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Investigation of the role of MAPK pathway genes in POP surgical complications

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ABSTRACT

Objectives: We hypothesized that the expressions of genes (*JUN, FOS, MAPK1, MAPK8, AKT1*) involved in the mitogen-activated protein kinase (MAPK) pathway would change in women with pelvic organ prolapse (POP), and we aimed to elucidate the relationship between this gene and the molecular mechanism of POP.

Materials and Methods: A total of 67 cases, including 36 patients (11 mesh, 25 native tissue) and 31 controls obtained from hysterectomy operations, were analyzed in our study. The relationship between MAPK-related genes and POP was investigated using the qRT-polymerase chain reaction method. In addition, we analyzed the genes *in silico* using Gencodis4 and Genemania web-based tools.

Results: The POP patients and control groups were analyzed, and the expression levels of MAPK8 (p=0.036), and AKT1 (p=0.010) genes were significantly higher in the POP group. Also, we have shown that the decreased expression level of the *MAPK1* gene was essential in complications (p=0.023). *In silico* analysis, we determine the biological processes, molecular functions, and biological pathways.

Conclusion: We have suggested that *MAPK1*, *MAPK8*, and *AKT1* genes are effective molecules for POP and POP-related complications. So, in further studies, these genes and related genes may be examined for the determination of the pathophysiological structure of POP disease.

Keywords: Gene expression; *in silico*; MAPK pathway; pelvic organ prolapse

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INTRODUCTION

Pelvic organ prolapse (POP) is the downward displacement of one or more organs in the pelvis (bladder, uterus, vagina, small intestine, rectum) by losing their anatomical support in their normal positions. Risk factors include age, birth, menopause, obesity, constipation, pelvic floor dysfunction, severe working conditions, socio-economic status, and hysterectomy.[1] POP is associated with urinary incontinence and defecation dysfunction, affects 10-25% of women, and often requires surgery. Surgical treatment aims to restore normal pelvic anatomy, normalize urinary and bowel functions, restore sexual functions, reduce the effects of symptoms, and improve quality of life. 30% of these operations are POP recurrences.[2,3]

POP's etiology is complex and multi-factor. The connective tissue, which is one of the most important structures that provide genitourinary support, consists of proteoglycans and glycoproteins that form a large extent of collagen, elastic fibrils, and viscoelastic matrix.[4] The predominant constituent of the connective tissue within the pelvic base is collagen, wherein Type I and Type III collagen assume primary responsibility for imparting tensile strength to the tissues. Concurrently, collagen variants of Type V and Type VI play a pivotal role in establishing interrelations between the extracellular matrix (ECM) and other essential tissue constituents.[5] Collagen and elastin are two basic protein components of the ECM of the pelvic base connective tissues. Changes in the metabolism of collagen and elastin may also change the tendency of the damaged connective tissues of the pelvic base and result in pelvic base relaxation.[6] It is stated in the previous studies that POP and other collagen-consulted disorders have a common etiology caused by the molecular level of collagen.[4]

ECM components mainly regulate cellular functions through integrin-mediated signal pathways. It is known that the receptors of the integrin family participate in various signal transmission pathways in mitogen-activated protein kinases (MAPKs)- such as extracellular signal-regulated kinase (MEK-ERK) and phosphoinositide 3-kinase (PI3K) (Figure 1).[7]

In this context, the expression levels of MAPK pathway genes in facial tissues taken during the surgical intervention of POP patients have never been investigated in the literature before. There is no previously defined information in the literature about the MAPK pathway genes, the surgical methods used in the treatment of POP, and the differences in the responses to treatment. We have selected the genes with tool of KEGG pathway and string database on MAPK pathway. Our study aimed to investigate the effects of *JUN, FOS, MAPK1, MAPK8*, and *AKT1* genes on collagen and ECM production and indirectly on POP treatment.

