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Fibrinogen-Aα THR312ALA Polymorphism is Associated to Chronic Thromboembolic Pulmonary Hypertension in Turkey

ABSTRACT

Background: Chronic thromboembolic pulmonary hypertension is a condition that occurs after mechanical obstruction of the pulmonary arteries by thrombus. Since the frequency and demographics of chronic thromboembolic pulmonary hypertension differ between countries, it is thought that genetic factors may play a role in its development. The aim of this study is to reveal the status of *VKORC1*, *CYP2C9*3*, *CYP2C9*7*, and *fibrinogen-Aα* THR312ALA gene polymorphisms in chronic thromboembolic pulmonary hypertension patients in Turkey.

Methods: In this prospective cross-sectional study, a total of 46 chronic thromboembolic pulmonary hypertension patients and 106 healthy volunteers were included. Polymerase chain reaction-restriction fragment length polymorphism method was used to determine candidate gene polymorphisms for chronic thromboembolic pulmonary hypertension. The general population parameters of each locus were calculated, and the relationship between dominant, co-dominant, and recessive genotype models and chronic thromboembolic pulmonary hypertension was analyzed.

Results: For the *fibrinogen-Aα* gene, those with the THR/THR genotype were found to have a 13.51 (95% CI: 2.688-33.333) times less susceptibility rate to the disease than those with the ALA/THR genotype, the susceptibility of THR/ALA genotype to the disease was 5.026 (95% CI: 1.774-14.242) times more than those with ALA/ALA genotype. There was no difference between patient groups for *VKORC1, CYP2C9*3* genes ($P > .05$). Since the *CYP2C9*7* patient group was monomorphic for the ILE allele, the patient/control odds ratio and 95% CI could not be calculated.

Conclusion: This study shows that there is an association between the *fibrinogen-Aα* gene ALA polymorphism at the amino acid position of 312 and the development of chronic thromboembolic pulmonary hypertension, but not between the *CYP2C9* and *VKORC1* gene polymorphisms.

Keywords: Chronic thromboembolic pulmonary hypertension, pulmonary arterial hypertension, anticoagulants, genetic polymorphism, fibrinogen alpha, RFLP

INTRODUCTION

Chronic thromboembolic pulmonary hypertension (CTEPH) is a type of pulmonary hypertension characterized by mechanical obstruction of the pulmonary arteries due to organized fibrotic thrombi tightly attached to the medial layer of elastic pulmonary arteries. Chronic thromboembolic pulmonary hypertension is a disease that generally develops after a single or recurrent pulmonary thromboembolism (PTE) and results from chronic obstruction of the pulmonary arteries by thromboembolic material.[1,2] Persistent occlusion of the pulmonary artery causes an increase in pulmonary artery pressure, an increase in surface tension behind the occlusion and in the vascular regions protected from thromboembolic occlusion. While open arteries experience the effects of pulmonary hypertension and structural changes are observed in these sections, the distal sections of occluded arteries may remain completely normal.[3] As a result of the disease, pulmonary hypertension and subsequent right heart failure develop.[1]

ORIGINAL INVESTIGATION

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The history of PTE, which constitutes the onset of CTEPH, may not be in every patient.[4] On the other hand, CTEPH develops in a minority of patients diagnosed with PTE. For example, the probability of developing CTEPH after PTE was reported as 3.8% in Italy,[5] while it was reported as 4.6% and 5.5% in 2 different studies conducted in Turkey.[6,7]

The source of thrombi coming into the pulmonary vascular bed is the deep veins, mostly in the legs.[8] However, deep vein thrombosis (DVT) may not be detected in every patient. The frequency of DVT was also found to be different between populations. For example, while the frequency of DVT in CTEPH patients in Japan varies between 12% and 38%,[9–11] it is reported as 35%-45% in the United States.[12] Similarly, the distribution between the genders also differs between ethnic groups. While the female/male ratio was 2.1 in Japan,[11] it was found as 0.7 in the United States.[13]

VKORC1 and *CYP2C*9* genes are associated with sensitivity to warfarin-derived drugs, a vitamin K antagonist frequently used in the treatment of patients with acute pulmonary embolism.[14] It was reported that *VKORC1* and *CYP2C*9* gene polymorphisms and CTEPH may be related in patients with insufficient anticoagulation, but as far as we know, no direct studies have been conducted on this subject.[15,16] It was reported that *fibrinogen-Aα* THR312ALA polymorphisms may also be associated with CTEPH, and this polymorphism may be a potential biomarker in identifying the development of CTEPH from PTE.[17]

