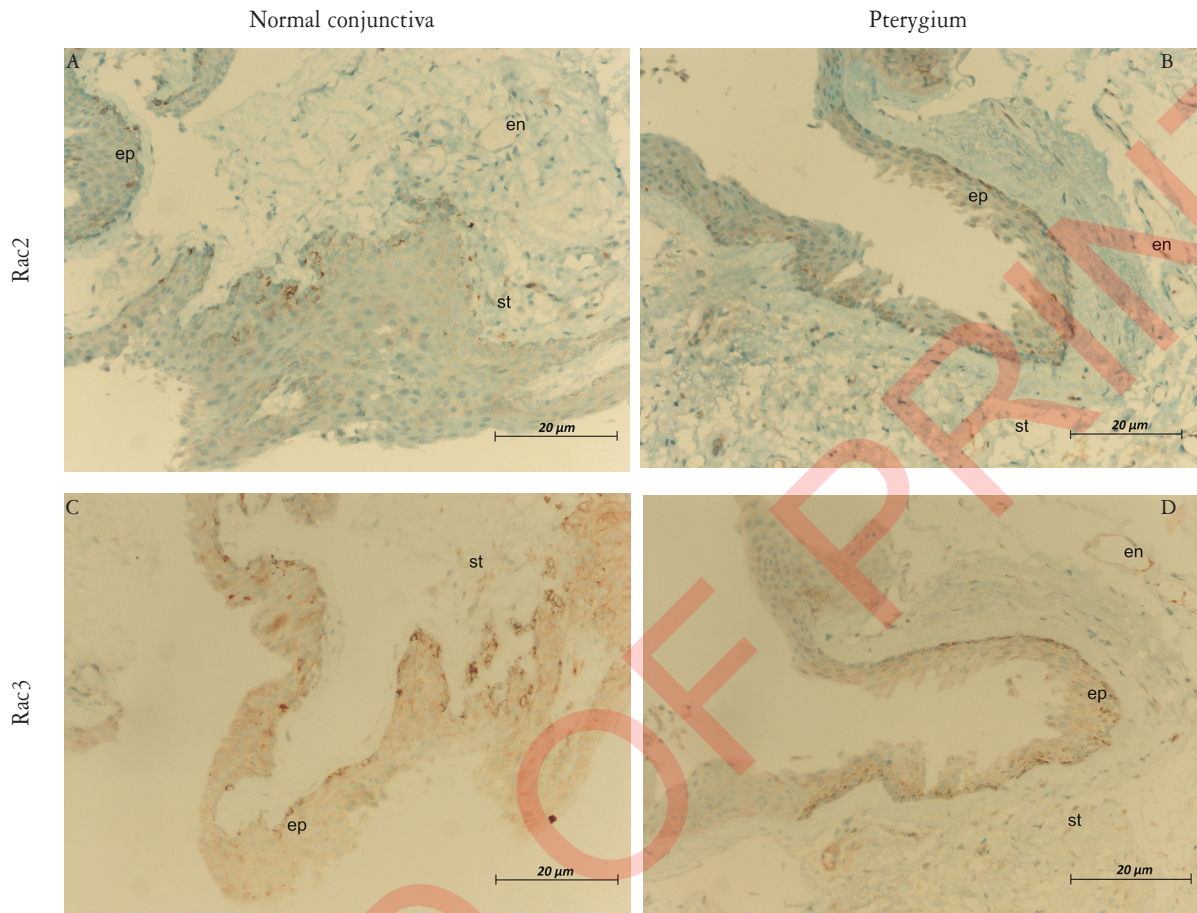


**Figure 1.** Comparison of the *RAC1*, *RAC2*, and *RAC3* gene expressions in pterygial tissues (n=30). Values are given as mean  $\pm$  SEM. SEM: Standard error of the mean

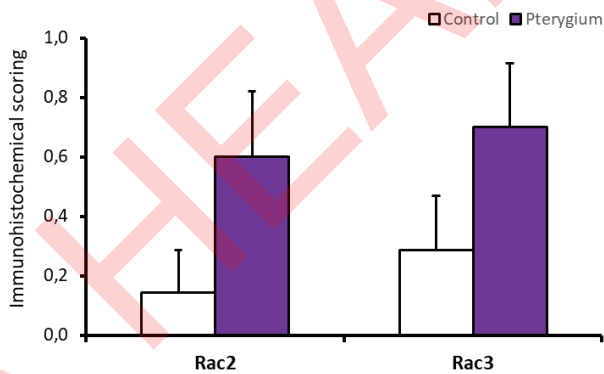


**Figure 2.** Representative western blotting bands (A) and comparison of Rac2 and Rac3 protein expression (B) in conjunctival autograft (control, n=36) and pterygial tissues (n=38). Values are given as mean  $\pm$  SEM. SEM: Standard error of the mean





**Figure 3.** Immunohistochemical images of Rac2 and Rac3 staining. Immunostaining of Rac2 (top) and Rac3 (bottom) in human normal conjunctiva (left) and pterygium (right)  
*ep: Epithelium, en: Endothelium, st: Ströma*



