DOI: 10.4274/vhd.galenos.2026.2026-1-5
Viral Hepat J 2026;32(1):19-25

Molecular Evaluation of Concomitant HBsAg, and Anti-HBs Positivity in Chronic Hepatitis B Patients

Kronik Hepatit B Hastalarında Eş Zamanlı HBsAg ve Anti-HBs Pozitifliğinin Moleküler Değerlendirmesi

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ABSTRACT

Objectives: The hepatitis B virus (HBV) has a high mutation rate during replication, leading to the production of different variants. However, such atypical profiles could also be due to variant strains resulting from mutations. This study aimed to determine the mutations responsible for this profile and the presence of mutant strains by performing sequence analysis of the HBV pol/S gene regions in patients with chronic HB infection who were found to have coexisting HB surface antigen (HBsAg) and anti-HBs positivity during their follow-up.

Materials and Methods: The study group consisted of patients who were found to be simultaneously positive for HBsAg and anti-HBs during routine follow-up for chronic HBV infection between 2021 and 2022. Mutations in the pol/S gene regions of HBV-DNA- positive patients were investigated using HBV-DNA sequencing technology.

Results: The coexistence of HBsAg and anti-HBs was observed in 33 patients (3.8%). Anti-HBe was positive in all cases. Thirteen (39.4%) patients were HBV-DNA positive (minimum 6.9+E1 IU, maximum 3.7+E6 IU). All patients had HBV genotype D, and the D1/D2 ratio was 45.5%/54.6%. Among HBV-DNA-positive patients, 11 (84.6%) had pol/S gene mutations. Sixty-eight HBsAg mutations (42.8%) and 91 reverse transcriptase (RT) mutations (57%) were detected in these cases, representing a total of 159 mutations. In total, three patients (27.2%) had clinically significant HBsAg mutations, and four (36.3%) had RT mutations. Drug resistance was detected in 18.2% of cases. A vaccine-escape HBsAg mutation was detected in two cases (18.1%).

ÖZ

Amaç: Hepatit B virüsü (HBV), replikasyon sırasında yüksek bir mutasyon oranına sahiptir ve bu durum çeşitli varyantların oluşmasına yol açmaktadır. Ancak, bu atipik profiller, mutasyonlardan kaynaklanan varyant suşlardan da kaynaklanabilir. Bu çalışma, kronik HB enfeksiyonu olan ve takipleri sırasında eş zamanlı HB yüzey antijeni (HBsAg) ve anti-HBs pozitifliği saptanan hastalarda HBV pol/S gen bölgelerinin sekans analizi yapılarak, bu profili oluşturan mutasyonları ve mutant suşların varlığını belirlemeyi amaçlamıştır.

Gereç ve Yöntemler: Çalışma grubu, 2021 ve 2022 yılları arasında kronik HBV enfeksiyonu için rutin takip sırasında eş zamanlı olarak HBsAg ve anti-HBs pozitifliği saptanan hastalardan oluşmuştur. HBV-DNA pozitif hastaların pol/S gen bölgelerindeki mutasyonlar, HBV-DNA sekanslama teknolojisi kullanılarak araştırılmıştır.

Bulgular: HBsAg ve anti-HBs'nin birlikte bulunması 33 (%3,8) hastada saptanmıştır. Tüm olgularda anti-HBe pozitifti. On üç (%39,4) hastada HBV-DNA pozitifliği saptandı (minimum 6,9+E1 IU, maksimum 3,7+E6 IU). Tüm hastalarda HBV genotipi D idi ve D1/D2 oranı %45,5/%54,6 idi. HBV-DNA pozitif hastaların 11'inde (%84,6) pol/S gen mutasyonu bulundu. Bu olgularda 68 (%42,8) HBsAg mutasyonu ve 91 (%57) ters transkriptaz (RT) mutasyonu saptandı ve toplamda 159 mutasyon tespit edildi. Toplamda üç hastada (%27,2) klinik olarak anlamlı HBsAg mutasyonu, dört hastada (%36,3) RT mutasyonu saptandı. Olguların %18,2'sinde ilaç direnci saptandı. İki olguda (%18,1) aşıdan kaçış HBsAg mutasyonu saptandı.