All these data show that CTEPH is associated with gene polymorphism. However, to the best of our knowledge, there is no study on candidate gene polymorphism that increases susceptibility to CTEPH in the Turkish population. Therefore, in this study, it was aimed to investigate the relationship between *VKORC1*, *CYP2C*9*, and *fibrinogen-Aα* THR312ALA gene polymorphisms and CTEPH in the Turkish population.

METHODS

Patient Selection

After local ethics committee approval, 46 CTEPH patients and 106 healthy volunteers were included in the study. Those with a known genetic disease and those younger than 18 years of age were excluded from the study. The patient group consisted of people who were admitted to the only expert pulmonary endarterectomy (PEA) center in our region according to the guidelines,[3] and our expertise on this field has been reported elsewhere,[7] 885 patients underwent PEA since 2009. Diagnosis of CTEPH was confirmed after 3 months of effective anticoagulation treatment, ventilation/perfusion scintigraphy, pulmonary computed tomographic angiography, and right heart catheterization. All patients were assessed by our multidisciplinary team of CTEPH experts, and all care was provided including detailed diagnostics and all forms of CTEPH therapy. This CTEPH expert team includes expert PEA surgeons, pulmonologists, cardiologists, and radiologists. The control group consisted of volunteers with similar demographic characteristics of the patient group.

No additional application or treatment changes specific to the study were made in the patient group. In the control group, only the appropriate venous blood sample was taken, and age, gender, and current and past disease histories, if any, were recorded. In the control group, no additional special radiological or laboratory examination was performed, except for blood collection.

About 5 mL of venous blood sample from the patient and control groups were taken into tubes with K3-EDTA and stored under appropriate conditions (–20°C).

DNA Isolation

DNA isolation was performed from blood samples using the standard phenol/chloroform method.[18] Quantity and quality of DNA samples checked at 260/280 nm UV. Primers used in the amplification of single nucleotide polymorphism (SNP) regions of *fibrinogen-Aα* (THR312ALA), *CYP2C9*3* (ARG144CYS), and *CYP2C9*7* (ILE359LEU) gene regions were obtained from previous literature.[19,20] Polymerase chain reaction primers (5′-AATGCTAGGATTATAGGCGTG AG-3′ and 5′-GCCAGCAGGAGAGGGAAATA-3′) covering *VKORC1* (1639 G>A) SNP region were designed using IDTDNA PrimerQuest Tool program (http://www.idtdna.com) and used for the first time in this study.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to determine *fibrinogen-Aα* THR312ALA, *VKORC1*, *CYP2C9*3*, and *CYP2C9*7* genotypes. The primers described in Table 1 and restriction endonuclease (RE) enzymes were used in the amplification and digestion of the SNP region of each candidate gene.

Polymerase chain reactions were prepared as 1× Mg^{2+} free PCR buffer, 1.5 mM $MgCl^{2+}$, 0.750 units of Taq polymerase, 200 µM dNTP, 10 pMol each primer pairs, and 50 ng DNA as template, in a total volume of 20 µL. Touchdown PCR profile was used. After denaturation for 4 minutes at 95°C, 94°C for 30 s, 60°C–0.5°C/cycle for 30 seconds, and at 72°C for 30 seconds for 16 cycles were used at stage I. At stage II, 25 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, elongation at 72°C for 30 seconds was applied.

In RFLP analysis, 10 µL of resulting PCR product was digested with 5 units of the relevant RE enzyme and the corresponding RE buffer at 1× final concentration in a total volume of 20 µL. Around 2-3% agarose gel electrophoresis was applied and

HIGHLIGHTS

- Gene polymorphisms may play a role in the etiology of chronic thromboembolic pulmonary hypertension (CTEPH) more frequently than expected.
- The *fibrinogen-Aα* THR312ALA polymorphism significantly increases the risk of CTEPH.
- No *VKORC1* and *CYP2C9* gene polymorphisms associated with warfarin sensitivity were detected in CTEPH patients.
- This is the first gene polymorphism study in CTEPH patients in our country.

