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ORIGINAL ARTICLE

Association of HIF-1α and TNFα single nucleotide polymorphisms with periodontal disease in diabetic patients

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Received: 24 September 2024, Accepted: 1 January 2025, Published online: 18 March 2025

ABSTRACT

Objective: Periodontal disease is a prevalent chronic inflammatory condition affecting the supporting structures of teeth and is considered one of the chronic complications of Type 2 diabetes mellitus (T2DM). Both diabetes and periodontal diseases are complex, multifactorial diseases to which genetic factors play a crucial role in susceptibility. The TNF-α/HIF-1 pathway might have a regulatory function in periodontal tissues. Several case-control studies have examined the association between TNF-α G308A or HIF-1α C1772T polymorphisms and diabetes complications, but the results have been inconsistent. We aimed to investigate the association between two specific genetic variants -HIF-1α C1772T and TNF-α G308A- and periodontal disease in patients with type 2 diabetes.

Methods: A total of 109 individuals were enrolled in the study including 24 chronic periodontitis with T2DM (group 1), 35 gingivitis with T2DM (group 2), 26 non-diabetic individuals with chronic periodontitis (group 3) and 24 periodontally healthy non-diabetic individuals (group 4). The normal allelic and genotype distribution of these variants was analyzed in healthy Turkish adults (n: 120), independent of the study cohort. Allele and genotype distribution of group 4 and healthy Turkish adults were similar. Allelic and genotypic comparisons between group 4 and other groups were evaluated by PCR-RFLP. Allelic, dominant, and recessive genetic models were calculated to assess the strength of the association.

Results: We found a significant association between the A allele at TNF-α G308A and the risk of gingivitis in T2DM (OR=3.75, CI:1.015–13.860, p=0.048). There was no association detected between HIF-1α C1772T polymorphisms and risk for periodontal diseases with T2DM.

Conclusion: These results suggest that TNF G308A polymorphism may be involved in the pathogenesis of periodontal disease in diabetics. Future studies may contribute to the investigation of the potential polygenic predisposition of the diseases and reinforce our findings.

Keywords: diabetes mellitus, periodontal disease, chronic periodontitis, gingivitis, single nucleotide polymorphism, HIF-1α, TNF-α, rs11549465, rs1800629

INTRODUCTION

Type 2 diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia. DM affects millions of people around the world with its rapidly increasing incidence and prevalence [1,2]. Along with the known macrovascular and microvascular complications, periodontal disease is considered to be one of the chronic complications of diabetes [3]. Periodontal disease is a multifactorial condition influenced by various genetic and environmental factors, and is characterized by the specific pathogenic bacteria colonized in the supporting tissues surrounding the teeth and the specific host response [4]. Diseases affecting the tooth-supporting structures are defined as gingivitis or periodontitis. The clinical features that distinguish periodontitis from gingivitis are the progressive destruction of the periodontal ligament and alveolar bone accompanied by periodontal pocket formation and/or gingival recession [5,6].

Inflammation is the key feature of both diabetes and periodontal diseases. Proinflammatory cytokines such as IL-1, and tumor necrosis factor-α (TNF-α) play a key role in the pathogenesis of periodontal diseases and inhibition of these cytokines reduces periodontitis-associated bone loss [7-10]. Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor consisting of alpha and beta subunits [11,12]. Many cells respond to hypoxia and ischemia by increasing the HIF-1-dependent transcription of vascular endothelial growth factor and other angiogenic growth factors [13]. On the other hand, TNF-α is the main pro-inflammatory cytokine, and hypoxia increases TNF-α expression in various cells, including osteoblasts, which in turn activates the HIF-1α pathway [14].

Both diabetes and periodontal diseases are complex, multifactorial diseases to which genetic factors play a crucial role in susceptibility [2,6]. HIF-1α and TNF-α are key molecules involved in inflammatory and angiogenic processes, both of which are critical in the pathogenesis of periodontal disease and diabetes. Single nucleotide polymorphisms (SNPs) are the most common form of polymorphism and affect the function of the gene [15]. Several case-control studies have examined the association between TNF-α G308A (rs1800629) or HIF-1α C1772T (rs11549465) polymorphisms and diabetes complications, but the results have been inconsistent [16,17].

