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Identifying Biomarkers for Cervical Neoplasia: A Label-free Proteomic Analysis of Cervicovaginal Fluid

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Abstract

Objective: Cervicovaginal fluid (CVF) is a rich reservoir of biomolecules reflective of the health status of the female reproductive tract. The intricate proteome of CVF contains potential biomarkers for various gynecological conditions, including cervical neoplasia. This study aimed to elucidate the proteome of CVF at different stages of cervical neoplasia using a label-free proteomics approach, employing liquid chromatography and tandem mass spectrometry. The primary purpose of this study was to identify proteins that could serve as biomarkers for the early detection of cervical cancer (CxCa).

Methods: A total of 60 CVF samples were analyzed, including 40 from women diagnosed with cervical intraepithelial neoplasia (CIN) (CIN-1, CIN-2, CIN-3) and 20 from healthy controls. Label-free spectral counting was used to quantify the relative abundance of proteins in these samples.

Results: Analysis of the CVF proteome from the study participants revealed 244 proteins. Among these, calmodulin-like protein-3, profilin-1, and annexin-A3 were significantly differentiated between the neoplastic and non-neoplastic samples. Calmodulin-like protein-3 showed a significant decrease in abundance in the neoplastic samples compared with the controls, with a $p<0.001$. Profilin-1 and annexin-A3 exhibited significant variations in expression levels, with p-values of 0.024 and 0.057, respectively, thereby distinguishing between the different groups.

Conclusion: The identification of significant proteins such as calmodulin-like protein-3, profilin-1, and annexin-A3 underscores the potential of CVF proteomics for the early detection of CxCa. These findings pave the way for further research into CVF as a source of diagnostic biomarkers for cervical neoplasia. Understanding the CVF proteome alterations associated with cervical neoplastic stages offers a promising avenue for non-invasive screening strategies. This approach could significantly enhance early detection efforts, ultimately facilitating timely intervention and prevention of CxCa progression.

Keywords: Proteomics, cervical intraepithelial neoplasia, protein biomarker, cervicovaginal fluid, cancer screening

INTRODUCTION

Human papillomavirus (HPV) infection is one of the most common sexually transmitted infections worldwide and is known as the major cause of cervical cancer (CxCa), despite the improvements in vaccines against multiple variants of the virus. CxCa represents one of the global health burdens of HPV-related cancers as being the fourth most common cancer in women, especially squamous cell carcinoma, with mainly subtypes HPV-16, 18, 45, and 56 (1). CxCa follows virus-related tissue dysplasia, progresses slowly (2-10 years), and has a high incidence and prevalence largely in younger sexually active women (1). The management of CxCa screening (see Figure 1A) follows current

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guidelines and universal classification; according to the Bethesda System (2), cervical intraepithelial neoplasias (CINs) are graded on a scale of 1 to 3: CIN-1, CIN-2, and CIN-3, which are also referred to as "mild dysplasia"; "moderate dysplasia"; and "severe dysplasia", respectively (2).

Body fluids represent a vital source of biomarkers for the early detection of diseases, offering a unique insight into the pathological state of the body (3,4). Cervicovaginal fluid (CVF), in particular, has emerged as a significant biological fluid for proteomic analyses because of its rich content of proteins that may serve as indicators for various gynecological conditions, including endometrial and ovarian cancers, as well as preterm birth (5-7). The exploration of CVF through proteomics can unveil distinctive biomarkers that reflect the up- or downregulation of proteins in response to specific pathological conditions (8-10).

Despite the advancements in proteomic technologies and their application to CxCa research, the complexity and variability of the proteome in body fluids present considerable challenges (5,11-13). Previous studies have identified numerous proteins associated with CxCa from various sample types; however, the dynamic nature of the proteome, especially in CVF, complicates the identification of reliable biomarkers (5). Unlike genomics, proteomics research faces hurdles due to the intricate interactions and modifications of proteins, which are not fully understood (14,15). CVF's unique composition, isolated from other bodily systems and easily collected, presents a promising avenue for non-invasive diagnostic and prognostic biomarker discovery (16,17). The role of this fluid in vaginal health and defense against pathogens further underscores its potential for disease marker identification (18). Given the limitations of current diagnostic methods, such as the inaccuracies associated with colposcopic biopsies and the invasive nature of these procedures, there is a pressing need for alternative, non-invasive screening methods (19,20).