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Received: 27.01.2026 **Accepted:** 11.05.2026 **Publication Date:** 01.06.2026

Cite this article as: Akdoğan Ö, Yapar D, Sayan M, Savcı Ü, Kaplan G, Baykam N, Kocagül Çelikbaş A. Molecular evaluation of concomitant HBsAg, and anti-HBs positivity in chronic hepatitis B patients. Viral Hepat J. 2026;32(1):19-25

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Conclusion: The mutations detected in the pol/S gene sequence analyses of patients with HBsAg and anti-HBs coexistence seen in the course of chronic HB infection are naturally occurring, and the coexistence of HBsAg and anti-HBs could not be explained by these mutations.
Keywords: HBsAg, anti-HBs, chronic hepatitis B, coexistence, mutation

Sonuç: Kronik HB enfeksiyonu sürecinde HBsAg ve anti-HBs birlikteliği görülen hastalarda pol/S gen sekans analizlerinde saptanan mutasyonlar genellikle doğal olarak oluşan mutasyonlardır ve HBsAg ile anti-HBs birlikteliği bu mutasyonlarla açıklanamayacağı düşünüldü.
Anahtar Kelimeler: HBsAg, anti-HBs, kronik hepatit B, birliktelik, mutasyon

Introduction

Hepatitis B virus (HBV) is a double-stranded DNA virus belonging to the family *Hepadnaviridae* and is part of the *Orthohepadnavirus* group. It has 10 different genotypes (A-J) and several subgenotype (1,2). Among the polymorphism patterns of genotype D, the model types D1, D2, and D6 were commonly observed. Genotypes influence the natural history and clinical outcomes of HBV and response to treatment in chronic patients (3,4,5). Chronic HB (CHB), characterized by HB surface antigen (HBsAg) positivity for more than six months, has emerged as a major public health problem worldwide (1). The most common genotype in Türkiye is genotype D (3,6,7,8).

The S region of HBV-DNA comprises the S gene and pre-S gene regions. This gene region encodes three envelope proteins of the HBsAg: small (S protein), medium (M protein), and large (L protein). Differences in these proteins are caused by synthesis starting from different codons within on the same gene (9). Serological and molecular tests, including HBsAg and anti-HBs antibodies, are used for the diagnosis and follow-up of HB infection. Although HBsAg is a marker of HBV infection, anti-HBs is a marker of immunity to HBV infection. Anti-HBs generally appear after the loss of HBsAg. Simultaneous HBsAg and anti-HBs positivity is not an expected condition (10). The mechanisms leading to simultaneous HBsAg and anti-HBs antibody positivity remain controversial. This atypical serological profile may result from laboratory error or be caused by viral and host-related factors (11).

Although the HBV is a DNA virus, it has a high replication capacity (>10^{12} virion/day). Because the viral polymerase enzyme lacks proofreading activity during the reverse transcription process, it is susceptible to mutation during replication and has a high mutation frequency (10^5 substitutions/base/cycle) (6). These mutations result in a large number of variants. Although most mutations are single nucleotide substitutions, they can sometimes occur as deletions or insertions. The coexistence of HBsAg and anti-HBs can be caused by mutant HBV strains escaping the immune system, point mutations occurring in the S gene, and rarely, pre-S and S gene deletion mutations, particularly mutations in the determinant "a" or polymerase region (12,13,14,15,16).

The aim of this study was to determine the presence of mutant strains by sequencing HBV gene regions and performing pol/S gene mutation analysis in patients with concurrent HBsAg and anti-HBs positivity who were followed up for CHB infection.

Materials and Methods

Between 2021 and 2022, patients aged >18 years who were diagnosed with CHB and followed up in the department of infectious diseases and clinical microbiology, and who simultaneously tested positive for both HBsAg and anti-HBs by ELISA (ARCHITECT HBsAg Qualitative II Reagent Kit, ARCHITECT Anti-HBs Qualitative II Reagent Kit, and ARCHITECT i2000SR Immunoassay Analyzer) were included in the study. Patients who did not simultaneously test positive for both HBsAg and anti-HBs or who had decompensated cirrhosis, delta virus, hepatitis C, or human immunodeficiency virus infection were excluded from the study. Mutation analysis was performed on serum samples from HBV-DNA positive patients, and the pol gene was amplified and sequenced. This is shown in the flowchart (Figure 1).