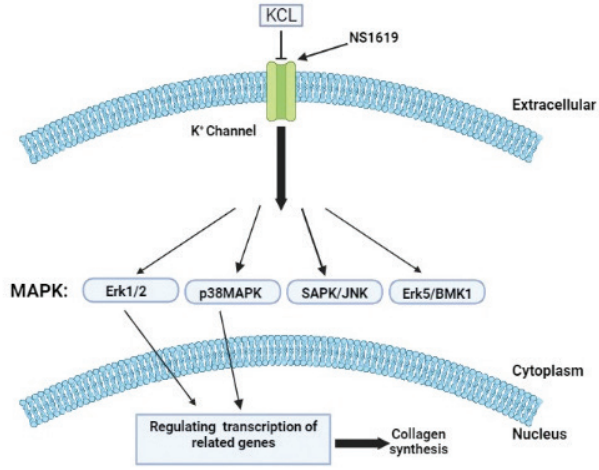

Figure 1. MAPK pathway in collagen synthesis

KCl: potassium chloride; MAPK: mitogen-activated protein kinase; NS1619, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazole-2-one; SAPK/JNK: stress-activated kinases/c-Jun N-terminal kinases; Erk: extracellular signal-regulated kinas; BMK1: big MAP kinase 1

MATERIALS AND METHODS

Collection of Sample Material

A total of 67 cases, including 36 patients (11 mesh, 25 natural tissue) who were diagnosed with POP and treated surgically, and 31 controls obtained from hysterectomy operations, who applied to the MSKU Faculty of Medicine, Department of Obstetrics and Gynecology Outpatient Clinic were included in our study. Tissues were obtained between June 2021 and October 2021 after the ethics committee decision dated 03.05.2018 and numbered 06/II. Knitting in which case and natural tissue surgery in which case was randomized and applied according to the results of a predetermined computer program. Two 0.5x0.5 cm tissues were taken from the pubocervicovaginal fascia (PSVF) during surgery from patients with POP. As the control group, 2 pieces of 0.5x0.5 cm tissue were taken from PSVF from patients who would undergo hysterectomy for benign reasons without POP.

Tissue samples taken were placed in RNA later (Hibrigen, Türkiye) solution and stored at -80 °C. The cases were evaluated in terms of pelvic pain, erosion, and genital organ prolapse degree [according to pelvic organ prolapse quantification, (POP-Q)] before POP surgery, and the same cases were re-evaluated in terms of complications 6 months after the treatment they were randomized to. Our study was evaluated and approved by the

Ethics Committee of Muğla Sıtkı Koçman University Faculty of Medicine, with decision number 06/II dated 03/05/2018, and an informed consent form was signed by all cases.

RNA Extraction and Quantitative Real-time Polymerase Chain Reaction

Total RNA isolation from tissue was performed with the Total RNA Isolation Kit (Cat. No: MG-RNA-01-250; Hibrigen Biotechnology R&D Industry and Trade Inc., Gebze, Kocaeli, Türkiye). For RNA isolation from each tissue sample, 50 mg was used. *JUN, FOS, MAPK1, MAPK8*, and *AKT1* gene expression levels were evaluated using the SYBR green real-time RT-PCR technique. Table 1 shows the primers used for the *JUN, FOS, MAPK1, MAPK8, AKT1,* and *GAPDH* genes. 2X One-Step SYBR Green RT-qPCR Mix (Cat. No: MG-OSSGM-01; Hibrigen Biotechnology R&D Industry and Trade Inc., Gebze, Kocaeli, Türkiye) kit was used to determine the gene expression levels The relative amount was normalized to the *glyceraldehyde 3-phosphate dehydrogenase* gene (GAPDH) expression tooth relative gene expression was evaluated by the 2-ΔΔCT method with at least three independent experiments.

Statistical Analysis

The expression results obtained were analyzed using the SPSS.22 program. The Kruskal-Wallis test was used for triple comparisons and Wilcoxon-Mann-Whitney U test for pairwise comparisons. *P*-value <0.05 was considered statistically significant in the analyses. In comparisons using Bonferroni correction, the significance level will be taken as 0.017. The results were expressed as mean ± standard deviation and median (minimum-maximum) and all statistical analyzes were performed using the R program.