Therefore, this study was designed to harness the potential of CVF in the search for biomarkers indicative of cervical neoplasia (see Figure 1B), using label-free tandem mass spectrometry to identify proteins associated with the early stages of CxCa development. We established a profile of neoplasia-associated proteins within CVF and identify markers that could signal the risk of cancerous transformation in the cervix. This two-fold objective seeks not only to contribute to the early detection of CxCa but also to enhance the accuracy and reliability of the population screening methods given in Figure 1A. This study hypothesized that specific proteins within CVF could serve as early indicators of neoplastic changes, offering a novel approach to CxCa screening and diagnosis.

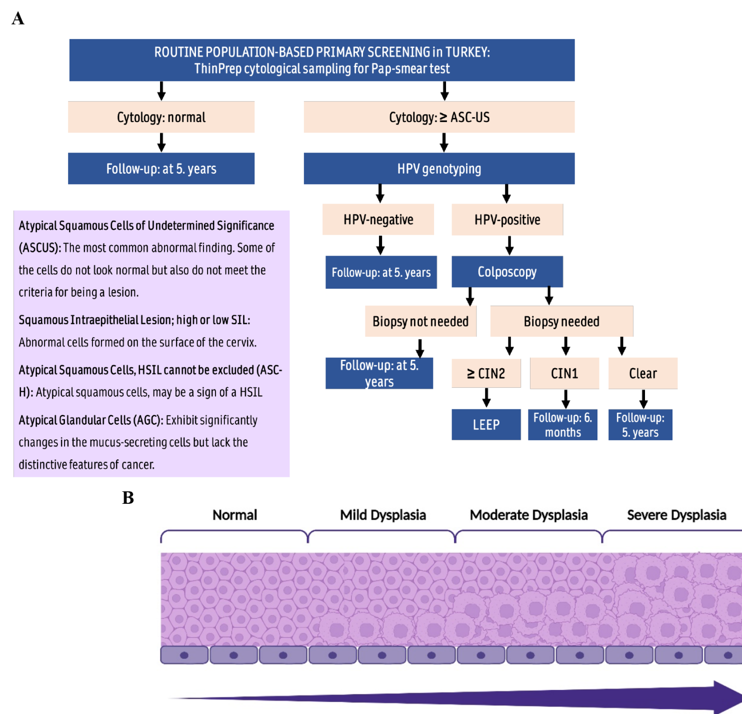

Figure 1. (A) Current cervical cancer population-based screening guideline in Turkey. (B) CIN development on the cervical layer.

ASC-US: Atypical squamous cells of undetermined significance, LSIL: Low-grade squamous intraepithelial lesions, HSIL: High-grade squamous intraepithelial lesions, AGC: Atypical glandular cells, AGUS: Atypical glandular cells of undetermined significance, CIN: Cervical intraepithelial neoplasia

METHODS

Design and Patient Selection

This study was meticulously planned to gather and analyze samples from a specific demographics group: women aged 20 to 49 years. The mean age of the participants was 34.46 years, with a standard deviation of 6.60, highlighting a median age of 34 and a mode of 32 years. Ethical considerations were paramount, and the study received approval from the Acıbadem Mehmet Ali Aydınlar University Medical Research Evaluation Board (ATADEK) (approval number: 2020-01/30, date: 09.01.2020). To ensure confidentiality and ethical compliance, each participant was assigned a unique identification code. This measure was critical in maintaining the anonymity of the participant's medical and demographic information throughout the study.

Patient Selection Criteria

The recruitment strategy targeted patients undergoing colposcopy because of either abnormal Pap smear test results or a positive test for high-risk HPV. This case-control cohort was divided into three groups based on their HPV status, presence or absence of intraepithelial lesions or malignancy, and histopathological examination results. The groups included individuals without HPV and lesions, patients with mild dysplasia (CIN-1), and those with moderate to severe dysplasia (CIN-2&3). The study's exclusion criteria were aligned with those typically applied to Pap smear sampling, excluding individuals experiencing menstruation, engaging in unprotected sexual intercourse, or using vaginal douches within 48 h before sampling. Notably, symptoms of gynecological infections did not disqualify participants from the study, ensuring a wide and inclusive participant base.