Written informed consent was obtained from all patients who agreed to participate in this study. Age, gender, HBsAg, anti-HBs, HBeAg, anti-HBe levels, HBV-DNA load, and current treatments were recorded. A 10-mL whole-blood sample was obtained from each patient. The separated sera were stored at -20 °C until the time of the study.

HBV-DNA Quantitation

A QIAsymphony SP magnetic particle isolation platform (QIAGEN GmbH, Hilden, Germany) was used to isolate HBV-DNA. HBV-DNA was quantified by real-time polymerase chain reaction using an artus HBV-DNA RGQ kit on the RotorGene platform (QIAGEN GmbH, Hilden, Germany).

HBV Genotyping and Resistance Analysis

HBV genotyping was performed by sequencing all known primary/compensatory nucleos(t)ide analogs, resistance mutations, and mutations of the S gene (HBsAg protein; amino acids 111-227) that overlap with the reverse transcriptase (RT) domain (RT region, amino acids 80-250) (17). Forward (F: 5'-TCGTGGTGGACTTCTCTCAATT-3') and reverse (R: 5'-CGTTGACAGACTTTCCAATCAAT-3') primers were used to amplify the HBV pol gene (742 bp). Phire Hot Start DNA polymerase (Finnzymes Oy, Finland) was used in the sequencing protocol. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc., USA) according to the manufacturer's recommendations. Sequencing was performed using the ABI PRISM 3130 platform (Applied Biosystems Inc., USA). Vector NTI v5.1 software (InforMax, Invitrogen, Life Science Software, USA) was used to generate electropherograms. Sequences were analyzed using the Geno2pheno Drug Resistance software (Center of Advanced European Studies and Research, Germany).

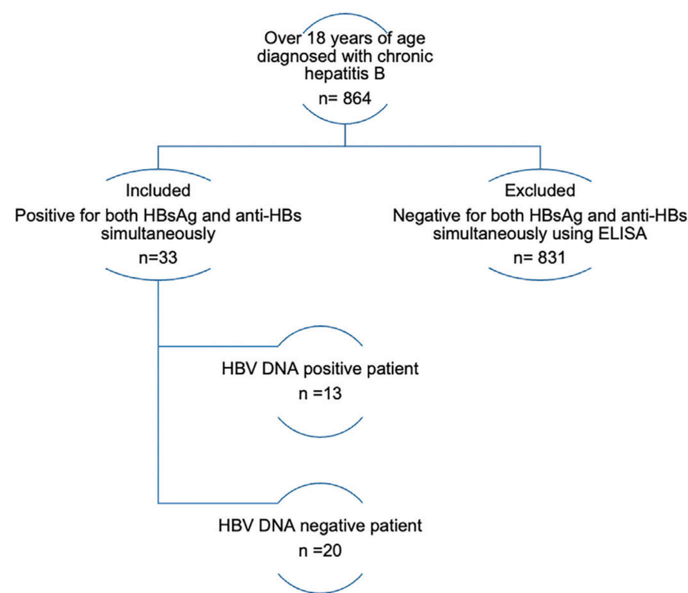

Figure 1. Patient selection flowchart

HBV: Hepatitis B virus, HBsAg: HB surface antigen, ELISA: Enzyme-linked immunosorbent assay

Statistical Analysis

Statistical analyses were performed using the SPSS (version 26.0) software package. Descriptive statistics were presented as mean ± standard deviation for normally distributed continuous data, median (minimum-maximum) for continuous variables with non-normal distribution, and numbers (n) and percentage (%) for categorical data.

Ethics Committee Approval

This study was approved by the Ethics Committee of the Faculty of Medicine, Hitit University (date: 28.04.2021, approval no: 457). This study was supported by the Scientific Research Project Unit of Hitit University (project number: TIP19001.21.004).

Results

A total of 864 patients diagnosed with CHB were followed up at the department of infectious diseases and clinical microbiology between 2021 and 2022. Of these, 33 patients (3.8%) with concurrent HBsAg and anti-HBs positivity were included in the study. Anti-HBe positivity was found in all cases (100%). HBV-DNA positivity was detected in 13 patients (39.4%). Among HBV-DNA-positive patients, 76.9% were male, and the mean age was 54.9 years. HBV-DNA results were negative in 20 of 33 (60.6%) patients with concurrent HBsAg and anti-HBs positivity (non-replicative) (Table 1).

