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Claudin 18.2 Expression in Gastric Adenocarcinomas in a Large Turkish Cohort

Geniş Bir Türk Kohortundaki Gastrik Adenokarsinomlarda Claudin 18.2 Ekspresyonu

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Abstract

Objective: Claudin 18.2 (CLDN18.2) is a tight junction protein expressed especially in gastric adenocarcinomas. The prognostic and clinicopathologic implications of CLDN18.2 expression is currently unknown. Zolbetuximab monoclonal antibody against CLDN18.2 is under investigation as a potential treatment for advanced gastric cancer (GC). We aimed to investigate the impact of CLDN18.2 expression in GC on prognosis and tumor features in a large Turkish cohort.

Materials and Methods: Seven tissue microarrays (TMAs) containing 263 cases of GC were constructed. Assessment of CLDN18.2 expression was performed by immunohistochemistry. The expression of CLDN18.2 was scored based on staining intensity and the percentage of staining. Staining intensity was classified as: 0, no reactivity; 1, weak; 2, moderate; and 3, strong. Percentage of staining was classified as: (0-39%), negative; (40-100%), positive. Cases with a percentage of staining score of (40-100%) with moderate to strong staining intensity (2 or 3) were defined as positive and cases with a percentage of staining score of (0-39%) with no to weak staining intensity (0 or 1) were defined as negative.

Results: 14.3% (37/258) of GCs were stained with anti-CLDN18.2 antibody. While 7.8% (20/258) of all cases were positive, 92.2% (238/258) were scored as negative. There was no statistically significant difference between the two groups in terms of patient features such as age or sex, tumor grade, TNM stage, histologic subtype, or overall survival.

Conclusion: CLDN18.2 expression was not associated with patient prognosis in the Turkish cohort. However, as this molecule is a potential therapeutic target, information about the impact of CLDN18.2 expression will be important in managing patients, therefore more studies are needed to learn more on the outcomes of CLDN18.2 expression on clinicopathologic features in GC.

Keywords: Gastric Cancer, CLDN18.2, Biomarker

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Öz

Amaç: Claudin 18.2 (CLDN18.2) özellikle gastrik adenokarsinomlarda (GC) eksprese edilen bir sıkı bağlantı proteinidir. CLDN18.2 ekspresyonunun prognostik ve klinikopatolojik etkileri halen bilinmemektedir. CLDN18.2'yi hedef alan Zolbetuximab monoklonal antikoru, ileri evre gastrik adenokarsinomlar için potansiyel bir tedavi olarak değerlendirilmektedir. Bu çalışmada, gastrik adenokarsinomlarda CLDN18.2 ekspresyonunun prognoz ve tümör özelliklerini geniş bir Türk kohortunda araştırmayı amaçladık.

Gereç ve Yöntemler: 263 GC vakası içeren yedi tane doku mikro dizini (TMA) hazırlanmıştır. CLDN18.2 ekspresyonu immünohistokimyal boyama yöntemi ile tespit edilmiştir. CLDN18.2 ekspresyonu, boyanma yoğunluğu ve boyanma yüzdesine göre puanlanmıştır. Boyanma yoğunluğu 0, reaktivite yok; 1, zayıf; 2, orta ve 3; güçlü. Boyanma yüzdesi şu şekilde sınıflandırılmıştır: (%0-39), negatif; (%40-100), pozitif. Boyanma yüzdesi (%40-100) olan ve orta ila güçlü boyanma yoğunluğuna (2 veya 3) sahip vakalar pozitif, boyanma yüzdesi (%0-39) olan ve hiç reaktivite olmayan veya zayıf boyanma yoğunluğuna (0 veya 1) sahip vakalar negatif olarak tanımlanmıştır.

Bulgular: GC'lerin %14,3'ü (37/258) anti-CLDN18.2 antikoru ile boyanmıştır. Tüm vakaların %7,8'i (20/258) pozitif, %92,2'si (238/258) negatif olarak değerlendirilmiştir. İki grup arasında yaş veya cinsiyet, tümör derecesi, TNM evresi, histolojik alt tip veya genel sağkalım gibi hasta özellikleri açısından istatistiksel olarak anlamlı bir fark bulunamamıştır.

