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Detection of Inherited Thrombophilic Mutations in Jordanian Children Suffering from Thrombotic Events

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Abstract

Inherited thrombophilia is a coagulation condition that is linked to increased risk of thrombosis. This study investigates the prevalence of FV Leiden (G1691A), FV H1299R (HR2), FV Y1702C, MTHFR C677T, MTHFR A1298C, FII G20210A and PAI-1 mutations among children with thrombotic events. This single-center study included 60 Jordanian children of both sexes (4 days-18 years) admitted to hospital and diagnosed with thrombotic event of any type. The control group consisted of 50 healthy subjects. The presence of thrombophilic mutations was detected using polymerase chain reaction strip assay. The majority of thrombotic events (38.3%) were reported in children at school age (6-12 years) and in adolescents (26.7%) (13-18 years). The most common thrombotic events were deep vein thrombosis (35%) followed by cerebrovascular accidents (18.3%) and cerebral vein sinus thrombosis (16.6%). The most common thrombophilic mutations among children diagnosed with thrombotic events were PAI-1(4G/5G), MTHFR A1298C, MTHFR C677T and FV Leiden (G1691A) constituting 63.3%, 56.7%, 48.3%, and 41.7%, respectively. A statistically significant difference in the occurrence of mutations between the control group and children with thrombotic events was found only in FV Leiden (G1691A), MTHFR A1298C and FV H1299R. This study revealed that neither children with thrombotic events nor subjects in the control group carried the FV Y1702C mutation. In conclusion, the presence of FV Leiden (G1691A), MTHFR A1298C and FV H1299R mutations may be considered as a risk factor for thrombosis in children. Genetic testing of children with family history could play an important role in detecting high-risk subjects.

Keywords: Deep vein thrombosis, inherited thrombophilia**,** FV Leiden, thrombophilic mutations**,** venous thromboembolism.

1. Introduction

Pediatric venous thromboembolism (VTE) is a severe health problem with mortality risk and possible complications such as pulmonary embolism, cerebrovascular events, and post-thrombotic syndrome (Monagle and Newall, 2018). Because of its growing recognition in the pediatric population, VTE is no longer considered an adult illness. In the early 1990s, the reported incidence of VTE was just 5.3 occurrences per 10,000 pediatric hospital admissions while in 2017 it had risen substantially to 30-58 incidents per 10,000 hospital admissions (Witmer and Takemoto, 2017). This increased risk in VTE cases may be due to greater survival of critically ill children as a result of increased use of central venous catheters, sophisticated therapies, advanced surgical techniques, and pediatricians' enhanced awareness of VTE (Faustino and Raffini, 2017).

The tendency to develop VTE is genetically determined, acquired or both (Lane et al., 1996). A systematic review on the impact of inherited thrombophilia on VTE in children has shown a significant association between thrombosis and the presence of inherited thrombophilic risk factors (Young et al., 2008). Yet, routine thrombophilia testing is controversial. The criteria for selecting who must be tested should be defined on a scientific basis to increase the chance of discovering a genetic risk factor (Vagdatli et al., 2013). It has been suggested that genetic testing of children is required if there is a family history of thrombophilia (Celkan and Dikme, 2018). In newborns, children, and adolescents with unprovoked and recurrent VTE, diagnostic thrombophilia testing is also advised, especially when no triggering factor is evident (Revel-Vilk et al., 2003). Because the prevalence of inherited thrombophilic mutations varies by population (Roberts et al., 2009), this study was designed to investigate the prevalence of inherited thrombophilia in Jordanian children suffering from any type of thrombotic event.

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2. Materials and Methods

2.1. Population of the study

Children of both sexes (4 days-18 years) admitted to Queen Rania Al-Abdullah Hospital for Children during the period from the 1st of January to 29th of November, 2021 and diagnosed with any thrombotic event with or without any associated disease were included in the study. The ethical approval was obtained from King Hussein Medical Centre, IRB number: 20/2021. The control group included 20 healthy Jordanian children (1 year -18 years) and 30 healthy Jordanian adults (19 - 45 years). Clinical and demographic data of patients (age, sex, thrombotic events, family history and the presence of any associated disease) was extracted from the electronic medical records.