The median anti-HBs titers were 51.4 mIU/mL in HBV-DNA-negative cases, 17.6 mIU/mL in HBV-DNA-positive cases, and 38.7 mIU/mL overall. Anti-HBs antibodies in HBV-DNA positive patients ranged from 11.1 to 25.9 mIU/mL (Table 1).

All HBV-DNA-positive had HBV genotype D (100%). An analysis of their sub-genotype showed that the D/D1 and D/D2 ratios were 45.5% and 54.6, respectively. Mutational analysis was performed on serum samples from 13 HBV-DNA-positive patients (3.8%) and the pol gene was amplified and sequenced. Codon analyses of the RT domain (polymerase/pol mutation) and of the HBsAg protein (S gene) were performed in all patients. In two of the 13 (15.4%) HBV-DNA-positive patients (P3, P7), sequence-based mutation analysis of the pol/S gene was not performed. Pol/S gene mutations were detected in 11 (84.6%) of the 13 patients. In these patients, 68 HBsAg mutations (42.8%) and 91 RT mutations (57.2%) were identified, resulting in a total of 159 mutations. While three patients (27.2%) were found to have a clinically significant HBsAg mutation, four patients (36.3%) were found to have an RT mutation. Three patients were found to have anti-HBs levels above 20 mIU/mL, and no pol/S gene mutation was detected in two of them. The mean HBV-DNA level was 2.8+E5 IU/mL (range, 6.2+E1-3.7+E6) (Table 1). Only one patient was found to have clinically significant concurrent HBsAg and RT mutations, and this patient had the highest viral replication (HBV-DNA: 3.7+E6) (Table 2).

Two patients (15.4%) received antiviral therapy, whereas 11 patients (84.6%) did not receive treatment. While one HBV-DNA-positive patient received entecavir, another received tenofovir disoproxil fumarate (Table 1). Drug resistance to nucleoside/nucleotide analogs was observed in 18.2% (n=2) of patients. While one patient (P1) had primary resistance to entecavir, telbivudine, and lamivudine, another patient (P4) had primary resistance to telbivudine and lamivudine. The same patient was moderately susceptible to entecavir. None of the patients with resistance mutations received antiviral therapy. Two cases (18.18%) were found to have the HBsAg vaccine escape mutation (Table 2).

Table 1. Demographic and laboratory characteristics of HBsAg, anti-HBs positive patients

Group	Age	Gender	Anti-HBs unit, mIU/mL	HBV-DNA load, IU/mL	Anti-HBe status	Treatment history
HBV-DNA positive patient						
P1	58	Female	12.4	6.9+E1	Positive	None
P2	51	Male	11.1	1.1+E3	Positive	None
P3	37	Female	34.2	4.3+E2	Positive	None
P4	68	Male	13.7	6.0+E2	Positive	None
P5	54	Male	14.8	1.6+E3	Positive	None
P6	51	Female	17.7	6.2+E3	Positive	TDF*
P7	50	Male	21.9	1.2+E3	Positive	None
P8	45	Male	17.2	4.0+E3	Positive	None
P9	76	Male	18.8	9.9+E2	Positive	None
P10	64	Male	11.8	1.4+E2	Positive	None
P11	38	Male	17.4	3.7+E6	Positive	None
P12	63	Male	25.9	3.4+E2	Positive	None
P13	58	Male	12.2	9.2+E2	Positive	ETV
			Median: 17.6	Median: 2.8+E5		
HBV-DNA negative patient						
P14	76	Male	46.4	Negative	Positive	None
P15	33	Female	276	Negative	Positive	None
P16	65	Female	17.9	Negative	Positive	None
P17	45	Male	10.9	Negative	Positive	None
P18	70	Male	12.9	Negative	Positive	None
P19	80	Male	22.5	Negative	Positive	TDF
P20	48	Female	16.7	Negative	Positive	None
P21	57	Female	18.4	Negative	Positive	None
P22	61	Female	42.1	Negative	Positive	None
P23	30	Female	20	Negative	Positive	None
P24	42	Female	12	Negative	Positive	None
P25	49	Female	11.5	Negative	Positive	None
P26	55	Male	11.9	Negative	Positive	None
P27	42	Male	14.6	Negative	Positive	None
P28	50	Female	16.5	Negative	Positive	None
P29	62	Female	11.4	Negative	Positive	None
P30	62	Male	25.4	Negative	Positive	None
P31	67	Female	19.9	Negative	Positive	None
P32	64	Male	19.3	Negative	Positive	None
P33	62	Female	401.5	Negative	Positive	TDF
			Median: 51.4			