Sonuç: CLDN18.2 ekspresyonu Türk kohortundaki GC'li vakalarda prognoz ile ilişkili değildir. Bununla birlikte CLDN18.2 potansiyel bir terapötik hedeftir ve CLDN18.2 ekspresyonu ile ilgili bilgiler hastalık yönetiminde önemli olacaktır. GC'lerde CLDN18.2 ekspresyonunun klinikopatolojik özellikler üzerindeki etkilerini anlamak için daha fazla çalışmaya ihtiyaç vardır.

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Anahtar Kelimeler: Gastrik Kanser, CLDN18.2, Biomarker

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Introduction

Gastric Cancer (GC) is one of the most common types of cancer worldwide, with high incidence and mortality rates (1). For decades, the standard treatment option for GCs has been perioperative and adjuvant chemotherapy/chemoradiation therapies. However, the 5-year disease survival rates' dramatic decline beyond Stage II, ranging from 61–63% for Stage IIIa to 30% – 35% for Stage IIIc, has led to the investigation and development of targeted treatments for advanced GCs (2). One of the first examples for targeted therapy in advanced GC is the use of the anti-HER2 antibody trastuzumab in patients bearing tumors with HER2 overexpression or amplification, which has been instituted in guidelines based on the results of the ToGA trial (3). Other targeted treatments used in the management of advanced GC include ramucirumab, a monoclonal antibody that targets the vascular endothelial growth factor receptor-2 (VEGF-R2) (4) and nivolumab/ pembrolizumab, immune checkpoint inhibitors that have shown benefit in clinical trials (5, 6).

Claudins are a family of 27 transmembrane proteins that form the major structural and functional components of tight junctions. Some subtypes of claudins are expressed in a tissue-specific manner (7). CLDN18.2 is a member of the claudin family and is specifically expressed in the stomach. It has been shown to be expressed in various types of cancers including GC, lung cancer, pancreatic cancer, ovarian and esophageal cancer (8). Targeting CLDN18.2 with zolbetuximab is a promising approach for treating advanced GC. Zolbetuximab was shown to significantly improve overall survival when added to standard chemotherapy, as investigated in the FAST trial (NCT03504397) (9). The initial results obtained from the global phase 3 study SPOTLIGHT(NCT03504397) were in concordance with the FAST trial. Currently, there are numerous clinical trials evaluating the efficacy of Zolbetuximab in addition to standard chemotherapy/immunotherapy regimens in locally advanced and metastatic gastric/gastroesophageal junction cancers (NCT 03504397, NCT03653507, NCT03505320) and one clinical trial studying the efficacy of Zolbetuximab in metastatic pancreatic adenocarcinoma (NCT03816163).

GC is a heterogeneous disease that shows significant differences in molecular characteristics among ethnic populations (10). The aim of this study was to evaluate the expression of CLDN18.2 in a large Turkish population with GC, and to compare it with prognostic parameters.

Materials and Methods

Patients and Gastric Cancer Tissue Microarrays

All cases diagnosed with GC at the Department of Pathology of Hacettepe University Faculty of Medicine between 2014-2022 were screened. Cases that received radiotherapy, chemotherapy before surgery, or any form of neoadjuvant therapy were excluded. The HE sections of total or partial gastrectomy materials of the cases were re-examined by two pathologists (AA, GG) and confirmed to be adenocarcinomas. The regions that best represented the tumor were identified in the HE sections. Recipient blocks necessary to create tumor microarrays (TMA) were prepared using a three mm Recipient Block Mold Kit (Quick Mold). One single core of 3 mm diameter Formalin-Fixed Paraffin-Embedded (FFPE) tissue sample was transferred to the recipient block for each case. A total of 7 tissue microarrays were prepared for 263 GC cases. Sections of 3.5-4.0 μm thickness were taken from the TMA blocks. Tumor foci were checked in the HE-stained sections. The study was approved by the Non-Interventional Clinical Research Ethics Committee of Hacettepe University (GO 21/603).

