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*Original Article*

# Analysis of prothrombotic mutations and polymorphisms in children who developed thrombosis in the perioperative period of congenital cardiac surgery

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**Abstract** In this study, we investigated some of the prothrombothic mutations and polymorphisms in 15 children with congenital cardiac malformations who developed severe thrombosis in the perioperative period following surgical repair. The mutations and polymorphisms included in the study were Factor V Leiden, prothrombin G20210A, methylentetrahydrofolate reductase C677T, endothelial nitric oxide synthase intron 4 VNTR, alpha-fibrinogen Thr312Ala, Factor XIII Val34Leu, and insertion or deletion of angiotensin 1 converting enzyme. Compared to the healthy Turkish subjects, our patients had a similar rate of mutation of Factor V Leiden, Factor XIII Val34Leu, and endothelial nitric oxide synthase a/b polymorphisms, but higher frequency of the prothrombotic angiotensin 1 converting enzyme deletion/deletion genotype, and lower frequency of the antithrombotic alpha fibrinogen Thr/Thr genotype. None of the patients exhibited mutations involving prothrombin G20210A or methylentetrahydrofolate reductase C677T. The results of our study suggest that, in addition to prothrombotic mutations such as Factor V Leiden, single-nucleotide polymorphisms should be considered in all children with congenital cardiac malformations who develop thrombosis.

Malformations of the heart are the most common of all serious lesions that are present at birth, with an incidence of 4 to 8 cases per 1,000 live births.[1] If needed, corrective surgery is usually the optimal treatment for these anomalies, but perioperative morbidity and mortality still remain high due to several factors. Arterial or venous thrombosis, or both varieties of thrombosis, is among these factors. Prior to surgery, the most frequent time at which these children develop thrombosis is during cardiac catheterization. Postoperative thrombosis in this group of patients is a more complex disorder, which can affect both small and large vessels, and is associated with a high morbidity and mortality.

Recent studies indicate that both point mutations and single-nucleotide polymorphisms of genes that encode proteins involved in the coagulative and anticoagulative cascades are important risk factors for development of thrombosis. Patients with these risk factors are most likely to develop thrombosis when triggering elements, such as placement of catheters, prolonged immobilization, or surgery, are also present. In this study, we investigated some of the above-mentioned mutations and polymorphisms in children who developed thrombosis in the perioperative period after correction of congenital cardiac malformations.

Keywords: Postoperative care; children; congenital heart malformations; point mutations

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## Brief Glossary

*Factor V Leiden*: Substitution of guanine with adenine at nucleotide 1691 in the Factor V gene that results in replacement of arginine by glycine at amino

acid position 506 of Factor V protein,which is the site that is recognized by activated protein C.

*Prothrombin G20210A*: Substitution of guanine with adenine at nucleotide 20210 in the untranslated region of the prothrombin gene, which causes prolonged survival of prothrombin messenger-ribonucleic acid, resulting in elevated levels of prothrombin in the plasma.

*Methylentetrahydrofolate reductase C677T*: Substitution of cytosine with thymine at nucleotide 677 in the methylentetrahydrofolate reductase gene, converting an alanine to a valine residue, and being responsible for the synthesis of a thermolabile form of methylentetrahydrofolate reductase enzyme.

*Endothelial nitric oxide synthase intron 4 VNTR alleles a and b*: Number of 27 base pair tandem repeats at the variable region of the 4th intron in the endothelial nitric oxide synthase gene. The wild-type product, the "b" allele, contained 420 base pairs, while the "a" allele contained 393 base pairs.

*Alpha-fibrinogen Thr312Ala*: A common polymorphism leading to a substitution of threonine with alanine at codon 312. It occurs within the carboxy-terminal end of the fibrinogen alpha chain, which is an important region for processes dependent on Factor XIII.

*Factor XIII Val34Leu*: Substitution of guanine with thymine at exon 2 of the gene for Factor XIII, which results in the replacement of leucine for valine at amino acid position 34 of the Factor XIII protein, this being a region close to the site of activation of thrombin.