This study aims to evaluate the association between two specific genetic variants -HIF-1α rs11549465 and TNF-α rs1800629- and periodontal disease in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Subject characteristics

The research was conducted as a single-center, cross-sectional, case-controlled cohort study at Hacettepe University. Fifty-nine patients with type 2 DM (T2DM) and 50 non-diabetic control individuals aged 30-65 were included in the study.

Patients were divided into four groups according to the presence of diabetes and periodontal disease;
Group 1: T2DM patients with chronic periodontitis (n:24),
Group 2: T2DM patients with gingivitis (n:35),
Group 3: Non-diabetic individuals with chronic periodontitis (n:26),
Group 4: Periodontally healthy non-diabetic individuals (n:24).

T2DM diagnosis was made according to American Diabetes Association criteria [1]. Patients were evaluated for microvascular and macrovascular complications. In the periodontology department, all parameters were evaluated in each patient for 6 teeth containing 6 mm or deeper periodontal pockets. If one of these teeth was absent in the mouth, the sampling was performed using the neighboring teeth with similar characteristics. Plaque index (PI), gingival index (GI), probing pocket depth (PPD), clinical attachment loss (CAL), and bleeding on probing (BOP) were recorded by a periodontal probe (Michigan O Color-Coded Probe, Hu-Friedy, Chicago, IL) for each tooth. The separated serum from the 10 ml blood was stored at -80 °C until tested for genotyping. This study (GO 14/250) was approved by the Non-Interventional Clinical Research Ethics Board of Hacettepe University. Informed consent was obtained from each patient.

Patients were excluded from the study if they had a diagnosed malignancy or paraproteinemia, were taking medications with known side effects on teeth or gums, had a history of preeclampsia or eclampsia during pregnancy, or had a history of ileus. Additional exclusion criteria included patients undergoing hemodialysis or peritoneal dialysis for chronic kidney disease, those with chronic liver disease, individuals outside the age range of 30-65 years, pregnant women, lactating women and active smokers.

Anthropometric parameters

Age, sex, height, weight, body mass index (BMI), blood pressure, and waist circumference were recorded.

Biochemical parameters

A1c level was measured by high-performance liquid chromatography method.

Genotyping analysis

In the Department of Medical Genetics, Hacettepe University, genotyping of TNF-α and HIF-1α was performed from the blood samples of a total of 109 participants.

To determine the genotype and allele distributions of HIF-1α, and TNF-α in the general population, polymorphism analysis of these genes was performed from the blood samples of 120 healthy individuals (37F/83M) in addition to these 109 participants. Thus, it was aimed at obtaining the normal allele and genotype distribution of these SNPs in healthy adults. Healthy individuals were selected among the hospital employees and the relatives of patients without any illness, who were referred to our department and willing to participate in the study.

1) DNA Extraction and PCR

Genomic DNA was extracted from peripheral blood using the salt precipitation method. The samples were stored at -80° C in an appropriate buffer solution. All Polymerase Chain Reactions (PCR) were performed using GoTaq Flexi DNA Polymerase (Promega, WI, ABD) at temperatures for optimal binding of primers.

2) Genotyping

a) TNFα (rs1800629)

Genotyping was performed by PCR + DNA Restriction Fragment Length Polymorphism (RFLP) method using 5'-AGG CAA TAG GTT TTG AGG GCC ATG-3' and 5'-ACA CAC AAG CAT CAA GGA TAC-3'. Obtained PCR products were subjected to StyI (Catalog no: R0500S, New England Biolabs, MA, ABD) DNA restriction enzyme.

b) HIF1 (rs11549465)

Genotyping was performed by PCR + DNA Restriction Fragment Length Polymorphism (RFLP) method using 5'-GAC TTT GAG TTT CAC TTG TTT-3' and 5'-ACT TGC GCT TTC AGG GCT TGC GGA ACT GCT T-3'. Obtained PCR products were subjected to NmuCI DNA restriction enzyme. The obtained PCR products will be subjected to Tsp45I (Catalog no: R0583L, New England Biolabs, MA, USA) DNA-cutting enzyme at 37 C.

All samples were genotyped by moving on agarose gel. The verification process was done for 20 samples by DNA sequence analysis method using the forward or reverse primers.