Immunohistochemistry

Immunohistochemistry was performed on 4-micron-thick unstained sections obtained from seven TMA blocks which were first deparaffinized in an incubator at 75°C for 40 minutes then deparaffinized with xylene and rehydrated through a series of graduated alcohols. Antigen retrieval was performed by heating the tissue sections in Citrate buffer (pH 6.0) in a microwave oven for 10 minutes. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide and methanol solution for 10

minutes. CLDN18.2 primary antibody (clone EPR19202, Abcam, Cambridge) was incubated at a dilution of 1:250 in antibody diluent for 1 hour at room temperature. After, the sections were incubated with a biotinylated secondary antibody and streptavidin-peroxidase (UltraVision Detection System Large Volume Anti-Polyvalent, HRP, Thermo Fisher Scientific) for 30 minutes each at room temperature. The signal was visualized with diaminobenzidine (DAB) chromogen, using hematoxylin as a counterstain. Normal gastric mucosa was used as a positive control.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 23.0 for Windows (IBM Corp. Released 2015, Armonk, NY: IBM Corp.). Pearson chi-square test was used to determine the association between CLDN18.2 protein expression and other variables such as gender, age, T stage, N stage and Lauren phenotype. Survival curves were generated using the Kaplan-Meier method to assess the relationship between CLDN18.2 expression and survival. The log-rank test was employed to evaluate the difference between the survival curves of the groups. p values <0.05 were considered statistically significant.

Results

CLDN18.2 expression in Gastric Cancer Samples

CLDN18.2 antibody was used to stain 3 mm core TMA samples, and all 263 samples except for 5 that had technical issues were used. Initially, CLDN18.2 staining intensity was categorized into four groups: 0, no reactivity in membrane or cytoplasm; 1+, weak reactivity in membrane or cytoplasm; 2+, moderate reactivity in membrane or cytoplasm; and 3+, strong reactivity in membrane or cytoplasm. 14.3% (37/258) of all cases showed staining with CLDN18.2. Then, the classification system was modified using the FAST criteria (≥40% staining intensity in tumor cells is considered positive); subsequently, samples were categorized into negative (0-1+) or positive (2+-3+) groups based on staining intensity. Of the total 258 samples, 92.2% (n=238) were negative for CLDN18.2 expression and 7.8% (n=20) were positive. (Figure 1).

The clinicopathologic features of the gastric cancer cases are summarized in Table 1.

Overall survival was defined as the time from the operation to the last follow up for censored patients, and as the time from the operation to death from any cause for deceased patients. 7 patients who died within a month after the operation were excluded from the survival analysis due to high likelihood of surgery-related mortality. The median survival of patients in the CLDN18.2 negative and CLDN18.2 positive group were 33.0 and 65.3 months, respectively. Although patients with positive CLDN18.2 expression had a longer median overall survival, the prognostic difference between these groups was not statistically significant (p=0.144) (Figure 2). In addition to these findings, we noted that CLDN18.2 expression was not significantly related to gender, age, T stage, N stage and Lauren phenotype (Chi square p values >0.05 for all) (Table 2).

Discussion

In this study we aimed to investigate the implication of CLDN18.2 expression on prognosis and tumor characteristics in patients with gastric cancer from a Turkish cohort in a retrospective manner. This large cohort was obtained from a large tertiary health care center, with patients from all around the country. This might, to an extent, increase the generalizability of our results. None of our patients had received any type of treatment before applying to our hospital, which reduces any sort of selection bias.

We did not note statistically significant differences between cases with and without CLDN18.2 expression in terms of patient characteristics, tumor grade, TNM stage, histologic subtype per Lauren classification and overall survival. However, when the Kaplan-Meier survival graphs of the two groups are compared, a tendency of patients with CLDN18.2 expression towards longer survival can be noticed (Figure 2). Yet,

as mentioned before this difference was not deemed statistically significant. The reason behind this seemingly erratic tendency is currently unknown.

