

# The prevalence of factor V (G1691A), MTHFR (C677T) and PT (G20210A) gene mutations in arterial thrombosis

Arteriyel trombozlarda faktör V (G1691A), metilentetrahidrofolat redüktaz (C677T) ve protrombin (G20210A) gen mutasyon sıklığı

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## BACKGROUND

Factor V (FV) [G1691A], methylenetetrahydrofolate reductase (MTHFR) [C677T] and prothrombin (PT) [G20210A] mutations are all well-recognized genetic risk factors for venous thrombosis. Although their prevalence in coronary artery disease has been established through debate, their role in patients with arterial thrombosis remains to be clarified. We investigated the prevalence rates of FV, MTHFR and PT gene mutations in patients with arterial thrombosis and in healthy controls.

## METHODS

All subjects and controls were from Central Anatolia. Thirty (8F) patients with median (range) age of 63 (16-88) years and 90 (52F) healthy controls with median (range) age of 31 (20-73) years were studied. DNA was extracted using conventional methods (proteinase K/phenol-chloroform) followed by PCR amplification and restriction endonuclease digestion (using Hinf I and Hind III). Digested PCR products were identified using agarose gel electrophoresis and stained with ethidium bromide.

## RESULTS

The prevalence rates of MTHFR and PT gene mutations were not significantly different between the groups. The prevalence rate of FV mutation was significantly higher in patients with arterial thrombosis. Coinheritance of FV and MTHFR was found in 67% of patients, which was significantly higher in arterial thrombosis, suggesting the MTHFR mutation as a synergistic risk factor for thrombosis in patients with FV mutation. PT gene mutation has no effect on arterial thrombosis.

## CONCLUSION

The increased prevalence rate and coexistence of both FV and MTHFR found in this group of patients suggest that these mutations might increase the risk of arterial thrombosis.

**Key Words:** Arterial thrombosis; factor V; gene mutation; MTHFR; prothrombin.

## AMAÇ

Faktör V (FV) [G1691A], metilentetrahidrofolat redüktaz (MTHFR) [C677T] ve protrombin [G20210A] gen mutasyonlarının venöz tromboz riskini artırdığı bilinmektedir. Bu mutasyonların, koroner arter hastalığındaki sıklıkları tartışmalı olmasına karşın, tek tek veya birlikte periferik arteriyel tromboz'lardaki (PAT) sıklıkları bilinmemektedir. Bu çalışmada, normal sağlıklı bireylerde ve PAT'si olan hastalarda bu mutasyonların sıklığı araştırıldı.

## GEREÇ VE YÖNTEM

Yaş ortalaması 31 yıl (dağılım 20-73) olan 90 (52K) sağlıklı bireyden ve yaş ortalaması 63 yıl (dağılım 16-88) olan 30 (8K) hastadan kan örnekleri alındı. Klasik proteinaz K / fenol-kloroform yöntemi ile DNA ayrıştırmasını takiben, PCR ürünü spesifik endonükleazlar (Hind III, Hinf I) kullanılarak kesildi. Elde edilen ürün, agaroz jel elektrofores de yürütülüp etidium bromid ile boyanarak ultraviole ışığı altında transilluminasyon yöntemi ile görüntülendi.

## BULGULAR

PAT'si olan hasta grubunda MTHFR ve protrombin gen mutasyon sıklığı kontrol grubundan farklı değildi. Ancak FV G1691A mutasyon sıklığı anlamlı ölçüde yüksekti. Yine arteriyel trombozlu olguların %67'sinde FV ve MTHFR gen mutasyonu birlikte bulunduğu, kontrol grubu ile karşılaştırıldığında aradaki farkın anlamlı olduğu ve FV pozitif olgularda MTHFR gen mutasyonu varlığının riski artıran sinerjistik bir faktör olduğu görüldü. Protrombin gen mutasyonunun tek başına ya da diğer gen mutasyonları ile birlikte, riski artıran önemli bir faktör olmadığı görüldü.

## SONUÇ

PAT'li hastalarda FV ve MTHFR mutasyonlarının artmış sıklığı ve birlikteliğinin, bu hastalarda tromboz oluşumundan sorumlu risk faktörlerinden birisi olabileceği kanaatine varıldı.