**Figure 4.** Comparison of the immunohistochemical scores for Rac2 and Rac3 protein expression in conjunctival autograft (control, n=7) and pterygial tissues (n=10). Values are given as mean ± SEM

*SEM: Standard error of the mean*

cell-matrix adhesions, and cellular transformation.<sup>21,22,23</sup> Rac is known to stimulate lamellipodia and membrane ruffles in fibroblasts, and Rac signaling is essential for efficient

cell migration.<sup>13</sup> It is known that fibroblasts involved in the scarring and fibrosis processes may contribute to the progression of pterygium.<sup>24</sup> Rac is thought to modulate the development of lamellipodial extensions in epithelial cells and facilitate interactions between adjacent cells.<sup>21,22</sup> Rac GTPases are also important to the maintenance or establishment of polarity in chemotactic migration.<sup>25</sup> Thus, Rac might control cell polarization and migration.<sup>12</sup> Rac is required for lamellipodium extension triggered by cytokines, extracellular matrix components, and growth factors.<sup>12</sup> All these features of Rac could contribute to the wing-shaped growth of conjunctival tissue onto the cornea seen in pterygium.

Rac1, the best-investigated Rac isoform, modulates gene expression, intercellular adhesion, cell cycle progression, the organization of the actin cytoskeleton to aid cell spreading and membrane ruffling.<sup>26</sup> The expression of Rac proteins differs considerably in terms of level and tissue distribution. Rac1 and Rac3 are ubiquitously expressed in different tissues and therefore modulate a wide variety

of cellular functions, whereas Rac2 is largely expressed in the hematopoietic cells.<sup>27,28,29</sup> Further, Rac2 is required for phagocytosis in macrophages.<sup>30</sup> Rac2 appears to specifically regulate chemotaxis, cellular differentiation, proliferation, actin remodeling, and generation of superoxide and kinase activation in neutrophils.<sup>29,31,32</sup> Rac2 plays a significant role in COX2 expression in macrophages,<sup>33</sup> and COX2 expression is also associated with the pathogenesis of pterygium.<sup>5</sup> Rac1 and Rac2 act as a regulatory component of the superoxide-producing NADPH oxidase and regulate the oxidative burst in phagocytic cells.<sup>15</sup> Rac directly contributes to ROS production.<sup>16</sup> There is evidence that ICAM-2 and ICAM-3 gene and protein expressions were significantly upregulated in pterygial tissues,<sup>17</sup> and ICAM-2 can regulate N-cadherin localization and vascular permeability through Rac1 signalling.<sup>34</sup> However, our findings in the present study showed that *RAC1* gene expression was not markedly modified in pterygial tissues.

Our data demonstrated that the *RAC2* gene and Rac2 protein were expressed in pterygial tissue, indicating that Rac2 is not restricted to hematopoietic cells. In support of this view, two reports describe Rac2 expression in vascular smooth muscle cells (VSMC).<sup>35,36</sup> Although Rac2 expression is undetectable under quiescent or normal conditions, its expression is stimulated upon induction of VSMC with inflammatory cytokines. Overexpression of Rac2 significantly increases VSMC migration and intracellular superoxide production.<sup>36</sup> It has been shown that tumor necrosis factor- $\alpha$  and transforming growth factor- $\beta$  are able to increase Rac2 expression in VSMC.<sup>36</sup> These growth factors are also present in the pterygial tissue.<sup>4</sup> Rac2 is expressed in endothelial cells and is also a requisite signaling component for endothelial cell migration.<sup>37</sup> Rac2 is also present in human bronchial epithelial cells and upregulation of Rac2 leads to increased NADPH oxidase activity and increased intracellular ROS generation.<sup>38</sup> Moreover, Rac2 is expressed in tumor cells. Although reduced expression of Rac2 was reported in malignant brain tumors, its overexpression was demonstrated in head and neck squamous cell carcinoma.<sup>39,40</sup> Pterygium can display tumor-like features and has been proposed to be a neoplastic-like growth disorder.<sup>41</sup>

Rac3 is the least studied Rac isoform. Our findings implicate for the first time that Rac3 expression is detectable in pterygial tissue. Although Rac3 may be involved in the stress activation pathway and tumor growth, its contribution to the pathogenesis of pterygium is currently unknown.<sup>9,42</sup>

#### Study Limitations

The main limitation of our study is the small sample size. Further studies with larger sample sizes may help shed more light on the cellular characteristics of this disease.

#### Conclusion

This study is the first to demonstrate gene expression of the small GTP-binding proteins Rac1, Rac2, and Rac3 in pterygium. However, gene and protein expressions were not modified compared to normal conjunctival tissue, suggesting that Rac proteins may not play a role in pterygium development. The

signaling pathways involved in Rac-mediated functions remain unknown in pterygium, and clarification of the participation of each of these proteins in pterygium requires further study.

#### Ethics

**Ethics Committee Approval:** The study was approved by the Gaziantep University Local Clinical Ethics Committee (decision no: 2017/312, date: 11.09.2017).

**Informed Consent:** Obtained.

**Peer-review:** Externally and internally peer-reviewed.

#### Authorship Contributions

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

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

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