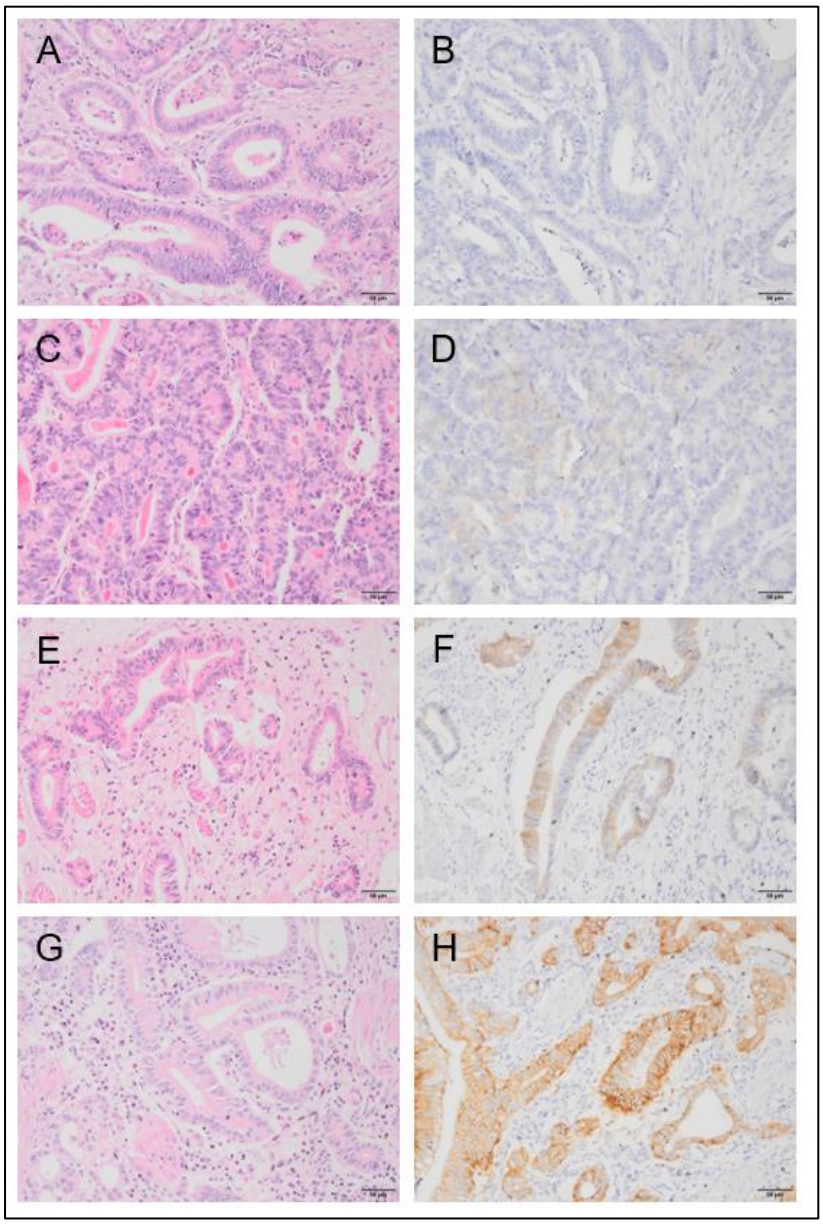

Figure 1. CLDN18.2 expression in gastric adenocarcinomas are demonstrated for negative (B), weak-focal (D), moderate (F) and strong (H) expression by immunohistochemistry (A, C, E, G; H&E staining images and B, D, F, H; CLDN18.2 immunohistochemistry of corresponding cases, respectively, scale bars: 50µm).

Previous studies conducted on the clinical implications of CLDN18.2 expression in GC have yielded conflicting results. The study conducted by Rohde et al. in 2019 found that CLDN18.2 was expressed in 87%, when the FAST criteria for positivity was applied this percentage dropped to 52%, of patients with GC in a Japanese cohort and CLDN18.2 expression was correlated with higher tumor grade and diffuse type GC per Lauren classification (10), Pelino et al. found that 45.1% of patients with advanced gastroesophageal cancers overexpressed CLDN18.2, in concordance with the FAST criteria. Although they found a correlation between lymph node metastasis, higher grade, peritoneal metastases and advanced stage, the overall survival between the two groups did not change (12). In another study, CLDN18.2

expression was higher in diffuse type GCs and HER-2 positive cancers, in a Korean cohort. However, they noted that the TNM stage and overall survival failed to differ according to CLDN18.2 expression (13).