*Angiotensin 1 converting enzyme insertion/deletion*: A polymorphism leading either to insertion or deletion of a 300 base pair deoxyribonucleic acid portion in intron 16 of the gene. Deletion is linked to high levels of angiotensin 1 converting enzyme.

## Patients and methods

*Patients*: We studied 15 children, 10 females and 5 males, with a median age of 3.5 years, who developed thrombosis in the perioperative period of surgery for congenital cardiac defects between July 2000 and March 2002. When thrombosis was suspected because of clinical signs and symptoms, further evaluation was performed by using Doppler ultrasonography, magnetic resonance imaging, or magnetic resonance angiography. For each case, we recorded the site of thrombosis, the outcome of thrombotic process, and the overall outcome. Catheterization was performed with bolus infusion of heparin at 50 units per kilogram. Patients who developed transient thrombosis after catheterization, and who were successfully treated with infusions of heparin or streptokinase, were not included in the study. None of the patients received anticoagulative treatment before surgery. Patients who had undergone surgical treatment were given 300 units per kilogram heparin before cardiopulmonary bypass. If the patient was under 1 year of age, the dose of heparin was increased to 400 units per kilogram. Baseline activated clotting time was recorded before giving the bolus of heparin. During cardiopulmonary bypass, activated clotting time was adjusted to 480 seconds, within a range between 460 and 500 seconds. After cardiopulmonary bypass, from 1.6 to 1.7 milligrams of protamine sulphate was given in order to neutralize each 1 unit of heparin. If the baseline activated clotting time was not achieved, additional doses of protamine were given. After construction of palliative shunts, between 10 and 20 units of heparin per kilogram heparin was given for between 48 and 72 hours after obtaining serous drainage. All the patients who had undergone construction of either a shunt or the Fontan circulation received 5 milligrams of salicilate per kilogram for 3 months after the operation.

The mutations and polymorphisms of the prothrombotic genes studied were Factor V Leiden, prothrombin G20210A, methylentetrahydrofolate reductase C677T, endothelial nitric oxide synthase intron 4 VNTR, alpha-fibrinogen Thr312Ala, Factor XIII Val34Leu, and angiotensin 1 converting enzyme insertion/deletion (see Brief Glossary for explanation of the genes).

*Protocol*: Families gave signed consent for their child to be involved in the study. We collected a sample of 5 milliliters of peripheral blood in ethylenediaminetetraacetic acid from each patient for isolation of genomic deoxyribonucleic acid. Analysis of mutations and polymorphisms was carried out as described previously.[2–7] In order to compare the frequencies of genotype with those in the healthy population, we also determined the distribution of the endothelial nitric oxide synthase intron 4 VNTR a/b genotype, the alpha-fibrinogen Ala/Thr genotype, and the Factor XIII Val/Leu genotype in healthy Turkish subjects who had no history of thrombosis. For this purpose, we included 167 subjects for evaluation of endothelial nitric oxide synthase, 119 subjects for alpha-fibrinogen, and 112 subjects for Factor XIII. For comparison of the Factor V Leiden, prothrombin G20210A, methylenetetrahydrofolate reductase C677T, and angiotensin 1 converting enzyme genotypes, we compared the results in our group of patients with results extant in the literature for healthy Turkish people.[3,8–10] The control groups were all unmatched.

Statistical analysis was performed by MINITAB 13.0 statistics packet program (Minitab Inc., Philadelphia, USA).