**Anahtar Sözcükler:** Arteriyel tromboz; faktör V; mutasyon; MTHFR; protrombin.

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Venous thrombosis is an important cause of morbidity and mortality and the pathogenesis is multifactorial, including both acquired and genetic factors. In the carriers of factor V (FV) mutation, inactivation of FVa by activated protein C (APC) is impaired, which results in prothrombotic state.<sup>[1-3]</sup> Risk of thrombosis increased five-fold with heterozygote mutations and 50 to 100 times with homozygote mutations.<sup>[4]</sup> The prothrombin (PT) G20210A mutation increases the risk of venous thrombosis via elevation of PT levels.<sup>[5-8]</sup> Methylenetetrahydrofolate reductase (MTHFR) is an enzyme involved in homocysteine metabolism and increased homocysteine levels, by depressing the activation of protein C, and was found to be a risk factor for venous thrombosis and stroke.<sup>[9-11]</sup> FV G1691A, MTHFR C677T and PT G20210A mutations are all considered risk factors for venous thromboembolism; their role in arterial thrombosis is not yet clarified. There have been some studies in patients with cardiac problems or cerebrovascular accidents and some studies on peripheral arterial disease, and their results are controversial.<sup>[12-14]</sup>

In view of the above, we aimed to investigate the prevalence rate of FV, MTHFR and PT gene mutations and the coexistence of these mutations in patients with arterial thrombosis and healthy controls from Central Anatolia.

## MATERIALS AND METHODS

This study was performed in the laboratories of Pediatric Molecular Genetics, Department of Pediatrics, Ankara University Medical School, Turkey. The study protocol was approved by the local ethics committee in accordance with the Declaration of Helsinki, and all participants gave informed consent.

### Patients

Thirty (8F) patients [median (range) age of 63 (16-88) years] with the diagnosis of acute occlusive

disease due to peripheral arterial thrombosis located at femoral, common iliac and popliteal arteries who were admitted to the Department of Surgery of Ankara Numune Hospital were included in the study. Ninety (52F) individuals with median (range) age of 31 (20-73) years without any comorbid disease, previous history of thrombosis or the use of any medications were included in the study as controls. Twelve of 30 patients had history of recurrent thrombosis. All patients were grade 2-3 and category 5-6 according to the updated Fontaine Classification.<sup>[15]</sup>

Blood samples were taken from patients and controls after an overnight fast for the investigation of FV (G1691A), MTHFR (C677T) and PT (G20210A) gene mutations.

### Methods

#### *Collection of Blood Samples and DNA Extraction*

10 ml blood was collected from both patients and controls by venipuncture into vacutainer tube with EDTA.

Total genomic DNA was obtained from peripheral blood after lysis with SDS (sodium dodecyl sulphate) and proteinase K treatment of buffy coat. DNA was purified using phenol/chloroform and ethanol precipitation. Extracted DNAs were frozen at -80°C until further assessment.

#### *Polymerase Chain Reaction (PCR) Conditions*

5 ml of extracted DNAs for each mutation were subjected to PCR by using two different specific primers for each mutation analysis.<sup>[2,15-17]</sup> The primer sequences and the size of the products are shown in Table 1.

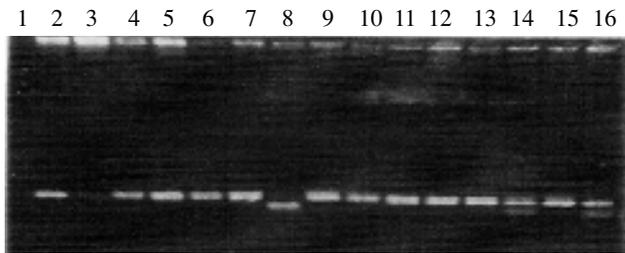
PCR reactions were performed according to standardized procedures on a Thermal Cycler (Ericomp, USA). In each run, marker and known homozygotes and heterozygotes for the tested polymorphisms were included to check for unspecific reactions and to confirm the correct genotyping, respectively.