Table 1. Primers Used in This Study

Gen	SNP	Sequence	PCR (bp)	RE
Fibrinogen-Aα	rs6050	CCTAGCAGTGCTGGAAGCTG	157	RsaI
		TTCCAGAGCTCCCAGAGTT		
*CYP2C9*3*	rs1799853	CACTGGCTGAAAGAGCTAACAGAG	372	AvaII
		GTGATATGGAGTAGGGTCACCCAC		
*CYP2C9*7*	rs1057910	AGGAAGAGATTGAACGTGTGA	130	StyI
		GGCAGGCTGGTGGGGAGAAGGCCAA		
VKORC1	rs9923231	AATGCTAGGATTATAGGCGTGAG	107	MspI
		GCCAGCAGGAGAGGGAAATA		

genotypes were determined after ethidium bromide staining (EtBr) staining.

Statistical Analysis

Descriptive statistics were obtained in the patient and control groups. The conformity of the variables with continuous variation to the normal distribution was checked with the Anderson–Darling test. Patients and control groups were compared in terms of demographic characteristics using Chi-square statistics and Mann–Whitney *U*-test. GenAlEx6[21] package program was used to calculate the general population parameters including allele and genotype frequencies, expected (He) and observed (Ho) heterozygosity levels, and deviation values from Hardy–Weinberg equilibrium (HWE) of each locus.

Analyses were carried out using dominant, additive, and recessive models via single-point regression analysis using R software (version 4.1.2) codes to determine the possible CTEPH relationship of each candidate gene with phenotypes.[22–24] *P*-value < .05 was considered statistically significant.

RESULTS

The study included 46 patients including 25 (54.35%) men and 106 healthy volunteers including 55 (51.89%) men. As a result of the Chi-square test, there was no statistically significant difference in terms of gender distribution in the patient and control groups. As a result of the Anderson–Darling test, it was understood that the ages of the individuals included in the study did not show a normal distribution ($P < .05$). While there was a significant difference between the median values for age of the patient (53.5) and control (39.0) groups, no significant difference was found in terms of other parameters. The details of the demographic information of the volunteers included in the study are given in Table 2.

Hardy–Weinberg Equilibrium analysis results are given in Table 3. While there was a statistically significant difference in genotype frequencies in the *fibrinogen-Aα* patient group ($P < .001$), no statistically significant difference was observed in the control group ($P > .05$). There was no statistically significant difference in the observed and expected genotype frequencies of the *VKORC1*, *CYP2C9*3*, and *CYP2C9*7* genes in the control and patient groups ($P > .05$).

Table 2. Demographic Data of the Volunteers in the Patient and Control Group Participating in the Study

Factor			Number of Individuals (n)	Age Median value (min.– max.)
Group	Patient		46	53.5 (19.0-76.0)
	Control		106	39.0 (18.0-71.0)
	P			**.000** ΨΨ
Gender	Male		80	41.0 (19.0-76.0)
	Female		72	41.0 (18.0-71.0)
	P		.570 Ψ	.806 ΨΨ
Group – Gender	Patient	Male	25	53.0 (19.0-76.0)
		Female	21	54.0 (27.0-71.0)
	P		0.66 Ψ	.921 ΨΨ
	Control	Male	55	39.0 (20.0-71.0)
		Female	51	40.0 (18.0-69.0)
	P		.698 Ψ	.907 ΨΨ

ΨObtained by Chi-square test.
ΨΨObtained by Mann–Whitney *U*-test.

Table 3. Observed Frequencies for Genotypes, Chi-Square Test Statistic, Degrees of Freedom, and *P*-Value for Hardy–Weinberg Equilibrium for Each Genotypes in Patient and Control Groups.

		Genotypes			
Gen	Group	G/G	A/G	A/A	HW *P*
VKORC1	Control	62	37	7	.90
	Patient	29	17	0	.31
		THR/THR	ALA/THR	ALA/ALA	
Fibrinogen-Aα	Control	31	45	29	.34
	Patient	2	39	5	.00
		ARG/ARG	ARG/CYS	CYS/CYS	
*CYP2C9*3*	Control	81	22	2	.94
	Patient	35	10	1	.96
		ILE/ILE	ILE/LEU	LEU/LEU	
*CYP2C9*7*	Control	92	11	2	.10
	Patient	46	0	0	

HW *P*: *P*-value for Hardy–Weinberg equilibrium
ALA, alanine; ARG, arginine; CYS, cysteine; ILE, isoleucine; LEU, leucine; THR, threonine.