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## Results

During the period of study, we performed in our hospital 819 cardiac catheterization, and 674 surgical procedures for congenital cardiac malformations. Weak or impalpable pulses were found in 104 patients (12.8%) after cardiac catheterization. All these children were treated with heparin, albeit that infusion of streptokinase was also needed in 43 of them. Severe thrombosis developed in 15 of these patients. We determined the clinical characteristics of thrombosis, and analysed the mutations and polymorphisms, in all these patients (Table 1). Of the malformations, 7 produced an acyanotic picture, while 8 produced more complex cyanotic disease. Thrombosis had occurred preoperatively in 6 of the children, but was found only postoperatively in the other 9 cases. Two of the children had Down's syndrome. The right femoral artery was the most common site of thrombosis, being found in 4 children, all developing after preoperative catheterization (cases #1–#4, Table 1). Pulmonary thromboembolism occurred in 2 patients, and 2 others developed purpura fulminans. An intracardiac thrombus was identified in one patient with complex congenital cardiac disease, while 2 patients developed thrombosis at anastomotic sites, one at the site of a Blalock–Taussig shunt, and the other involving a bidirectional cava-pulmonary anastomosis. Of the 15 patients, 6 died in the postoperative period, with causes of death given in Table 1.

Molecular analysis revealed that only 1 patient was heterozygous for mutation of Factor V Leiden. None of the patients exhibited mutations of prothrombin G20210A or methylentetrahydrofolate reductase C677T. When analyzing the genotypes for endothelial nitric oxide synthase intron 4 VNTR, 1 patient was a/a, 3 were a/b, and 11 were b/b. Corresponding analysis for alpha-fibrinogen revealed 2 patients with the Ala/Ala genotype, 11 with Ala/Thr, and 2 with Thr/Thr, while for Factor XIII, 10 patients had the Val/Val genotype, 4 were discovered with Val/Leu, and 1 with Leu/Leu. The results for insertion or deletion of angiotensin 1 converting enzyme were 10 patients with the deletion/deletion genotype, 4 with insertion/deletion, and 1 with insertion/insertion. Table 2 shows a comparison of the findings concerning mutations and polymorphisms when comparing our patients with healthy Turkish subjects. Mutations of Factor V Leiden, and rates of polymorphism for Factor XIII Val34Leu and endothelial nitric oxide synthase intron 4 VNTR, were similar with the normal population. The deletion/deletion genotype for prothrombotic angiotensin 1 converting enzyme, however, was significantly higher, and the frequency of the Thr/Thr genotype for antithrombotic alpha fibrinogen was significantly lower, than in the normal Turkish population (p value is less than 0.05 for both).

## Discussion

Most studies of genetic mutations and polymorphisms associated with venous thrombosis have focused on genes that encode proteins in either the coagulative or the anticoagulative pathways. In our study, we investigated the effects of some point mutations and genetic polymorphisms on the risk for thrombosis in children who developed this complication in the perioperative period of congenital cardiac surgery.

A recent study conducted in Turkey revealed a very high percentage of heterozygosity for mutation of Factor V Leiden, this being found in one-quarter of patients with congenital cardiac malformations who developed thrombosis.[11] This rate is much higher than in the normal Turkish population, with less than one-tenth showing this feature.[8] In our cohort of children, however, the rate of heterozygosity for Factor V Leiden mutation was comparable to the normal population, and none of the children exhibited mutations of prothrombin G20210A or methylentetrahydrofolate reductase C677T. Compared to the groups of normal healthy Turkish subjects we studied, our children had similar rates of polymorphisms for Factor XIII Val34Leu and endothelial nitric oxide synthase intron 4 VNTR. We observed, however, significantly higher frequency of the deletion/deletion genotype for prothrombotic angiotensin 1 converting enzyme, and a lower frequency of the Thr/Thr genotype for our children with thrombosis when compared to normal healthy Turkish subjects. These genotypes are known to be associated with increased risk of thrombosis.[6,12]

Endothelial production of nitric oxide is regulated by the enzyme endothelial nitric oxide synthase intron 4 VNTR.[13] The a/b polymorphism is one of three novel polymorphisms of this gene that have recently been documented. These single-nucleotide polymorphisms are known to alter the activity of the enzyme, and hence reduce the production of nitric oxide. In one study, comparison of findings in cultured human umbilical vein endothelial cells between subjects having the a/b and b/b genotypes revealed significantly lower mean levels of both the protein and its activity in the group with the a/b genotype.[14] There is evidence that these lower levels may lead to increased aggregation of platelets and thrombosis. A recent report noted a strong association between myocardial infarction and allele a of intron 4 VNTR.[15] In our investigation,