**Table 1.** Specific primers for each mutation and PCR products

Mutations	Primers	PCR Products	Restriction Endonuclease Digestion	
Factor V G1691A	5' TCAGGCAGGAACAACACC 3' 5' GTTACTTCAAGGACAAAATACCTGTAAAGCT 3'	241 Bp	241/209Bp 209Bp	Heterozygote Homozygote
MTHFR C677T	5' TGAAGGAGAAGGTGTCTGCGGGA 3' 5' AGGACGGTGCGGTGAGAGTG 3'	198Bp	198/175Bp 175Bp	Heterozygote Homozygote
PT G20210A	5' TCTAGAAACAGTTGCCTGGC 3' 5' ATAGCACTGGGAGCATTGAAGC 3'	345Bp	345/322 Bp 322 Bp	Heterozygote Homozygote

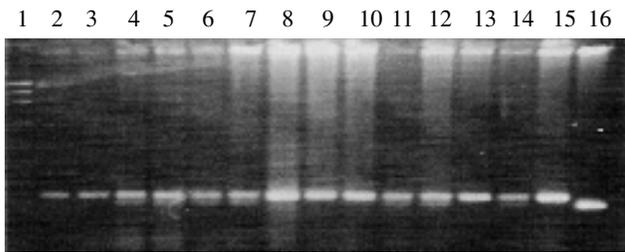
*Restriction Endonuclease Digestion (RED)*

In order to detect genetic mutations, PCR products were subjected to RED by using Hind III enzyme for FV and PT and Hinf I enzyme for MTHFR (Promega, Madison, USA) as explained previously.<sup>[2,16-18]</sup> Digested DNA samples were analyzed in agarose gel electrophoresis in 2% for PCR and 3% for RED and the separated DNA was visualized by ethidium bromide staining. Mutations were identified by the presence of the predicted restriction



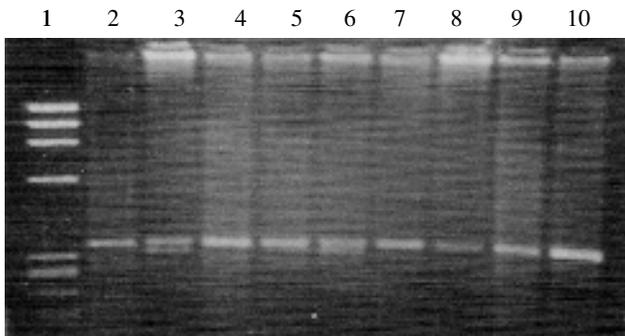
**Fig. 1.** FV G1691A gene mutation.

Row 1: OX-174-HaeIII (marker)  
 Row 2: Un-cut PCR product (241 bp)  
 Rows 3-7; 9-13; 15: Homozygote normal control (241bp)  
 Rows 14-16: Heterozygote FV G1691A mutation (241-209 bp)  
 Row 8: Homozygote FV G1691A mutation (209 bp)



**Fig. 2.** MTHFR C677T gene mutation.

Row 1: OX-174-HaeIII (marker)  
 Rows 2, 3, 8-10, 13, 15: Homozygote normal control (198bp)  
 Rows 4-7, 11-12, 14: Heterozygote MTHFR C677T mutation (198-175bp)  
 Row 16: Homozygote MTHFR C677T mutation (175bp)



**Fig. 3.** Prothrombin G20210A gene mutation.

Row 1: OX-174-HaeIII (marker)  
 Rows 2, 4, 5, 7-10: Homozygote normal control (345bp)  
 Rows 3, 6: Heterozygote PT G20210A mutation (345bp-322bp)

fragment length polymorphisms associated with FV1691, MTHF677 and PT20210 (Figures 1-3).

**Statistical Analyses**

Statistical analyses were performed by using SPSS 10.0 for Windows (SPSS Inc, Chicago, IL, USA). The ages of the patients are shown as median (range) and the prevalences as percent (%). All the comparisons between groups were performed using chi-square, Yates' correction and Fisher's exact tests. Risk analyses, Odds Ratio (OR) and 95% Confidence Interval (CI) were calculated using Logistic Regression Analysis. p values less than 0.05 were considered as significant.

**RESULTS**

**Controls**

Ninety (52F) healthy controls with a median (range) age of 31 (20-73) years, without any comorbid disease, previous history of thrombosis or use of any medications were included in the study. The prevalence rates of FV and MTHFR gene mutations and their distributions according to gender are shown in Table 2.