Table 4. Allele Frequencies for Patient and Control Groups

		Allele frequency (%)			
		Allele 1		Allele 2	
Gen	Group	Frequency	%	Frequency	%
		G		A	
VKORC1	Patient	75	81.52	17	18.48
	Control	161	75.94	51	24.06
		THR		ALA	
Fibrinogen-Aα	Patient	43	46.74	49	53.26
	Control	107	50.95	103	49.05
		ARG		CYS	
*CYP2C9*3*	Patient	80	86.96	12	13.04
	Control	184	87.62	26	12.38
		ILE		LEU	
*CYP2C9*7*	Patient	92	100.00	0	0.00
	Control	195	92.86	15	7.14

A, adenine; ALA, alanine; ARG, arginine; CYS, cysteine; G, guanine; ILE, isoleucine; LEU, leucine; THR, threonine.

Allele frequencies in the patient and control groups for each SNP are given in Table 4. All individuals in the *CYP2C9*7* patient group were observed to carry only the ILE allele.

Allele 1 versus allele 2 patient/control odds ratios and 95% CIs are given in Table 5. Since the *CYP2C9*7* patient group is monomorphic for the ILE allele, the patient/control odds ratio and 95% CI could not be calculated. For other genes, patient/control odds ratios were found to be statistically insignificant ($P > .05$).

Patient/control odds ratios and 95% CIs of genotypes for each gene are given in Table 6. Statistics could not be made for the *CYP2C9*7* gene because the patient group is monomorphic for the ILE genotype. It was observed that genotype frequencies of *VKORC1* and *CYP2C9*3* genes did not differ significantly according to patient/control group ($P > .05$).

For the *fibrinogen-Aα* gene, those with the THR/THR genotype were found to have a 13.51 (95% CI: 2.688-33.333) times less susceptibility rate to the disease than those with the ALA/THR genotype. The susceptibility of THR/ALA genotype to the disease was 5.026 (95% CI: 1.774–14.242) times more than those with ALA/ALA genotype. The disease susceptibility of THR/THR + THR/ALA genotype was 3.39 times (95% CI: 0.024-0.475, $P < .001$) times more than ALA/ALA genotype.

Table 5. Odds Ratios and 95% CIs for Allele Frequencies in the Patient and Control Group

Gen	Allele 1 vs. Allele 2 Patient/ Control Odds Ratio (95% CI)	*P*
VKORC1	1.350 (0.748-2.438)	.159
Fibrinogen-Aα	0.888 (0.552-1.431)	.314
*CYP2C9*3*	1.070 (0.518-2.212)	.427
*CYP2C9*7*	Inf ( )	Na

Allele 1/Allele 2 for VKORC1, Fibrinogen-Aα, CYP2C9*3 and CYP2C9*7 are G/A, THR/ALA, ARG/CYS and ILE/LEU, respectively.

Table 6. Patient/Control Odds Ratios and Confidence Intervals in Each Genotypes

SNP	Genotypic Model	Patient/Control Odds Ratio (95% CI)	*P*
VKORC1	G/G vs. G/A	1.018 (0.493-2.099)	.48
Fibrinogen-Aα	THR/THR vs. THR/ALA	0.074 (0.016-0.331)	**.0003**
*CYP2C9*3*	ARG/ARG vs. ARG/CYS	0.950 (0.407-2.215)	.453
*CYP2C9*7*	ILE/ILE vs. ILE/LEU	-	-
VKORC1	G/G vs. A/A	7.08 (0.391-128.165)	.92
Fibrinogen-Aα	THR/THR vs. ALA/ALA	0.374 (0.067-2.081)	.130
*CYP2C9*3*	ARG/ARG vs. CYS/CYS	0.864 (0.075-9.846)	.453
*CYP2C9*7*	ILE/ILE vs. LEU/LEU	-	-
VKORC1	A/G vs. G/G	7.0 (0.378-129.588)	.095
Fibrinogen-Aα	THR/ALA vs. ALA/ALA	5.026 (1.774-14.242)	**.001**
*CYP2C9*3*	ARG/CYS vs. CYS/CYS	0.870 (0.705-10.729)	.453
*CYP2C9*7*	ILE/LEU vs. LEU/LEU	-	-
VKORC1	G/G vs. G/A + A/A	1.21 (0.593-2.468)	.299
Fibrinogen-Aα	THR/THR vs. THR/ALA + ALA/ALA	0.108 (0.024-0.475)	**.001**
*CYP2C9*3*	CYS/CYS vs. CYS/ARG + CYS/CYS	0.942 (0.416-2.132)	.443
*CYP2C9*7*	LEU/ILE vs. LEU/ILE + LEU/LEU	-	-
VKORC1	G/G + G/A vs. A/A	7.613 (0.426-135.991)	.084
Fibrinogen-Aα	THR/THR + THR/ALA vs. ALA/ALA	3.390 (1.225-9.380)	**.009**
*CYP2C9*3*	ARG/ARG + ARG/CYS vs. CYS/CYS	0.942 (0.083-10.644)	.48
*CYP2C9*7*	ILE/ILE + ILE/LEU vs. LEU/LEU	-	-