Table 1. *JUN, FOS, MAPK1, MAPK8,* and *AKT1* genes primers used for RT-qPCR reaction

Gene	Primers
JUN	**F** 5' GAGCTGGAGCGCCTGATAAT 3' **R** 5' CCCTCCTGCTCATCTGTCAC 3'
FOS	**F** 5' ATACACTCCAAGCGGAGACA 3' **R** 5' GGTGAGCTGCCAGGATGAAC 3'
MAPK1	**F** 5' GATCTTAAATTTGTCAGGACAAGGG 3' **R** 5' CAGAAACCGCCCCTCCAAA 3'
MAPK8	**F** 5' ACGACGCGGCTTGGATTG 3' **R** 5' AAGGCTGCAAGACCGGC 3'
AKT1	**F** 5' ATTTCCCTCTTTGGAGGCTGT 3' **R** 5' ATAGCCACGTCGCTCATGG 3'
GAPDH	**F** 5' GAAGGTGAAGGTCGGAGTC 3' **R** 5' GAAGATGGTGATGGGATTTC 3'

RT-PCR: real-time polymerase chain reaction

In Silico Analysis

Prediction of gene-gene interactions

GeneMANIA (https://genemania.org/) tool was used to investigate the relationship between the *AKT1* and *MAPK8* genes, which gave significant results in the statistical analyzes made as a result of RT-qPCR (Access Date 15.10.2022). Proteins co-expressed and physically interacting with the *AKT1* and *MAPK8* genes were investigated by GeneMANIA.[8]

Gene ontology

Gene ontology search for the 5 genes (*JUN, FOS, MAPK1, MAPK8, AKT1*) selected for the study was performed using the GeneCodis 4 (https://genecodis.genyo.es/) tool (Accessed 15.10.2022). This tool examined the biological processes, molecular functions and pathways that the 5 genes have in common.[9]

RESULTS

qRT-PCR

A total of 67 cases were included in our study, including 11 cases for POP repair using mesh, 25 cases for POP repair using natural tissue, and 31 cases in the control group. Expression levels of *JUN, FOS, MAPK1, MAPK8,* and *AKT1* genes were compared in patients diagnosed with POP and control groups (Table 2).

As a result of the comparison of POP patients and control groups, a statistically significant difference was found in the expression levels of *MAPK8* and *AKT1* genes (p=0.036, p=0.010). The expression levels of *JUN, FOS, MAPK1, MAPK8,* and *AKT1* genes in the MAPK pathway in POP patients and controls are given in Figure 2.

Expression levels of *JUN*, *FOS*, *MAPK1*, *MAPK8*, and *AKT1* genes in the MAPK pathway were compared between patient tissues and control tissues. *JUN* gene expression level did not differ between the two groups and this result was not statistically significant (p=0.407). Although the *FOS* gene expression level was higher in the patient group, this result was not statistically significant (p=0.950). *MAPK1* gene expression level was found to be lower in the patient group. However, this decrease is not considered statistically significant (p=0.023). Expression levels of *MAPK8* and *AKT1* genes were found to be statistically significantly higher in the patient group (p=0.036, p=0.010).

Six months after the surgical intervention, feedback was received from the patients about whether there were any complications. The MAPK1 level was significantly higher in the uncomplicated group (Figure 2f) (p=0.017). In this case, it has been determined that MAPK1 has a protective effect on the development of complications.

Table 2. Comparison of expression levels of *JUN, FOS, MAPK1, MAPK8,* and *AKT1* genes in POP patients and control groups

Gene	n (%)	Median	Mean	Standard deviation	χ^2 *p*-value
JUN	POP patients (n=36)	0.07	1.01	3.270	0.407
	Control groups (n=31)	0.09	1.10	1.896	
FOS	POP patients (n=36)	0.30	5.48	22.675	0.950
	Control groups (n=31)	0.32	1.60	3.152	
MAPK1	POP patients (n=36)	0.59	4.18	10.429	0.223
	Control groups (n=31)	0.75	28.97	149.197	
MAPK8	POP patients (n=36)	1.29	2.12	1.887	**0.036**
	Control groups (n=31)	1.00	1.33	0.878	
AKT1	POP patients (n=32)	5.86	8.32	8.233	**0.010**
	Control groups (n=30)	2.16	4.75	5.912	