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Table 1. Clinical characteristics and the results of analysis for mutations and polymorphisms in our cohort of patients.

| Patient | Site of thrombosis | Time of thrombosis | Congenital cardiac defect | Factor V Leiden | Prothrombin G20210A | Methylentetra-hydrofolate reductase C677T | Endothelial nitric oxide synthase intron 4 VNTR | Alpha-fibrinogen | Factor XIII | Angiotensin 1 converting enzyme | Outcome or cause of death |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Right femoral artery | Preoperative | Supravalvar aortic stenosis | Wild type | Wild type | Wild type | a/b | Ala/Thr | Val/Leu | Insertion/Deletion | Alive with patent circulation |
| 2 | Right femoral artery | Preoperative | Atrioventricular septal defect | Wild type | Wild type | Wild type | a/b | Ala/Thr | Val/Leu | Insertion/Insertion | Alive |
| 3 | Right femoral artery | Preoperative | Double outlet right ventricle | Wild type | Wild type | Wild type | b/b | Thr/Thr | Leu/Leu | Insertion/Deletion | Alive |
| 4 | Right femoral artery | Preoperative | Aortic coarctation Patent arterial duct | Wild type | Wild type | Wild type | b/b | Ala/Thr | Val/Leu | Insertion/Deletion | Alive |
| 5 | Pulmonary thrombo-embolism | Postoperative | Atrial septal defect Patent arterial duct Pulmonary hypertension | Wild type | Wild type | Wild type | b/b | Thr/Thr | Val/Leu | Insertion/Deletion | Died with pulmonary insufficiency |
| 6 | Pulmonary thrombo-embolism | Postoperative | Tetralogy with pulmonary atresia | Wild type | Wild type | Wild type | b/b | Ala/Thr | Val/Val | Deletion/Deletion | Alive on warfarin treatment |
| 7 | Sagittal sinus thrombosis | Postoperative | Atrial septal defect Ventricular septal defect Pulmonary hypertension | Heterozygous | Wild type | Wild type | b/b | Ala/Thr | Val/Val | Deletion/Deletion | Died with multi-organ dysfunction |
| 8 | Sigmoid sinus thrombosis | Postoperative | Tetralogy of Fallot | Wild type | Wild type | Wild type | b/b | Ala/Thr | Val/Val | Deletion/Deletion | Alive |
| 9 | Purpura fulminans | Postoperative | Ventricular septal defect Double outlet right ventricle Patent oval foramen | Wild type | Wild type | Wild type | b/b | Ala/Ala | Val/Val | Deletion/Deletion | Died with purpura fulminans and multi-organ dysfunction |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 | Purpura fulminans | Postoperative | Down's syndrome<br>Atrioventricular septal defect | Wild type | Wild type | Wild type | b/b | Ala/Thr | Val/Val | Deletion/Deletion | Died with purpura fulminans |
| 11 | Intracardiac | Postoperative | Common atrium<br>Common atrio-ventricular orifice<br>Double inlet left ventricle<br>Pulmonary stenosis | Wild type | Wild type | Wild type | b/b | Ala/Thr | Val/Val | Deletion/Deletion | Died with haemodynamic and respiratory insufficiency |
| 12 | Left femoral artery | Preoperative | Tetralogy of Fallot<br>Patent oval foramen | Wild type | Wild type | Wild type | b/b | Ala/Thr | Val/Val | Deletion/Deletion | Died with systemic inflammatory response syndrome and multi-organ dysfunction |
| 13 | Right popliteal and superficial veins | Preoperative | Down's syndrome<br>Ventricular septal defect<br>Patent oval foramen | Wild type | Wild type | Wild type | a/a | Ala/Ala | Val/Val | Deletion/Deletion | Alive, warfarin treatment completed |
| 14 | Bidirectional cava-pulmonary anastomosis | Postoperative | Tricuspid atresia<br>Double outlet right ventricle<br>Ventricular septal defect<br>Pulmonary stenosis | Wild type | Wild type | Wild type | b/b | Ala/Thr | Val/Val | Deletion/Deletion | Alive |
| 15 | Blalock–Taussig shunt | Postoperative | Transposition<br>Ventricular septal defect<br>Pulmonary stenosis | Wild type | Wild type | Wild type | a/b | Ala/Thr | Val/Val | Deletion/Deletion | Alive |