Statistical analysis

All statistical analyses were performed using the SPSS20.0 (SPSS software package, Chicago, USA). The Hardy Weinberg equation of the gene polymorphisms were analyzed by chi-square test. Linkage disequilibrium was evaluated with R software (genetics package). No relationship was detected between the SNPs. The comparison of the data of other frequency types by allele or genotype was done by chi-square test and odds ratio was calculated. Allelic, dominant and recessive genetic models were calculated to assess the strength of the association. A difference with a P value of <0.05 was considered as statistically significant. First of all, the normal distributions for all the continuous data were tested and logarithmic transformation was applied to non-normally distributed data. Continuous variables were expressed as mean and standard deviation. The differences between the groups were evaluated using the Student's t-test for normally distributed variables and the Mann-Whitney-U test for non-normally distributed variables. On the other hand, the Kruskal-Wallis test was used for the analysis of more than three

non-normally distributed groups, and paired comparisons were made with the Dunn test in case of a difference between the groups. Chi-square and Fisher's exact test were used for the evaluation of the categorical variables.

RESULTS

Comparison of Different Groups

Fifty-nine diabetic patients (36F/23M, mean age: 53.6±6.4) and 50 non-diabetic individuals (27F/23M, mean age: 41.0±8.5) were included in the study. As expected, the body weight (86.9±16.4 and 74.4±14.1 kg), BMI (34.6±7.2 and 26.8±4.7kg/m^2), and waist circumference (102.8±12.4 and 89.3±11.7 cm) in the diabetic group was statistically significantly higher than the non-diabetic group ($p<0.001$).

Eighty five patients with periodontal disease and 24 periodontally healthy patients were also compared. As expected, periodontal PD (4.0±1.9 mm), CAL (4.5±2.1 mm), PI (1.2±0.7), GI (1.6±0.6), BOP (95.8%) in the group with periodontal disease was statistically significantly higher than the patients without periodontal disease ($p<0.001$).

Thirty-five T2DM patients with gingivitis (27 F/8 M, mean age: 54.0±5.4) and 24 T2DM patients with chronic periodontitis (9 F/15 M, mean age: 51.3±7.8) were compared. While the age at diagnosis was 44.4±7.6 in the group with diabetic gingivitis, it was 45.2±7.7 in the group with diabetic periodontitis ($p=0.694$). There was no significant difference between fasting plasma glucose (158.2±61.7 mg/dL), postprandial glucose (184.4 ± 78.5 mg/dL), A1c levels (7.5±1.8%) of patients in the gingivitis group and those in the periodontitis group with fasting plasma glucose (173.0±60.7 mg/dL), postprandial glucose (219.2±89.2 mg), A1c levels (8.4±1.9%) ($p=0.174$; 0.132; 0.063, respectively). The mean BMI of the diabetic gingivitis group was 34.6±8.3 kg/m^2 and this is the group with the highest number of patients among the groups. The mean BMI was 33.0±5.8 kg/m^2 in the diabetic periodontitis group. On the other hand, the mean BMI of the non-diabetic periodontally healthy group, which we accepted as a reference and made the genotype and allele comparison of all groups accordingly, was found to be 25.3±2.7 kg/m^2. The mean PPD, CAL, and PI were higher in the diabetic periodontitis group than the non-diabetic periodontitis group, but there was no statistically significant difference.

Genotype and Allele Frequencies of HIF-1α, TNF-α in Adults

The normal frequency of genotype and allele distribution of HIF-1α and TNF-α in adults was intended to be seen with the 120 healthy individuals in the Medical Genetics Department's pool. Genotype and allele distribution of HIF-1α and TNF-α in the general population are shown in detail in Table 1. Each SNP was tested for Hardy-Weinberg equilibrium and all were in equilibrium. No association was detected between the HIF-1α – TNF-α SNPs.

Evaluation of TNF-α Polymorphism According to the Study Groups

The GG genotype, which is frequent in the population, was detected to be significantly less frequent in the diabetic gingivitis group ($p=0.026$). Similarly, the frequent G allele in the population was significantly less frequent in the diabetic gingivitis group ($p=0.013$) (Table 2). We showed that TNF-α G308A polymorphism is associated with an increased risk for gingivitis in T2DM patients. In other words, the risk of gingivitis was found to be 4 times higher in GA+AA carrier diabetic patients (OR=4.13, CI:1.0-16.6, $p=0.045$). In line with this, the risk of diabetic gingivitis was detected to be increased by approximately 4 fold in mutant A allele carriers (OR=3.75, CI:1.015-13.860, $p=0.048$). There was no statistically significant difference between the other groups.