A, adenine; ALA, alanine; ARG, arginine; CYS, cysteine; ILE, isoleucine; LEU, leucine; G, guanine; THR, threonine.

When the relationship between deep vein thrombosis and *fibrinogen-Aα* gene polymorphism was examined, it was observed that the frequency of DVT increased as the number of ALA alleles increased. Two patients having THR/THR genotype did not have DVT. DVT was present in 5 (13.5%) of 32 patients with the THR/ALA genotype and in 3 (60%) of 5 patients with the ALA/ALA genotype. However, statistical analysis could not be performed because the number of patients was insufficient.

DISCUSSION

In the natural course of acute pulmonary thromboembolism, it is expected that the thrombus will lysis and eventually the pulmonary circulation will return to its normal course. On the other hand, despite the use of anticoagulants for 3 months following the acute phase, in some patients, the thrombus does not dissolve and pulmonary hypertension develops. However, mechanical obstruction of the non-lysed thrombus is not the only cause of developing pulmonary hypertension. Also, remodeling in the non-thrombotic pulmonary vascular bed is thought to be a factor.[5] Since open arteries are exposed to the effects of pulmonary hypertension, structural changes are also observed in these sections, while the distal sections of arteries occluded by thrombus may remain completely normal.[3] Mechanical obstruction and remodeling process in the pulmonary artery bed results in pulmonary hypertension and subsequently right heart failure.[1]

Approximately 3-5% of patients develop CTEPH after PTE. However, it is not known beforehand which patient will develop CTEPH. It was reported that thrombophilia, splenectomy, clot size, and autoimmunity may contribute to this process in patients who develop CTEPH.[25]

In studies conducted with CTEPH patients from different ethnic groups, differences were found in terms of the frequency of CTEPH developing after PTE, gender, and accompanying deep vein thrombosis.[26] Variable demographic data reported from different countries suggest that the genetic structure may be different in the development of CTEPH, in the absence of lysis of thrombus occurring in the pulmonary vascular bed in some patients. Although there was no definitive familial or genetic association in CTEPH in various studies, some previous research suggested that there may be a genetic predisposition. For example, Kominami et al[4] found a relationship between *HLA-DPB1* and *NFKBIL1* in DVT-negative CTEPH patients. In another study conducted in China, it was reported that the results suggest that the gene mutation that causes pulmonary arterial hypertension (PAH) may play an important role in the development of CTEPH.[27]

Fibrinogen-Aα THR312ALA is a well-known polymorphism and causes the conversion of amino acid 312 from threonine (THR) to alanine (ALA) in the Aα region of the polypeptide chains.[28] Although the functional significance of this polymorphism is not clear, this area is associated with Factor XIII activation in its immediate vicinity and the center of Factor XIII-dependent α-chain cross-linking and α2-antiplasmin binding.[29]

An in vitro study comparing CTEPH patients with a control group showed that polypeptide fibrin chains from CTEPH patients cleaved significantly more slowly.[30] Therefore, genetic studies suggested that THR312ALA polymorphism may play an additional pathophysiological role in CTEPH, in addition to predisposing patients to embolic events. Standeven et al[31] reported that the clot formed in those with the ALA/ALA genotype had wider a-chain cross-linking, increased fiber thickness, and a harder clot structure than the THR/THR genotype. Thus, we show that the presence of the ALA allele increases the lateral aggregation of fibrin protofibrils and promotes the formation of a more tightly packed fibrin structure. This evidence suggests that delayed fibrin degradation mediated by the THR312ALA polymorphism and organized clot development may be an important component of scar tissue development in acute PE thrombus in some patients with CTEPH.