Sample Collection and Preliminary Processing

As seen in Figure 2A, the current study started with sample collection, followed by a series of experiments and ended with statistical analysis. All participants provided written informed consent before sampling, and the experiments were performed following the approved institutional guidelines. Enrollment and sampling were performed at Acıbadem Altunizade Hospital and Acıbadem Maslak Hospital in İstanbul, Turkey. The criteria of eligibility were women with (patient group) and without (control group) cervical neoplasia development. Exclusion criteria were unprotected sexual intercourse within 72 h of sampling, vaginal bleeding within the current or previous week before sampling, or any vaginal pomade or wick use within 72 h.

The procedure for sample collection was rigorously designed to prevent contamination. Each sample was collected at the beginning of the colposcopic examination, immediately labeled with the patient's identification number, and refrigerated at +4 °C. Upon arrival at the laboratory, the samples underwent

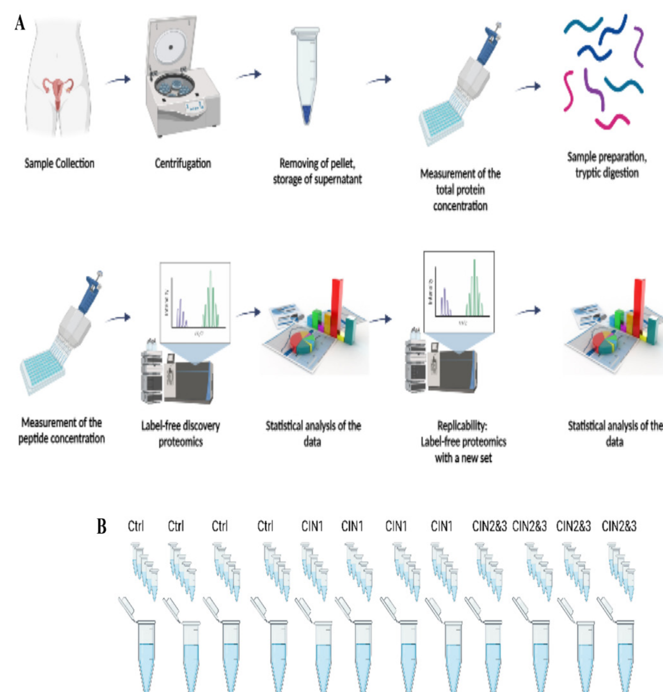

Figure 2. (A) Schematic representation of the study workflow (Created with BioRender.com). (B) Samples pooled into 12 (4×3) tubes by 20 samples × 3 groups, each of 5 samples

CIN: Cervical intraepithelial neoplasia

a standardized preparation process involving the addition of a storage buffer, followed by centrifugation to remove cell debris. This process was critical for isolating a clear supernatant for subsequent analysis. The study meticulously measured the total protein concentration of each sample, acknowledging the inherent variability in biological materials. This step was essential in preparing the samples for the analytical phase, ensuring that each sample was handled with the utmost care to maintain its integrity for accurate analysis.

Detailed Liquid Chromatography and Tandem Mass Spectrometry Analysis Methodology

The analytical phase employed a label-free liquid chromatography and tandem mass spectrometry (LC-MS/MS) technique, emphasizing a pooling method (Figure 2B) to reduce individual sample variability. This phase was underpinned by a detailed protocol that began with the mixing of universal protein extraction solution with a protease inhibitor cocktail, followed by the application of heat to denature the proteins. The samples then underwent a series of reduction and alkylation reactions within protein low-bind tubes, culminating in a desalting and digestion process designed to release the peptides for analysis. This study leveraged advanced centrifugation techniques to prepare the sample lysate, ensure detergent depletion, and facilitate the isolation of peptides. Quantification of peptide concentration and optimization of loading volumes were critical steps, enabling precise analysis of tryptic peptides. The use of a Symmetry C18 column for peptide trapping and elution, followed by the application of an acetonitrile gradient, was pivotal in separating and fractionating peptides based on their hydrophobic characteristics. This detailed methodology underscores the study's commitment to achieving high-resolution peptide profiles, providing a robust foundation for subsequent statistical analysis.