There were no homozygote FV mutations in controls. The prevalence rate of heterozygote FV mutation was 6.6% overall, and 13% in males and 2% in females (p=0.0348 chi-square; p=0.0464 Fisher's exact). The prevalence rate of homozygote MTHFR mutation was 6.6% overall, and 5% in males and 7.7% in females (p=0.64 chi-square; p=0.497 Fisher's exact). The rate of heterozygote MTHFR mutation was 33.3% overall and the distribution demonstrated no significant difference between males and females (40% vs 28.8%, respectively).

The PT G20210A mutation was studied in only 42 healthy subjects (18M, 24F) with a median (range) age of 32 (21-73) years. Only two females aged 26 years had the mutation (4.8%). None of the controls in this group had homozygote mutation for FV and heterozygote FV mutation was seen in only 4 (1F) subjects (9.5%). Homozygote MTHFR mutation was seen in 3 (2F) subjects and heterozygote mutations in 17 (8F) subjects, and distribution did not differ significantly according to gender. Three of 6 (50%) FV-positive subjects also had MTHFR mutations and 1 subject (16.6%) had mutations in all three genes.

**Patients**

Thirty (8F) patients with arterial thrombosis with

**Table 2.** The prevalence rates of FV and MTHFR gene mutations and their distributions according to gender

	Males n (%)	Females n (%)	Total n (%)
FV (+/+)	0 (0)	0 (0)	0 (0)
FV (+/-)	5 (13)	1 (2)	6 (6.6)
FV (-/-)	33 (87)	51 (98)	84 (93.3)
Total	38 (100)	52 (100)	90 (100)
MTHFR (+/+)	2 (5)	4 (7.7)	6 (6.6)
MTHFR (+/-)	15 (40)	15 (28.8)	30 (33.3)
MTHFR (-/-)	21 (55)	33 (63.5)	54 (60)
Total	38 (100)	52 (100)	90 (100)

a median (range) age of 63 (16-88) years were included in the study. All patients were diagnosed to have acute arterial ischemia due to thrombosis. The prevalence rates of all mutations are shown in Table 2. There was no patient with homozygote FV mutation, whereas 6 patients (3F) had heterozygote mutation [6/30 (20%)]. While the prevalence was 13.6% in men (3/22), which was parallel to the rate in controls, it was 37.5% (3/8) in women and this was 19 times higher than the rate seen in control females (2%) ( $p=0.002$  chi-square;  $p=0.002$  Yates' correction;  $p=0.0061$  Fisher's exact). The prevalence rate of FV mutation in controls was 6.6% and in patients with thrombosis was 20% ( $p=0.03$  chi-square).

The prevalence of MTHFR gene mutation was 14/30 in patients [11 males and 3 females (46.6%)], and only 1 of those (3.3%), a male, had homozygote mutation. While the rate of mutation in males was 50% (11/22), it was found to be 37.5% in females (3/8). These distributions were similar to those seen in controls.

PT G20210A mutation was seen in only 1 out of 30 patients (3.3%) and this was not different from the prevalence rate in control subjects (4.7%). In both patients and controls, the distributions of FV 1691 G/A and MTHFR 677 C/T gene mutations, with ORs and 95% CIs, are shown in Table 3.

**Table 3.** The distributions and risk evaluations of mutations in patients and controls

	Patients (n=30)	Controls (n=90)	OR	95% CI
FV 1691				
GG	24	84	1	
GA	6	6	0.286	0.08-0.967
MTHFR 677				
CC	16	54	1	
CT	13	30	0.654	0.281-1.522
TT	1	6	2.07	0.239-17.938
PT 20210				
GG	29	40	1	
GA	1	2	1.45	0.25-16.73

OR: Odds ratio; CI: Confidence interval.

When we searched for the coexistence of FV and MTHFR mutations in patients, we found that 4 of 6 patients with FV mutations also had heterozygote MTHFR mutation (66.6%). This rate is significantly higher than the rate seen in controls [ $p=0.043$  chi-square; OR: 0.224, 95%CI (0.047-1.066)]. The coexistence of both mutations was 100% in females with thrombosis, whereas it was only 33% in males [ $p=0.083$  chi-square; OR: 0.25, 95%CI (0.046-1.365)].