Table 1. Genotype and Allele Frequencies of Hypoxia Inducible Factor-1α (HIF-1α) and Tumor Necrosis Factor-α (TNF-α) in the Turkish Population

HIF-1α (rs11549465) (n:120)		**TNF-α (rs1800629) (n:120)**	
Genotype		**Genotype**	
CC	76.6% (n:92)	**GG**	67.5% (n:81)
CT	20.8% (n:25)	**GA**	31.7% (n:38)
TT	2.5% (n:3)	**AA**	0.8% (n:1)
Allele		**Allele**	
C	87.1% (n:209)	**G**	83.3% (n:200)
T	12.9% (n:31)	**A**	16.7% (n:40)

HIF-1α: Hypoxia Inducible Factor-1alpha, TNF-α: Tumor Necrosis Factor-alpha, G: Guanine, C: Cytosine, A: Adenine, T: Thymine, n: Number of patients.

Table 2. Allele and Genotype Frequencies of TNF-α G308A polymorphisms (rs1800629) in the study groups

GENOTYPES	GG	GA	AA	P*
Non-DM/Healthy PS (Group 4)	87.5% (n:21)	12.5% (n:3)	0% (n:0)	
Non-DM/Periodontitis (Group 3)	80.8% (n:21)	19.2% (n:5)	0% (n:0)	0.445; 0.723
DM/Gingivitis (Group 2)	62.9% (n:22)	34.3% (n:12)	2.9% (n:1)	**0.026**; 0.140
DM/Periodontitis (Group 1)	75% (n:18)	25% (n:6)	0% (n:0)	0.237; 0.511
GROUPED GENOTYPES	**GG**	**GA + AA**		
Non-DM/Healthy PS (Group 4)	87.5% (n:21)	12.5% (n:3)		
Non-DM/Periodontitis (Group 3)	80.8% (n:21)	19.2% (n:5)		0.723
DM/Gingivitis (Group 2)	62.9% (n:22)	37.1% (n:13)		0.090
DM/Periodontitis (Group 1)	75% (n:18)	25% (n:6)		0.511
ALLELES	**G**	**A**		
Non-DM/Healthy PS (Group 4)	93.8% (n:45)	6.3% (n: 3)		
Non-DM/Periodontitis (Group 3)	90.4% (n:47)	9.6% (n:5)		0.438; 0.811
DM/Gingivitis (Group 2)	80% (n:56)	20% (n:14)		**0.013**; 0.221
DM/Periodontitis (Group 1)	87.5% (n:42)	12.5% (n:6)		0.228; 0.663

DM: Diabetes mellitus, PS: Periodontal status, n: Number of patients, G: Guanine, A: Adenine.

*All genotypes, grouped genotypes, and alleles were compared relative to Group 4, and p values were written in order.

Evaluation of HIF-1α Polymorphisms According to the Study Groups

HIF-1α genotyping was performed using the DNA samples of 109 participants in the study groups. First of all, the genotype and allele distribution in groups were analyzed. Because the HIF-1α TT genotype is very rare in the population, it was also grouped with CT heterozygotes. Our main objective was to evaluate the relationship between periodontal diseases in diabetes and HIF-1α. Allele and genotype frequencies of HIF-1α in the study groups are shown in Table 3. Intergroup comparisons were made between the non-diabetic, periodontally healthy group (group 4) and the other groups. All genotypes, grouped genotypes and alleles were compared relative to Group 4 and p values were written in order (Table 3). No statistically significant difference was observed between the groups (group 3 and others) in terms of the genotype and allele distribution of HIF-1α C1772T polymorphisms. Odds ratio and confidence interval (CI) were calculated for all groups setting HIF-1α CC genotypes (dominant model) as reference. There was no statistically significant difference between the groups.