Statistical Analysis

The statistical analysis was designed to rigorously evaluate the discriminative power of the identified biomarkers. By employing ANOVA tests and box plots, this study revealed and visualize the clinical performance of potential biomarkers. The use of MedCalc software facilitated a sophisticated analysis, allowing for the detailed examination of biomarker efficacy across different clinical groups. This analytical approach was instrumental in assessing the statistical significance of the findings, ensuring that the conclusions drawn from the study were both reliable and robust.

RESULTS

Overview of Sample Demographics and Classification

The study successfully analyzed 60 samples, which were meticulously categorized into three clinical groups based on their diagnostic criteria (see Figure 1B). The "control" group included samples from 20 individuals deemed healthy after rigorous screening. The "CIN-1" group comprised samples from 20 individuals diagnosed with mild dysplasia, indicating the initial stages of CIN. Furthermore, the "CIN-2&3" group was assembled from samples of 20 individuals, split evenly between moderate and severe dysplasia diagnoses, showcasing the study's commitment to covering a broad spectrum of the disease's progression. Each sample's corresponding colposcopic punch biopsy pathology report was meticulously collected and analyzed, adhering to the ethical guidelines set forth by the relevant committee.

Clinicopathological Characteristics

The clinicopathological profiles of the participants, which are vital for the comprehensive analysis, are detailed in Table 1. This included a broad range of data meticulously collected to ensure a robust understanding of each group's specific characteristics. Notably, the average age across all individuals from whom samples were collected was 34 years, highlighting a homogeneous age distribution that enhances the study's relevance to its target demographic.

Proteomic Analysis Findings

The proteomic exploration conducted through Nano LC-MS/MS analysis was a cornerstone of this study, yielding significant insights into the protein composition of the collected samples. A total of 244 protein groups were identified across the samples with high confidence, achieving a false discovery rate of less than 1%. This achievement underscores the precision and reliability of progenesis QIP software in parsing complex proteomic data to unveil proteins with potential relevance to the pathophysiology of CIN and its varying degrees of severity.

The identification of these protein groups marks a pivotal step toward understanding the molecular underpinnings of cervical dysplasia and its progression. The detailed analysis of these proteins, set against the backdrop of the clinical classifications of the samples, provides a rich dataset for further investigation into potential biomarkers for early detection, prognosis, and personalized treatment strategies for cervical dysplasia and cancer.

Table 1. Patient information

Sample chart (n=60)						
Age	**Protein (ug/mL)**	**Medical history**	**Infection**	**Cytology**	**Biopsy**	**Study group**
39	122.54	None	Acute vulvitis	Normal	Clear	Control
36	177.39	None	None	Normal	Clear	Control
38	5454.24	None	None	Normal	Clear	Control
34	428.38	None	Acute vaginitis, vaginal candidiasis	Normal	Clear	Control
43	1033.11	Conization	None	Normal	Clear	Control
33	631.22	None	None	Normal	Clear	Control
46	682.77	Ovarian cyst rupture	None	ASC-US	Clear	Control
25	666.46	None	Acute vaginitis	Normal	Clear	Control
32	1606.34	None	Vaginal candidiasis	Normal	Clear	Control
20	537.43	None	None	Normal	Clear	Control
32	507.60	None	None	Normal	Clear	Control
36	1132.90	None	None	Normal	Clear	Control
28	1322.01	None	None	Normal	Clear	Control
38	1092.81	None	Vaginal candidiasis	Normal	Clear	Control
43	990.53	Breast cancer	None	Normal	Clear	Control
27	1547.43	None	None	LSIL	Clear	Control
26	727.65	CIN-I	None	LSIL	Mucinous metaplasia	Control
40	1721.85	None	Cronic cervicitis	Normal	Squamous metaplasia	Control
38	366.30	None	Acute vaginitis	LSIL	Squamous metaplasia	Control
42	679.15	None	Chronic cervicitis	LSIL	Clear	Control
35	952.43	None	Acute vaginitis	HSIL	CIN-1	Mild dysplasia
39	21.80	None	None	ASC-US	CIN-1	Mild dysplasia
42	43.54	None	None	LSIL	CIN-1	Mild dysplasia
49	611.64	None	None	ASC-US	CIN-1	Mild dysplasia
27	509.82	None	Acute vaginitis	ASC-US	CIN-1	Mild dysplasia
32	610.09	None	None	LSIL	CIN-1	Mild dysplasia
33	276.17	LSIL	None	Normal	CIN-1	Mild dysplasia
39	262.22	LSIL	None	ASC-US	CIN-1	Mild dysplasia
30	605.67	None	None	ASC-H	CIN-1	Mild dysplasia
40	582.54	ASC-H	None	ASC-US	CIN-1	Mild dysplasia
39	580.23	None	None	ASC-H	CIN-1	Mild dysplasia
24	589.17	None	Acute vaginitis	HSIL	CIN-1	Mild dysplasia
43	101.86	ASC-US	None	LSIL	CIN-1	Mild dysplasia
37	598.36	None	None	-	CIN-1	Mild dysplasia
30	349.56	None	None	LSIL	CIN-1	Mild dysplasia
26	611.78	ASC-US	Acute vaginitis	ASC-US	CIN-1	Mild dysplasia
38	605.02	None	None	-	CIN-1	Mild dysplasia
30	587.50	None	None	LSIL	CIN-1	Mild dysplasia
46	560.42	None	None	ASC-US	CIN-1	Mild dysplasia
43	1849.82	Uterine polyps	Acute vaginitis	LSIL	CIN-1	Mild dysplasia
32	1253.50	ASC-H	None	ASC-H	CIN-2	Moderate dysplasia
28	749.86	None	Acute vaginitis	ASC-H	CIN-2	Moderate dysplasia