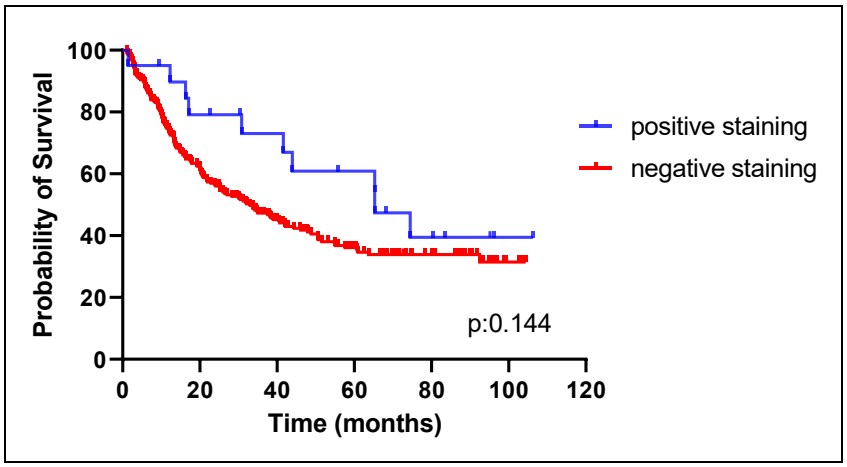

Figure 2. Kaplan-Meier curves depicting the postoperative survival of CLDN18.2 negative and positive gastric adenocarcinoma cases.

Table 1
Main Clinicopathological Characteristics of Patients with Gastric Adenocarcinoma Represented in Tissue Microarrays

Characteristics	**n (%)**
Age (years) [n=263], median (range)	62 (21-88)
Gender	
Female	97 (36.9)
Male	166 (63.1)
Histologic subtype	
Adenocarcinoma	123 (46.8)
Diffuse type carcinoma	62 (23.6)
Other/undetermined	78 (29.6)
Total	263
Size [mean (range)] (cm)*	6.4
*known for 255/263 cases (96.96%)	
Stage**	
**known for 262/263 cases (99.61%)	
I	1 (0.4)
II	26 (9.9)
III	56 (21.4)
IV	179 (68.3)
Status	
Alive	104 (39.5)
Deceased	159 (60.5)
CLDN18.2 immunostaining***	
***known for 258/263 cases (98.08%)	
Negative	221 (85.6)
Focal	17 (6.6)
Moderate	12 (4.7)
Strong	8 (3.1)

Table 2

Patient Characteristics and CLDN18.2 Expression

			CLDN18.2				
		Total	**Negative staining**	**Positive staining**	**% negative staining**	**% positive staining**	**p value**
Age	**≤60**	117	110	6	46.0	30.0	0.242
	>60	146	129	14	54.0	70.0	
Gender	**Male**	166	149	16	62.3	80.0	0.148
	Female	97	90	4	37.7	20.0	
T stage	**T1 or T2**	27	25	1	10.5	5.0	0.505
	T3 or T4	235	213	19	89.5	95.0	
N stage	**N0 or N1**	96	84	9	35.4	45.0	0.469
	N2 or N3	165	153	11	64.6	55.0	
Subtype	**Adenocarcinoma**	123	110	11	47.8	57.9	0.612
	Diffuse type carcinoma	62	56	3	24.3	15.8	
	Other/ undetermined	78	64	5	27.8	26.3	

Dottermusch et al., studied the clinicopathologic features of 481 GCs with and without CLDN18.2 expression in a Caucasian cohort. They showed that CLDN18.2 expression was not correlated with any clinical findings of patients, stage of tumor, Lauren phenotype or overall survival. Their results showed that when the FAST criterion for positivity was applied, the CLDN18.2 expression percentage failed to exceed 10%. Their studies also revealed a strongly positive correlation between EBV genome positivity and CLDN18.2 expression (14). A study done by Arnold et al. in 2020 also failed to show a correlation between tumor histologic subtype, tumor grade, stage or overall survival in a Caucasian cohort. After staining and analysis, 17.1% of gastric and esophageal adenocarcinomas showed overexpression of CLDN18.2, however there was no statistically significant correlation in clinical features of patients or overall survival with respect to CLDN18.2 expression (15). The works of Hong et al. in 2020, showed conflicting results with previous studies. They studied the characteristics of various tumor types with CLDN18.2 overexpression. Among 85 GC specimens, 12 showed CLDN18.2 expression, which approximates to 14%. Although they failed to show a difference in patient characteristics, EBV genome positivity, TNM stage, HER2 expression and overall survival between the two groups, they noticed that CLDN18.2 expression was higher in intestinal type of GCs when compared with diffuse type GCs per Lauren phenotype (16).