POP: pelvic organ prolapse

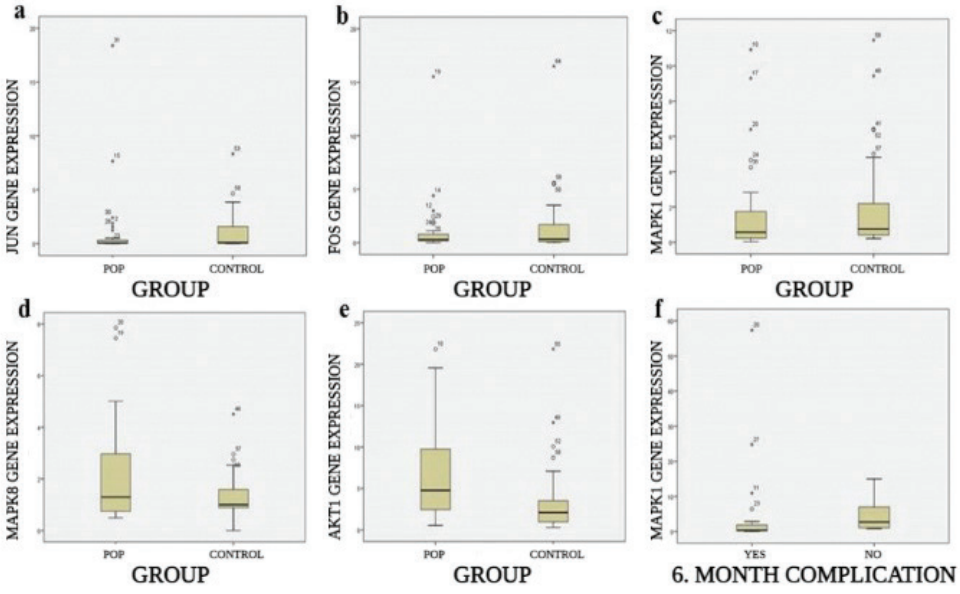

Figure 2. Expression levels of JUN **(a)**, *FOS* **(b)**, *MAPK1* **(c)**, *MAPK8* **(d)** and *AKT1* **(e)** genes involved in the MAPK pathway in POP patients and controls, and expression level of the *MAPK1* gene 6 month complication **(f)**
POP: pelvic organ prolapse; MAPK: mitogen activating protein kinase

Table 3. Complication rates after surgical interventions (mesh, native tissue)

Tissue	Complication		*p*-value
	Yes n (%)	No n (%)	
Mesh	9 (32.1)	2 (25.0)	0.699
Native tissue	19 (67.9)	6 (75.0)	

Responses from patients treated with different surgical interventions (Mesh, Native Tissue) after 6 months were evaluated (Table 3). There was no statistical difference between the probability of complications and mesh and native tissue treatment (p=0.699).

In Silico Analysis

Prediction of gene-gene interactions

The results obtained as a result of *in silico* analysis show that the *AKT1* gene is expressed together with 6 genes (*GRK2, PHLPP2, RPS6KB2, AKT2, PRKDC, FOXO1*). In addition, it was concluded that 18 genes (*PHLPP1, PHLPP2, RGCC, APPL1, FOXO4, RPS6KB2, AKT2, PTEN, THEM4, RICTOR, PRKDC, FOXO1, MAP3K14, NR4A1, TCOF1, PIK3R1, NO3, MTOR*) physically interact with AKT1 (Figure 3a).

In silico analysis for the *MAPK8* gene shows that 6 genes *(MAPK8IP1, REL, MAP3K1, MAP2K4, DUSP8, PAK1*) are