Table 1. Continued						
Sample chart (n=60)						
Age	Protein (ug/mL)	Medical history	Infection	Cytology	Biopsy	Study group
23	1431.25	None	None	HSIL	CIN-2	Moderate dysplasia
35	821.40	None	None	Normal	CIN-2	Moderate dysplasia
29	656.61	None	None	LSIL	CIN-2	Moderate dysplasia
29	510.83	ASC-US	None	ASC-US	CIN-2	Moderate dysplasia
34	611.34	LSIL	None	LSIL	CIN-2	Moderate dysplasia
29	408.94	ASC-US	None	LSIL	CIN-2	Moderate dysplasia
32	522.59	None	None	LSIL	CIN-2	Moderate dysplasia
36	406.07	None	Acute vaginitis	HSIL	CIN-2	Moderate dysplasia
34	1440.49	ASC-US	None	ASC-US	CIN-3	Severe dysplasia
27	1063.41	None	None	ASC-H	CIN-3	Severe dysplasia
40	549.59	None	Acute vaginitis	LSIL	CIN-3	Severe dysplasia
36	638.98	HSIL	None	HSIL	CIN-3	Severe dysplasia
45	359.97	None	None	LSIL	CIN-3	Severe dysplasia
24	1332.13	None	None	LSIL	CIN-3	Severe dysplasia
29	425.82	None	Acute vaginitis	ASC-US	CIN-3	Severe dysplasia
43	377.74	LSIL	None	HSIL	CIN-3	Severe dysplasia
33	434.10	None	None	ASC-H	CIN-3	Severe dysplasia
32	451.30	None	Acute vaginitis	LSIL	CIN-3	Severe dysplasia

CIN: Cervical intraepithelial neoplasia, LSIL: Low-grade squamous intraepithelial lesions, ASC-US: Atypical squamous cells of undetermined significance, ASC-H: Atypical squamous cells, HSIL cannot be excluded, HSIL: High-grade squamous intraepithelial lesions

DISCUSSION

Considering the extensive research conducted over the past 15 years, as outlined in our review article (21), the investigation into the proteome of CVF has been a focal point in understanding gynecological health and disease. Previous studies, such as those by Van Ostade et al. (14), Zegels et al. (17), and Van Raemdonck et al. (22,23), Boylan et al. (24), Starodubtseva et al. (13), Ma et al. (25), and Gutiérrez et al. (26), have identified a varying number of proteins in CVF, reflecting the fluid's potential as a diagnostic tool for gynecological diseases. Despite differing methodologies across studies, the pursuit to delineate a "healthy core proteome" of CVF has been consistent, underscoring the fluid's variability and the challenges in using it for biomarker identification.

The pivotal discovery of our study was the identification of 244 protein groups with high confidence, highlighting the significant variability of the CVF proteome. Among these, three proteins -calmodulin-like protein-3 (CALL3, P27482), profilin-1 (PROF1, P07737), and annexin A3 (ANXA3, P12429)- were found to significantly differentiate between the clinical groups studied, with statistical significance shown in Figure 3B. This differentiation is crucial for understanding the molecular basis of cervical neoplasia and underscores the potential of these proteins as biomarkers for the disease.