Risk evaluation for the coinheritance of two different gene mutations was performed by using OR and 95% CI and the results are shown in Table 4. We found that 51 (57%) of the controls and 14 (47%) of the patients carried no mutations for FV and MTHFR ( $p=0.341$  chi-square). Thirty-six (40%) control subjects and 12 (40%) patients carried only one mutation (either the FV or MTHFR), while 3 controls (3%) and 4 patients (13%) carried both FV and MTHFR mutations ( $p=0.043$  chi-square).

## DISCUSSION

Atherosclerotic peripheral arterial disease is a progressive condition characterized by arterial stenoses and occlusions in the peripheral arterial bed of the extremity. Thrombophilic conditions including FV, PT and MTHFR mutations have been inves-

**Table 4.** The risk evaluations of the coexistence of FV 1691 G/A and MTHFR 677 C/T mutations

FV	MTHFR	Patients	Controls	OR	95%CI
G (-)	C (-)	14	51	1.495	0.652 - 3.426
G (-)	T (+)	10	33	1.158	0.484 - 2.769
A (+)	C (-)	2	3	0.483	0.77 - 3.037
A (+)	T (+)	4	3	0.224	0.047 - 1.066

OR: Odds ratio; CI: Confidence interval.

tigated, but current evidence does not support the hypothesis that one or all of these mutations might be a risk factor for peripheral arterial disease.<sup>[19-23]</sup>

In this study, the incidence of FV, MTHFR and PT gene mutations was investigated in 90 healthy subjects and 30 hospitalized patients with peripheral arterial thrombosis.

The prevalence of the FV mutation in normal populations was reported as 2-14% and the differences in distribution were explained by ethnicity and geographic differences.<sup>[24]</sup> Though the incidence is higher in Europe and North America, no mutations have been detected in China, Japan, Africa or Native Americans (Indians).<sup>[25]</sup> The prevalence is around 3-5% in European countries, 14% in Greece, and between 7.1-10.3% in Turkey.<sup>[26-28]</sup> It is reported to be 12.2% in Northern Cypriots.<sup>[28]</sup> Greece and Cyprus are the most common places for the mutations in Europe.<sup>[29]</sup> Azerbaijan is the only country outside Europe, with the prevalence rate of 14%.<sup>[30]</sup>

While no mutations are noted in Japan, China, Africa and Native Americans, the higher prevalence rate in Europe and the Middle East and especially in Anatolia, which is considered a bridge between Asia and Europe, suggests that this mutation might have been distributed from Anatolia. Gurgey<sup>[30]</sup> explains in her hypothesis that FV mutation developed in the Middle East and was distributed via immigrants to Azerbaijan, Daghestan, Turkey, India and Europe.

The previous studies investigating the prevalence of FV mutations in Turkey have reported a range between 7.4 and 10.8%.<sup>[26-28]</sup> The discrepancy between studies might partly be due to the geographical differences, since Gül et al.<sup>[27]</sup> studied the population from Istanbul and Gurgey<sup>[26]</sup> and Akar<sup>[28]</sup> investigated the populations from Ankara. The other factors might be differences in control groups and gender. As we found in our study, the prevalence rate in control males was around six times higher than in females (13% vs 2%). Therefore, the unequal gender distributions in the studies might result in higher or lower prevalence rates.

It has previously been reported that the risk of venous thrombosis is increased 7-10 times with the heterozygote FV mutation, and the risk is found to be increased around 100 times with the homozygote FV mutation; together with the other risk factors, risk is increased more.<sup>[4]</sup> The FV G1691A and PT G20210A mutations are widely accepted as risk fac-

tors for the development of venous thromboembolism; however, their role in arterial thrombosis is controversial, and association of the FV and PT gene polymorphisms with arterial thrombosis is not well documented.<sup>[13,14]</sup>

In the present study, there was no homozygote FV mutation in the patient group or in controls. While the incidence of heterozygote FV G1691A mutations in controls was 6.6%, it was 20% in patients, and its distribution according to gender was interesting. The incidence was parallel between males in the control and patient groups (13% vs 13.6%, respectively), but was significantly higher in the female patients than female controls (37.5% vs 2%, respectively).