Table 3. Allele and Genotype Frequencies of HIF-1α C1772T polymorphisms (rs11549465) in the study groups

GENOTYPES	CC	CT	TT	P*
Non-DM/Healthy PS (Group 4)	66.7% (n:16)	33.3% (n:8)	0% (n:0)	
Non-DM/Periodontitis (Group 3)	80.8% (n:21)	15.4% (n:4)	3.8% (n:1)	0.117 / 0.394
DM/Gingivitis (Group 2)	57.1% (n:20)	42.9% (n: 15)	0% (n:0)	0.397 / 0.465
DM/Periodontitis (Group 1)	66.7% (n:16)	20.8% (n:5)	12.5% (n:3)	>0.9 / 0.529 / 0.580
GROUPED GENOTYPES	**CC**	**CT + TT**		
Non-DM/Healthy PS (Group 4)	66.7% (n:16)	33.3% (n:8)		
Non-DM/Periodontitis (Group 3)	80.8% (n:21)	19.2% (n:5)		0.468
DM/Gingivitis (Group 2)	57.1% (n:20)	42.9% (n:15)		0.465
DM/Periodontitis (Group 1)	66.7% (n:16)	33.3% (n:8)		>0.9
ALLELES	**C**	**T**		
Non-DM/Healthy PS (Group 4)	83.3% (n:40)	16.7% (n:8)		
Non-DM/Periodontitis (Group 3)	88.5% (n:46)	14.3% (n:6)		0.282 / 0.837
DM/Gingivitis (Group 2)	78.6% (n:55)	21.4% (n:15)		0.393
DM/Periodontitis (Group 1)	77.1% (n:37)	22.9% (n:11)		0.372

DM: Diabetes mellitus, PS: Periodontal status, n: Number of patients, C: Cytosine, T: Thymine.

*All genotypes, grouped genotypes, and alleles were compared relative to Group 4, and p values were written in order.

DISCUSSION

Our study provides valuable insights into the genetic basis of periodontal disease in Turkish diabetic patients. Our study showed that TNF-α G308A polymorphism is associated with an increased risk for gingivitis in T2DM patients. The risk of diabetic gingivitis was found to be increased by approximately 4-fold in mutant A allele carriers. These results suggest that TNF G308A polymorphism may be involved in the pathogenesis of periodontal disease in diabetics. Our study emphasizes the importance of considering genetic factors in the management of periodontal complications in diabetic individuals.

In a meta-analysis including the analysis of 31 studies, Ding et al. evaluated the potential effect of TNF-α G308A polymorphism on periodontitis. As a result, the AA genotype in Asians was associated with an increased risk for aggressive periodontitis [18]. On the other hand, a meta-analysis including the analysis of 52 studies suggested that the TNF-α G308A polymorphism could be a protective factor against chronic periodontitis and aggressive periodontitis in Asians [19]. In the meta-analysis by Shi et al. [16], TNF-α G308A polymorphism was associated with chronic periodontitis in T2DM patients in the Asian population, while no significant risk was detected among Caucasian populations [16]. In our study, we found a significant relationship between TNF-α G308A polymorphism and diabetic gingivitis, but we did not find a significant relationship between TNF-α rs1800629 polymorphism and diabetic chronic periodontitis.

Many previous publications have suggested that the A allele is a possible marker of the severity of periodontal disease and the authors attempted to explain this by the increased regulatory effect of the A allele on TNF-α production [16]. The result of our study suggests that the increase in the frequency of A allele may be an early indicator of the progression of periodontal disease in diabetic patients. Smoking is a major risk factor for periodontitis [20]. While actively smoking individuals were excluded from our study, those with a history of smoking were included. Environmental factors play an important role in the progression of periodontitis and smoking history may have masked the effect of this variation.

Brand et al. [21] investigated the effect of TNF G308A polymorphism on obesity in 176 Caucasian cases. TNF 308-A allele carriers were detected to have significantly higher BMI than the G allele carriers [21]. In our study, the frequency of A allele was found to be significantly higher in the diabetic gingivitis group. At the same time, BMI was higher in the diabetic gingivitis group compared to other groups. In light of these findings; the statistically significant increase in the frequency of TNF 308-A allele in the diabetic gingivitis group may be related to BMI rather than gingivitis. On the other hand, Modéer et al. investigated the periodontal status of obese adolescents. It was shown that more gingivitis and pathological periodontal pocket depth (>4mm) were present in obese patients compared to normal-weighted individuals, however there was no difference in incipient alveolar bone loss. So, the risk of gingivitis increases in obese patients. There is a positive correlation between obesity and periodontal risk indicators [22]. In our study, the A allele frequency was increased in the diabetic gingivitis group, it may be related to raising the risk of both obesity and gingivitis.