Study Limitations

The study's methodology, while comprehensive, is not without its limitations. The variability inherent to CVF as a sample material introduces potential biases and uncertainties in protein identification. This variability, coupled with the diverse methods of sample collection and analysis used in previous studies, underscores the challenges in achieving a standardized approach to CVF proteomics. This study meticulously addressed these concerns through rigorous sample processing and advanced analytical techniques, but the potential for variability remains a critical consideration.

The differential protein expression profile analyzed in this study provides a nuanced understanding of cervical neoplasia at the molecular level. The significant proteins identified not only offer insights into the disease's pathophysiology but also align with findings from similar studies (23,26-28), reinforcing the relevance of these proteins in the context of cervical health. The careful interpretation of these results, considering the study's

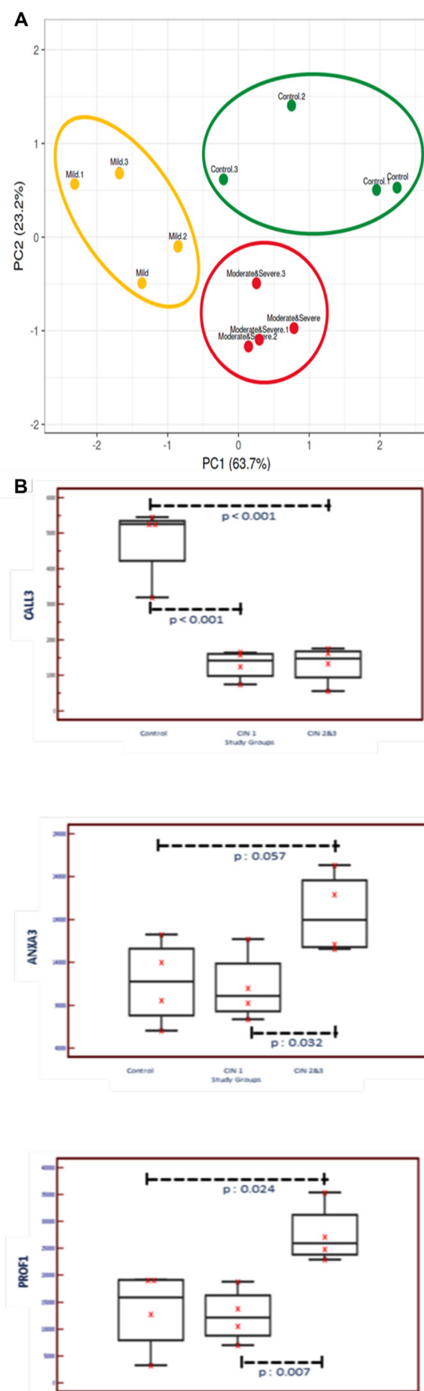

Figure 3. (A) Principal component analysis (PCA) plot with all identified proteins in the samples. (B) Box plot charts of the significant proteins for discrimination of the clinical groups
CIN: Cervical intraepithelial neoplasia

objectives and limitations, suggests a promising direction for future research in identifying reliable biomarkers for cervical neoplasia.

Before concluding, it is imperative to discuss the external validity of our findings. The study's results, while promising, are derived from a specific cohort, and their generalizability to broader population warrants careful consideration. The inherent variability of CVF (29,30) and the methodological differences across studies pose challenges in applying these findings universally. Nonetheless, the identified proteins provide a valuable foundation for further investigation into their roles in cervical neoplasia.

CONCLUSION

In summary, our study identified key proteins that significantly differ across various stages of cervical neoplasia, offering insights into the disease's molecular landscape. These findings highlight the potential of CVF proteomics in advancing the diagnosis and understanding of gynecological diseases. However, to confirm these findings and fully understand their implications, further randomized controlled trials are necessary.

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Ethics

Ethics Committee Approval: The study received approval from the Acıbadem Mehmet Ali Aydınlar University Medical Research Evaluation Board (ATADEK) (approval number: 2020-01/30, date: 09.01.2020).

Informed Consent: Informed written consent was obtained from all participants.

Authorship Contributions

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

Conflict of Interest: No conflicts of interest were declared by the authors.

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