HBV: Hepatitis B virus, HBsAg: HB surface antigen, anti-HBe: HB e-antibody, TDF: Tenofovir disoproxil fumarate, ETV: Entecavir, P: Patient

Table 2. Mutations in the pol/S gene in HBsAg/anti-HBs antibody-positive patients

Case	The HBV genotype	HBV sub-genotype	Clinically significant RT mutation	Clinically significant HBsAg mutation	Antiviral resistance	Clinical significance
P1	D	D1	ND	ND	Lamivudin, entecavir and telbivudin resistant	
P2	D	D2	ND	ND	ND	
P4	D	D1	ND	ND	Lamivudin and telbivudin resistance, entecavir is moderately susceptible	
P5	D	D1	ND	**W172L**	ND	W172L; mutations in the HBsAg caused by acyclic phosphonate. Naturally occurred
P6	D	D2	**Q215H**	ND	ND	Q215H; accessory mutation. Repair of HBV replication/ viral fitness
P8	D	D1	ND	ND	ND	
P9	D	D2	ND	**S193L**	ND	S193L; HBsAg vaccine escape mutation. Naturally occurred
P10	D	D2	**Q149K**	ND	ND	Q149K; accessory mutation. Repair of HBV replication/ viral fitness
P11	D	D2	**Q149K, A194T**	**S193L**	ND	S193L; HBsAg vaccine escape mutation. Naturally occurred
P12	D	D2	**A194T**	ND	ND	A194T; mutation occurring in TDV treatment; repairs HBV replication/viral fitness
P13	D	D1	ND	ND	ND	

P3 and P7 were not sequenced. HBV: Hepatitis B virus, HBsAg: HB surface antigen, ND: Not detected, P: Patient, TDV: Tenofovir disoproxil, RT: Reverse transcriptase

Discussion

In patients with CHB, the coexistence of HBsAg and anti-HBs may be detected during follow-up. Studies conducted in Turkey and other parts of the world have reported that the prevalence of serological profiles with simultaneous HBsAg and anti-HBs positivity ranges from 2.8% to 9% (10,18,19,20). In our study, the coexistence of HBsAg and anti-HBs was observed in 3.8% of patients with CHB infection, which is consistent with the literature.

Although the underlying molecular mechanisms for the coexistence of HBsAg and anti-HBs in patients with CHB infection are not clear, it may be due to the selection of immune escape mutants of HBV during chronic process, and pre-S and S gene deletion mutations have been identified in such cases (9,21,22). Some studies have shown that this is usually caused by S gene point mutations, but it is rarely associated with pre-S and S gene mutations (18,23). In our patients with HBsAg and anti-HBs positivity who were followed up with CHB, mutation analysis of pol/S genes by sequence analysis of HBV gene regions showed that a total of 159 mutations were observed, including sixty-eight HBsAg mutations (42.8%) and 91 RT mutations (57.2%); of these, a clinically significant HBsAg mutation was detected in three cases (27.27%) and an RT mutation in four cases (36.36%). The presence of these mutations is important in the context of treatment and immunosuppression.

When the relationship between the observed mutations and the HBV-DNA result was evaluated, a patient with a combination of clinically significant mutations in HBsAg and RT had the highest rate of viral replication (HBV-DNA: 3.7+E6). It has been reported that the pre-S/S and polymerase genes sometimes overlap during replication, and some codons may overlap. Increased HBV replication leading to a severe clinical course has been observed in HBV variants with combinations of HBsAg and RT mutation (18). This case was closely monitored clinically. Mutations were not analyzed in 60.6% of patients with negative HBV-DNA and in two patients with positive HBV-DNA (P3 and P7) because sequencing could not be performed. The presence of these mutations in individuals testing negative for HBV-DNA suggests they may originate from remnants of a previously formed viral variant or a low-level persistent mutant virus.