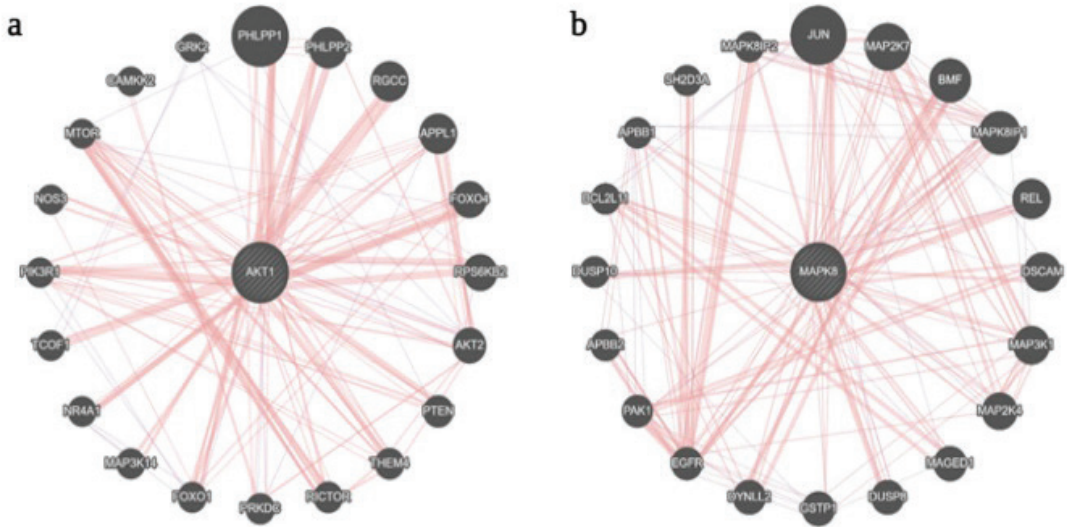

Figure 3. GeneMANIA server **a**) *AKT1* **b**) *MAPK8* gene-gene interactions

co-expressed with MAPK8. In addition, 19 genes (*JUN, MAP2K7, BMF, MAPK8IP1, REL, DSCAM, MAP3K1, MAP2K4, MAGED1, DUSP8, GSTP1, DYNLL2, EGFR, PAK1, APBB2, DUSP10, BCL2L11, APBB1, MAPK8IP2*) are in physical interaction with MAPK8 (Figure 3b).

Gene ontology

When the biological process is examined as a result of the gene ontology study for *JUN, FOS, MAPK1, MAPK8*, and *AKT1* genes, it is seen that these proteins are involved in the cellular response to reactive oxygen species, muscle tension response, and cellular response to cadmium ion.

In molecular function analysis, four of the five proteins studied (JUN, FOS, AKT1, MAPK1) were found to have identical protein binding and three proteins (MAPK1, MAPK8, AKT1) had kinase activity.

JUN, FOS, MAPK1, MAPK8, and AKT1 proteins appear to be involved in RANKL/RANK signaling pathway, prolactin signaling pathway, chromosomal and microsatellite instability in colorectal cancer, osteoclast signaling, toll-like receptor signaling pathway, gastrin signaling pathway, and IL-3 signaling pathway.

DISCUSSION

In our study, we compared the expression levels of *JUN, FOS, MAPK1, MAPK8* and *AKT1* genes in the MAPK pathway in patients with POP and control groups. Expression levels of *MAPK8* and *AKT1* genes were statistically significant between the patient and control groups (p=0.036 and p=0.010, respectively). There was no significant difference between the two groups for *JUN*, *FOS* and *MAPK1* genes (p=0.407, p=0.950 and p=0.223, respectively).

It was observed that MAPK1 expression level was higher in the patients who were evaluated for complications after surgical treatment compared to the group without complications. These results reveal that low MAPK1 level prevents complications that may occur after treatment. At the same time, when the patients treated with two different methods, namely mesh and native tissue, were evaluated in terms of complications, it was seen that there was no difference between these two treatment methods. In addition, as a result of gene-gene interaction analysis, genes in which *AKT1* and *MAPK8* genes are expressed together were determined by *in silico* approaches and GO analysis of these genes was performed.

In POP, the vagina and uterus may protrude.[10] Hysterectomy, which is a surgical method, is usually applied for the treatment of POP. However, the removal of the uterus does not cure the deficiency in the pelvic floor. Hence, uterus sparing surgeries either thanks to mesh or the natural tissue have also been applied in recent years.

The molecular mechanism underlying POP is very complex and has not been fully elucidated at present. Decreased estrogen infiltration, decreased ECM, activation of oxidative stress, genetic predisposition, and decreased neuropeptide and vasoactive intestinal peptide are among the molecular changes that occur in the pelvic floor of individuals diagnosed with POP. The predominant pathogenic mechanism of POP is decreased ECM metabolism.[11] Collagen and elastin in the ECM provide strength and flexibility to the connective tissue. Changes in collagen and elastin metabolism are observed in the pelvic tissues of individuals with POP. As a result of the research, it was concluded that the decrease in the amount of collagen and elastin was due to

the increased amount of MMP and increased metalloproteinase tissue inhibitor.[12] Among the collagens, it was observed that the amount of collagen type I in particular decreased in POP.[13] The ERK pathway, which is involved in regulating ECM components, contains several proteins involved in the MAPK pathway. The MAPK pathway includes 3 subfamilies; ERKs, c-JUN N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs), and p38 and ERK1/2 and ERK5. ERK3/4 and ERK7/8.[14] Micro-sequencing studies have shown that there is an abnormal expression of the MAPK pathway in POP patients.[15]