Periodontal disease arises from a complex interplay of factors, including genetic and epigenetic variations, lifestyle influences such as smoking and diet, systemic conditions like diabetes, and local dental or stochastic factors. Over 60 genetic variants have been implicated in periodontitis, highlighting pleiotropic links with conditions like cardiovascular diseases. Despite advances, further research is needed to deepen our understanding of how genetic and inflammatory pathways contribute to the disease's pathogenesis and to solidify cause-and-effect relationships [23]. On the other hand, a study in minipigs by Li et al. has shown that epigenetic changes, such as DNA methylation, contribute to increased susceptibility to periodontal disease in diabetic patients [24].

DM and periodontal diseases are common, chronic, multifactorial diseases. In many studies, diabetes was shown to be an important risk factor for the development of gingivitis and periodontitis [23]. Poorly controlled diabetes (A1c≥7) is associated with the progression of periodontal disease. In addition, periodontal diseases also adversely affect

glycemic control in diabetics [25]. Compatible with literature, blood glucose regulation was found to be worse in diabetics with periodontal disease in our study. A1c level was detected to be higher in the diabetic chronic periodontitis group (8.4%) than in the diabetic gingivitis group (7.5%). This finding indicates the adverse effect of poor glycemic control on periodontal tissues.

T2DM patients are known to have more gingival inflammation than non-diabetics [26]. PPD, CAL and PI levels are risk indicators for periodontal disease. In our study, the periodontal risk indicators of individuals with diabetic and non-diabetic periodontitis were examined. Mean PPD, CAL, and PI values were found to be higher in the group with diabetic periodontitis than in the non-diabetic periodontitis group, but this difference was not statistically significant.

Our study is the first in the literature, we examined the association between HIF-1α C1772T polymorphism and periodontal disease in diabetic patients. When we compared diabetic gingivitis and diabetic periodontitis groups with the non-diabetic periodontally healthy control group; no significant association was detected between the HIF-1α C1772T polymorphism and the development of periodontal diseases. In light of this information, we can emphasize that HIF-1α C1772T polymorphism does not play an important role in the development of diabetic periodontal disease. Nevertheless, the variation in this locus may have moderate effects on the development of periodontal diseases. However, environmental factors are dominant in both T2DM and periodontitis progression and may have caused this variation to be overlooked.

Our study had several limitations: (1) we had a small sample size, (2) it didn't include diabetic periodontal healthy individuals, and (3) the potential influence of demographic and clinical features, such as the duration of diabetes, presence of complications, and medications, on the development of periodontal disease could not be evaluated due to the limitations of our study design. This inability to perform subgroup comparisons among diabetes groups should be acknowledged as a limitation in interpreting the results.

CONCLUSION

TNF G308A polymorphism may be involved in the pathogenesis of periodontal disease in diabetics. Being a carrier of GA + AA, namely the presence of mutant A allele, increases the risk of diabetic gingivitis approximately 4-fold. To elucidate the importance of these SNPs in the pathogenesis of periodontal diseases, which occur more frequently in diabetes; more comprehensive studies, considering the effect of environmental factors that impact diabetes and periodontal diseases, should be conducted. Further research with larger cohorts and diverse populations is warranted to validate and expand upon these findings. Understanding the genetic underpinnings of periodontal disease in diabetic patients could pave the way for personalized therapeutic approaches and targeted interventions.

Author contribution

Study conception and design: SKK, YDİ, MA, RN, and TE; data collection: SKK, YDİ, NH, YÖ, AD, SD, RN, and TE; analysis and interpretation of results: SKK, SK, EK, MA, RN, and TE; draft manuscript preparation: SKK, MA, RN, and TE. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study was approved by the Non-Interventional Clinical Research Ethics Board of Hacettepe University (Protocol no. GO 14/250 / 30.04.2014). Informed consent was obtained from each patient

Funding

This work was supported by H. U. B. A. B. THD-2015-5408. The authors declare that there is no duality of interest associated with this manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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