Analysis of the relationship between these mutations and genotype shows that pre-S/S mutations (HBsAg mutation) are more common in genotype D infection, which is associated with fulminant

hepatitis (18). Despite this study, other studies have reported that genotype C has more mutations (16,24). In our study, genotype D was found in all included patients, and no mixed isolates were observed. In patients with genotype D, fewer clinically significant HBsAg mutations (27.2%) were detected than RT mutations (36.3%). D2 was the dominant sub genotype in our cohort (54.6%). In Türkiye, genotype D is dominant, as in Southern Europe, the Middle East, and India. The sub-genotypes observed in Türkiye range from D1 to D4. D1 is the most common sub-genotype. Although HBeAg seroconversion is observed at lower rates in patients infected with genotype D (7,25), all of our patients had HBeAg seroconversion. We must always consider the presence of these mutations when monitoring our patients.

Mutant HBV variants can also infect vaccinated individuals and are not neutralized by vaccine-derived anti-HB antibodies; these are known as vaccine escape mutations. The emergence and predominance of vaccine escape mutants may pose a serious threat to the control of HBV infection. Some common immune escape mutants are G145R, D144A, P142S, Q129H, I/T/126N/A and M133L (18,24). A study in which genotypes D (81.3%) and E (17.3%) were dominant reported that vaccine escape mutations (P120T, D144E/A and G145R) were detected in genotype D isolates (19). All patients in our study were genotype D, and 18.2% had the S193L HBsAg mutation, a vaccine escape mutation. This mutation can occur with entecavir use (24), but it was not associated with drug use in our study because the patients were not receiving antiviral drugs. This mutation developed naturally. This suggests that HB infection can develop despite the presence of immunoglobulin.

Nucleoside analogs can induce point mutations in the pol gene. The formation of point mutations can lead to drug resistance (17). These mutations can be divided into two main groups: primary drug resistance mutations, which result in non-response to treatment, and compensatory mutations, which affect viral fitness (increased viral load and restored replication capacity). The W172L mutation in HBsAg caused by acyclic phosphonate has been frequently observed in patients receiving lamivudine (17,26). In our study, two patients who developed drug resistance had no prior exposure to antiviral therapy. This situation suggests to us that it may be an infection caused by a resistant strain or it may have developed spontaneously through mutation. Close monitoring of these patients is important.

When the relationship between the observed mutations and the anti-HBs titers was evaluated, the anti-HBs levels of our patients ranged from 11.1 to 25.9 mIU/mL. The median anti-HBs titer was 51.4 in the HBV-DNA-negative group and 17.6 in the HBV-DNA-positive group. Anti-HBs titers were lower in the HBV-DNA- positive group. This interpretation may be supported by the increase in anti-HBs levels, which suggests a weak immune response. No mutation was detected in two of the three cases (P3, P7, P12) with anti-HBs levels above 20 mIU/mL (P3 and P7). Thus, in proportion to the anti-HB titer, more amino acid mutations are observed in HBsAg and anti-HBs positive patients (27).

In our study no correlation was found between the number of mutations observed and the anti-HB titer. Given the small number of cases, this approach should be supported by studies with larger sample sizes.

Study Limitations

1. No patients under the age of 18.
2. The number of cases is small.

Conclusion

During the follow-up of patients with CHB, HBsAg and anti-HBs may be observed together. The mutations detected in the pol/S gene analyses of the HBV genomes of these patients are not clinically significant; these mutations are naturally occurring, and the association of HBsAg and anti-HBs could not be explained by these mutations.

Ethics

Ethics Committee Approval: This study was approved by the Ethics Committee of the Faculty of Medicine, Hitit University (date: 28.04.2021, approval no: 457).

Informed Consent: Written informed consent was obtained from all patients who agreed to participate in this study.

Footnotes

Authorship Contributions

Concept: Ö.A., N.B., A.K.Ç., Design: Ö.A., M.S., N.B., A.K.Ç., Data Collection or Processing: Ö.A., D.Y., Ü.S., G.K., Analysis or Interpretation: Ö.A., M.S., Ü.S., N.B., A.K.Ç., Literature Search: Ö.A., D.Y., M.S., N.B., A.K.Ç., Writing: Ö.A., D.Y., M.S., N.B., A.K.Ç.

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

Financial Disclosure: This study was supported by the Scientific Research Project Unit of Hitit University (project number: TIP19001.21.004).

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