A meta-analysis done by Ungureanu et al. in 2021, which included only 6 studies due to the scarcity of studies, concluded that there was no correlation in histologic subtype, grade, TNM stage, HER2 expression between patients bearing tumors with or without CLDN18.2 expression. They also failed to find a difference between the overall survival rates of the two groups (17).

When the aforementioned studies are evaluated, the percentage of CLDN18.2 expression of GC differs considerably from one study to another. Though these conflicting results might seem counter-intuitive at first, there may be several reasons as to why they vary. The previous studies analyzed tumor samples from various different populations from distinct ethnic origins. The present study was done on a Turkish cohort,

which is largely of Caucasian origin, and yielded a CLDN18.2 overexpression percentage of 7.8%. Dottermusch et al. (14) also evaluated CLDN18.2 expression in GCs in a large Caucasian cohort, and their results were similar to ours, CLDN18.2 expression of 10% after applying the FAST eligibility criteria. However, when the works of Rohde et al. (10) and Baek et al. (13) are scrutinized, it is observed that the population they worked in is not of Caucasian but of Japanese and Korean origin, respectively. Although these two populations have different origins, they are closest to one another when compared with others (18). CLDN18.2 expression positivity in GCs from these two cohorts are much higher than in Caucasian cohorts; 52% for Rohde et al. and 29.4 % for Baek et al. The difference of CLDN18.2 expression might be explained by the varying genetic polymorphisms among populations.

Nonetheless, different ethnic origins do not suffice to explain the discrepancy among the results of the studies, since Pelino et al. also investigated a large Caucasian cohort and yielded a CLDN18.2 expression percentage of 45.1%. This might be attributed to the different antibodies used in the studies. As mentioned in the meta-analysis done by Unugaru et al., there are two major antibody kits used in the mentioned studies: The CLAUDETECT 18.2, which is not specific for claudin 18.2 but rather recognizes the C-terminal end of claudin 18, and the Anti-CLDN EPR19202 (Abcam, Cambridge), which is specific for 100 amino acids of the human claudin 18.2 isoform. Rohde et al. and Pelino et al. used the former whereas Dottermusch et al, Baek et al. and the current study used the latter, which might help to explain the higher CLDN18.2 expression positivity in the works of Rohde et al and Pelino et al.

A recent study done by Kayıkcıoglu et al. in a small Turkish cohort yielded a CLDN18.2 positivity of 73.8% (48/65). Although they also failed to show a correlation between clinicopathologic features of patients and CLDN18.2 expression, CLDN 18.2 expression was much higher than our study. Reason as to why, might be that the study only evaluated samples from patients with metastatic gastric adenocarcinomas excluding locally advanced gastric and gastroesophageal adenocarcinomas (19).

Claudins make up a crucial part of tight junctions which promote cell-cell adhesion. Loss of cell-cell adhesions have been well known to be an important part of the epithelial-mesenchymal transition which ultimately leads to invasion and metastasis. Matsuda et al. have validated this process in which they showed decreased expression of claudins in the invasive front of GCs when compared to the non-invasive front (20). This heterogenous expression of claudins may result in varying CLDN18.2 expression in samples taken from different parts of the tumor, even in the same patient, leading to discrepant results between studies.

Conclusions

As of now there is no consensus on the prognostic or clinicopathologic implications of CLDN18.2 expression in patients with GC. With the introduction of Zolbetuximab, a monoclonal antibody against CLDN18.2, as a therapeutic option for advanced GC, CLDN18.2 expression analysis may gain importance in clinical practice. As the data grows and more studies are conducted in different and larger cohorts, we might reveal the true face of CLDN18.2, when it comes to the prognosis of GC patients.

Ethics Committee Approval: The study was approved by the Non-Interventional Clinical Research Ethics Committee of Hacettepe University (GO 21/603).

Informed Consent: Written consent was obtained from the participants.

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