In a previous study, clinically homozygous adenine (A; THR) genotype was associated with acute pulmonary embolism, but not with DVT.[20] However, to our best knowledge, there have so far been 2 studies of the *fibrinogen-Aα* THR312ALA polymorphism in patients with CTEPH. In a study with 214 CTEPH and 200 control groups reported by Suntharalingam et al[32] in the United Kingdom with the Caucasian population, the presence of the ALA allele was shown to significantly increase the risk of CTEPH. In another study conducted in China, with 101 CTEPH subjects, 102 PTE subjects, and 108 healthy control groups, *fibrinogen-Aα* THR312ALA (G/A) polymorphism was associated with CTEPH but not PTE, and it was reported that GG (ALA/ALA) genotype polymorphism contributed to CTEPH by increasing fibrin resistance.[17] In our study, for the *fibrinogen-Aα* gene, those with the THR/THR genotype were found to have a 13.51 (95% CI: 2.688-33.333) times less susceptibility rate to the disease than those with the ALA/THR genotype. The susceptibility of THR/ALA genotype to the disease was 5.026 (95% CI: 1.774-14.242) times more than ALA/ALA genotype, and the disease susceptibility of THR/THR+THR/ALA genotype was 3.39 times (95% CI: 1.225-9.380) times more than the ALA/ALA genotype. Our findings show that ALA allele increases the risk of developing CTEPH, which is consistent with two other studies reported in the literature.

The origin of thrombi coming into the pulmonary vascular bed is thought to be deep veins, mostly in the legs.[8] In a previous study, ALA/ALA genotype was clinically associated with acute pulmonary embolism, but not with DVT.[20] In our study, the frequency of ALA allele and the frequency of DVT increased; however, definite data could not be presented because there was not enough sample size for statistical analysis.

The *VKORC1* and *CYP2C*9* genes are associated with the metabolism of coumarol drugs. In a study conducted in Morocco, it was shown that there is great interindividual variability in maintenance dose requirement in patients who are anticoagulated with acenocoumarol for various reasons, and *VKORC1* and *CYP2C*9* polymorphisms significantly affect the dose of acenocoumarol.[33] In a study comparing CTEPH patients with PAH patients, it was reported that *VKORC1*-1639 AA genotypes were more common in PAH patients and higher phenprocoumon dose was required in CTEPH patients.[16] Besides, it was shown that in the *VCORC1* gene, AA carriers have less pulmonary vascular resistance than GG genotypes, thus it may have an effect on hemodynamic variability among CTEPH patients.[15] Our study shows that there is no difference in *VKORC1*, *CYP2C9*3*, and *CYP2C9*7* genotypes in CTEPH patients compared to

healthy individuals. *CYP2C9*7* is monomorphic, with only the isoleucine (ILE) allele found.

Study Limitations

A limitation of this study is that patients with pulmonary embolism were not included in the study. Also, although it is desirable to have a higher number of CTEPH patients, the study was conducted with 46 patients, since the number of proven CTEPH patients was limited and the number of patients included in the study was statistically sufficient.

CONCLUSION

This study shows that there is an association between ALA polymorphism at amino acid position 312 of the *fibrinogen-Aα* gene and the development of CTEPH, but not between *CYP2C9* and *VKORC1* gene polymorphisms. It is thought that the *fibrinogen-Aα* THR312ALA polymorphism confers resistance to fibrinolysis in the resulting thrombus. We think that conducting similar studies in a way to include other VTE patients and with a larger number of patients will be beneficial for us to understand the development of chronic diseases and ultimately prevent them.

Ethics Committee Approval: Approval was obtained from the Clinical Research Ethics Committee of Abant İzzet Baysal University. The decision number is 2015/37 and the decision date is 02/07/2015.

Informed Consent: Written informed consent was obtained from all participants who participated in this study.

Peer-review: Externally peer-reviewed.

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