Table 2. The distributions of the mutations and polymorphisms in our patients compared with healthy Turkish subjects.

| Mutation/ polymorphism | Genotype | Patients | Healthy Turkish subjects |
|---|---|---|---|
| Factor V Leiden | G1691A | 6.7%* | 7.2%*[1] |
| Prothrombin | G20210A | 0* | 2.3%*[2] |
| Methylentetrahydrofolate reductase | C677T | 0* | 28.8*[3] |
| Endothelial nitric oxide synthase | a/a | 7.7% | 2.4%[4] |
| | a/b | 23% | 29.3%[4] |
| | b/b | 69.3% | 68.3%[4] |
| Alpha-fibrinogen | Thr/Thr§ | 13.3% | 32.6%[4] |
| | Ala/Thr | 73.4% | 59%[4] |
| | Ala/Ala | 13.3% | 8.4%[4] |
| Factor XIII | Val/Val | 61.5% | 71.5%[4] |
| | Val/Leu | 30.8% | 27.6%[4] |
| | Leu/Leu | 7.7% | 0.9%[4] |
| Angiotensin 1 converting enzyme | Deletion/ Deletion§ | 66.6% | 37.4%[5] |
| | Insertion/ Deletion | 26.7% | 47.2%[5] |
| | Insertion/ Insertion | 6.7% | 15.4%[5] |

§ p value is less than 0.05
* Percentage of heterozygous cases
[1] Reference 8
[2] Reference 9
[3] Reference 3
[4] Percentages from our analyses
[5] Reference 10

though statistically insignificant, the frequency of the a/a genotype is twice as high in our patients than in the healthy Turkish population we investigated. The number of patients in our cohort was inadequate to draw a conclusion.

Polymorphism of alpha-fibrinogen Thr312Ala occurs in a region of the gene that is important for Factor XIIIa-dependent crosslinking of fibrinogen. Research has shown that presence of the Ala allele is associated with formation of more stable fibrin clots. Thus, individuals with this allele might be at increased risk for thromboembolic events than those who carry only the Thr allele.[6] We found a significantly lower frequency of the Thr/Thr genotype that prevents thrombosis in our cohort of patients than in the healthy Turkish population we investigated. Specifically, 13 of our 15 patients were either homozygous or heterozygous for the Ala allele, possibly indicating an increased susceptibility to thromboembolic events.

Genetic alterations involving the renin-angiotensin system have been reported to play roles in myocardial infarction and hypertension.[16] There is also recent evidence to suggest that disruption of this system is strongly linked with venous thrombosis.[12] Angiotensin-converting enzyme cleaves the carboxy terminal of angiotensin, and degrades the vasodilator bradykinin. Recent studies have demonstrated that the deletion allele is associated with higher levels of angiotensin 1 converting enzyme, and an increased risk of thrombosis.[16] In our analysis, the frequency of the deletion/deletion genotype in our cohort of children was almost twice the rate we observed in the healthy subjects, this being significant at a level of less than 0.05.

The results of our study suggest that, in addition to prothrombotic mutations such as Factor V Leiden, Prothrombin G20210A and methylentetrahydrofolate reductase C677T, single-nucleotide polymorphisms related to thrombosis need to be kept in mind when caring for children with congenital cardiac malformations who develop thrombosis. Immobilization, catheterization, and the surgical procedures themselves, are some of the factors suspected to induce a thrombotic process in the perioperative period of congenital cardiac surgery. In our study, we investigated only the 15 patients who developed severe thrombosis from the 819 patients who had undergone cardiac catheterization, and 674 who had undergone congenital cardiac surgery. We believe that increasing the number of subjects with thrombosis in further multicentric studies would give more accurate results.

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