The relation between thrombosis and FV mutations might be established hypothetically, since 20% of FV is located in platelets, which play a very important role in the pathogenesis of atherosclerosis. Though the normal and mutant platelets and whole of the mutant FVa are inactivated by APC, the single mutation in FV may result in resistance to proteolytic degradation by APC.<sup>[2,30,31]</sup> This phenomenon results in a hypercoagulable state that is seen in 50% of thrombophilic families and in 10% of venous thrombosis patients. The risk of venous thrombosis is also increased with PT G20210A mutation.

Most of the studies related to arterial thrombosis have actually involved patients with myocardial infarction or cerebrovascular accidents and they produced conflicting results.<sup>[30,32-35]</sup> Previous studies describing the possible role for FV and/or MTHFR or PT gene mutations as risk factors for arterial thrombosis also revealed conflicting results.<sup>[15,19-21,36-38]</sup> Kim et al.<sup>[13]</sup> in their previous meta-analyses pointed out that most of the studies on peripheral arterial disease have several limitations, including size, methods of matching and spectrum or delineation of cases and controls.

In the present study, the median age of the patients was 63 years and the prevalence of FV mutation was found to be 20%, which is higher than the rate in all previous studies in patients with arterial disease and also the rate in our control group (6.6%). Although the significantly higher rate in female patients may partly be due to the overestimation resulting from the small size of the patient population, it still needs careful evaluation. The very low rate of prevalence in female controls (37.5% vs 2%) might be important and protective for the continua-

tion of our species as the risk of thrombosis is significantly increased in females carrying this mutation.

The incidence of MTHFR C677T gene mutation was not different between control and patient groups (40% vs 46.6%), and the distribution was also similar between male and females in both groups. While the rate of homozygote mutations was 6.6% in controls, it was found to be 3.3% in patients. While Kluijtsman et al.<sup>[12]</sup> showed that the risk of cardiovascular disease increased three-fold with the presence of C677T mutations, we failed to show any relation with the increased risk of thrombosis in the present study.

On the other hand, we found that 67% of patients with FV mutation also had MTHFR mutations; this rate was 50% in controls. Interestingly, all of the female patients with FV mutation also had MTHFR mutation, whereas only 33% of male patients with FV mutations had MTHFR mutation. Coexistence of FV and MTHFR mutations especially in female patients as compared to controls might be an important risk factor for peripheral arterial thrombosis. The same situations were also noted by Akar<sup>[7]</sup> previously in patients with deep vein thrombosis.

The incidence of PT G20210A mutation was also similar between the controls and patients (4.7% vs 3.3%). In the previous studies, the risk of venous thrombosis was found to be increased.<sup>[6,38]</sup> Previous studies in Turkish populations revealed a prevalence rate of 2.6% in controls and 6-26% in patients with venous thrombosis.<sup>[7,8]</sup> Despite the increased risk of venous thrombosis with this mutation, we failed to show any relation between PT gene mutation and increased risk of arterial thrombosis, since there was only one patient in the present study with PT mutation, who was negative for the other two mutations.

Mueller et al.<sup>[14]</sup> recently investigated the prevalence and risk factors of all three mutations including FV G1691A, MTHFR C677T, and PT G20210A in chronic lower limb ischemia and concluded that peripheral arterial disease is not associated with the increased prevalence of these mutations.

The aim of this study was to investigate the prevalence rate of FV G1691A, MTHFR C677T and PT G20210A mutations in patients with arterial thrombosis and also to evaluate the risk related to presence of these mutations. We were able to demonstrate that the prevalence of FV G/A mutation is higher in patients with arterial thrombosis, especially in female patients, with the rate increased to

19 times more than normal level, suggesting that FV G1691A mutation is an independent risk factor for thrombosis and increases the risk of thrombotic events especially in females. Despite its being harmless when alone, MTHFR C677T gene mutation also increases the risk when it is seen together with FV mutation. As the PT G20210A was very uncommon in our patients, it is difficult to make any comment on its effect on arterial thrombosis with our study; wider studies are needed for this purpose.

We conclude that genetic risk factors, especially FV, MTHFR and PT, should be evaluated in all patients with arterial thrombosis and taken into consideration with other risk factors. In patients carrying these mutations, antithrombotic therapies might prevent or decrease the risk of recurrent attacks.

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