In 1996, Jackson et al.[16] showed that genitourinary prolapse is associated with a decrease in total collagen content, supporting the findings of another study. Ruiz-Zapata et al.[17] found that the pyridinoline collagen cross-links, which reflect the mature collagen level in the prolapse region, were significantly increased compared to the non-prolapsed group. Vulic et al.[18] reported that matrix metalloproteinase 1 expression was increased and collagen I expression decreased in the uterosacral ligaments of women with POP compared to women without POP. Studies have also shown that inhibition of the MAPK pathway significantly reduces the level of collagen.[19] The mechanism of action of the MAPK pathway on collagen synthesis led us to investigate the effect of this pathway in the POP patient group. According to a study published in 2017; in primary culture of human vaginal fibroblast cells, silenced MAPK and nuclear factor-kB pathways were found to decrease the expression level of collagen I.[20] In addition, Vetuschi et al.[21] showed that protein levels of advanced glycation end products, ERK1/2, Smad-2/3, MMP-3, and collagen III molecules were higher in POP samples compared to the control group. Selected genes in TGF-β, SMAD pathway, another pathway associated with collagen production, were examined by us and meaningful data were obtained.[22]

Based on the results of gene-gene interactions, it is seen that the *GRK2, PHLPP2, RPS6KB2, AKT2* and *PRKDC* genes expressed together with the *AKT1* gene have not been investigated in POP studies before. In previous studies, only the *FOXO1* gene has been associated with POP.[23] This shows that the *GRK2, PHLPP2, RPS6KB2, AKT2* and *PRKDC* genes can be studied in further studies for POP. Also, there are no studies on *MAPK8IP1, REL, MAP3K1, MAP2K4, DUSP8*, *PAK1* genes and POP co-expressed with the *MAPK8* gene. This shows that there are genes that guide us in future studies.

As a result of the gene ontology study, it was determined that *JUN, FOS, MAPK1, MAPK8*, and *AKT1* genes are involved in the cellular response to reactive oxygen species (ROS). Previous studies have shown that oxidative stress markers due to the increase in ROS levels are high in women with POP.[24] It has also been shown that the studied genes are involved in the biological process of muscle tension. It is assumed that POP may occur in the future due to excessive stretching of the levator ani muscle in the pelvic floor, especially in women who have had a vaginal delivery.[25] The estrogen signaling pathway is among the biological pathways in which *JUN, FOS, MAPK1, MAPK8*, and *AKT1* genes are common. Studies show that estrogen and estrogen receptor expression level regulates the connective tissue components collagen and elastin.[26]

CONCLUSION

It was concluded that the expression levels of *MAPK8* and *AKT1* genes from the *JUN, FOS, MAPK1, MAPK8,* and *AKT1* genes we examined in our study were higher in patients with POP. In this context, investigation of molecules regulating these genes and epigenetic regulation analysis can be done for further studies. As a result of Gene-gene interactions analysis, other genes associated with *AKT1* and *MAPK8* may be potential genes to be investigated for the pathogenesis of POP. In addition, the gene ontology analysis sheds light on the biological processes and pathways that *JUN, FOS, MAPK1, MAPK8*, and *AKT1* genes have in common, and the association of different biological processes and pathways with the POP disease. There is a need to study with a larger sample group to better understand the relationship of the MAPK pathway with POP and evaluate different treatment types regarding the occurrence of complications in POP.

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ETHICS

Ethics Committee Approval: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Muğla Sıtkı Koçman University Faculty of Medicine (date: 03.05.2018/no: 06/II).

Informed Consent: The written informed consent forms were obtained from individuals who agreed to participate in the study.

Peer-review: Externally peer-reviewed.

Contributions

Surgical and Medical Practices: A.A.S., B.K., M.N.A., E.A.; Concept: A.A.S., B.K., M.N.A., E.A., A.D.B., M.K., S.K.Ç., T.G.E.; Design: A.A.S., B.K., M.N.A., E.A., A.D.B., M.K., S.K.Ç., T.G.E.; Data Collection or Processing: A.A.S., T.G.E.; Analysis or Interpretation: A.D.B., M.K., S.K.Ç., T.G.E.; Literature Search: A.D.B., S.K.Ç., T.G.E.; Writing: A.A.S., A.D.B., T.G.E.

DISCLOSURES

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

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