2.2. Testing genetic mutations

Blood samples were collected in EDTA tubes for DNA extraction. Polymerase chain reaction (PCR) was used for genetic testing. The test is based on the reverse-hybridization principle, where specific oligonucleotide probes immobilized as parallel lines on membrane-based strips hybridize with biotinylated PCR products. Genomic DNA was extracted according to a standard procedure using Wizard Genomic DNA Purification kit (Promega, USA, Cat. No. A1125). Thermal Cycler (BIO-RAD iCycler Thermal**)** was used to amplify the DNA. DNA polymerase, PCR mix solution containing oligonucleotides and nitrocellulose membrane strips coated with oligonucleotideswere were obtained from Nuclear Laser Medicine Srl-Viale Delle Industrie, 3 – 20090**,** Settala (MI), Italy. The incidence of factor V (FV) Leiden (G1691A), FV H1299R (HR2), FV Y1702C, methylene-etrahydrofolate reductase (MTHFR) C677T, MTHFR A1298C, Factor II (FII) G20210A and plasminogen-activator inhibitor-1 (PAI-1) (4G/5G) polymorphism was studied.

2.3. Statistical analysis

Using descriptive statistics, the data was presented as numbers (percentages) for categorical variables and mean ± standard deviation for numeric variables. A web-version of Fisher's exact test (Univariate analysis) was used to compare categorical variables (gender and the presence of mutation). SPSS version 22 was used to study the correlation between the type of thrombotic event and age. A p-value of ≤ 0.05 was considered as statistically significant.

3. Results

3.1. Demographic and clinical characteristics of the study population

This study included 60 Arab Jordanian children (32 males and 28 females) admitted to Queen Rania Al-Abdullah Hospital for Children, Amman, Jordan and diagnosed with any thrombotic event. Their ages ranged from 4 days to 18 years with an average of 9 years. The control group included 50 healthy Jordanian subjects (25 males and 25 females). Their ages ranged from 1 year to 45 years: 20 children with a mean age of 9 years and 30 adults with a mean age of 30 years.

A 4-day old male newborn had right ischemic brain lesion and cerebral artery occlusion. The only mutation he had was 4G/4G. The majority of thrombotic events (23 cases; 38.3% of total cases) were at school age (6-12 years) and adolescents (13-18 years) (16 cases; 26.7% of total cases). The most common thrombotic event was deep vein thrombosis (DVT): 21 patients (11 females and 10 males; 35% of total cases). The second most common thrombotic event was cerebrovascular accident (CVA): 11 patients (4 females and 7 males) that constituted 18.3% of the total cases. The third was cerebral vein sinus thrombosis (CVST): 10 patients (4 females and 6 males; 16.6% of the total cases) followed by renal vein thrombosis (RVT) and portal vein thrombosis (PVT) (Table 1). No correlation between the type of thrombotic event and age was found.

Table 1. Thrombotic events reported in the studied children with thrombotic events.

Thrombotic event episode(s)	Sex			
	M	F	Total	Percent (%)
DVT (deep vein thrombosis)	10	9	19	31.6
DVT+ PE **(**pulmonary embolism**)**	0	2	2	3.3
cerebrovascular accident (CVA)	7	4	11	18.3
cerebral vein sinus thrombosis (CVST**)**	6	4	10	16.6
renal vein thrombosis (RVT)	4	3	7	11.6
portal vein thrombosis (PVT)	2	4	6	10
Hepatic vein thrombosis (HVT)	2	0	2	3.3
Thrombosis at AV graft	1	0	1	1.6
Internal jugular vein thrombosis (IJVT)	0	1	1	1.6
Progressed ischemic toes foot and left forearm cubital area	0	1	1	1.6
TOTAL	32	28	60	

None of the children who were diagnosed with thrombotic event died. Forty-three (70% of children with thrombotic events) had a thrombotic event for the first time without a family history or an associated disease except for one child who had a confirmed family history of DVT in several members of his family. Fourteen children (21.7%) had a thrombotic event with an associated disease such as nephritic syndrome (NS) and systemic lupus erythematosus (SLE) (Table 2). Twelve of them were catheterized; one had arteriovenous graft (AV graft) and eleven had central venous catheter (CVC). Five children (8.3%) had recurrent thrombotic episodes: one of them had recurrent DVT and the other child had DVT at the age of 1 year and developed cerebral vein sinus thrombosis at the age of 11 years. The other three children had recurrent thrombotic events with an associated disease (Table 2).

Table 2: Associated diseases with thrombotic events in the studied population of children

Thrombotic event	Associated diseases/Family history	Recurrent thrombosis	Family history	No. cases	Gender	Age
CVA	Single ventricle	-	-	1	M	4Y
CVA	Arthralgia	-	-	1	F	8Y
CVA	Press syndrome	-	-	1	F	9Y
CVA	Down syndrome	-	-	1	M	5Y
CVA	Microcephaly	-	-	1	M	1Y
DVT +PE	Nephrotic syndrome + family history of recurrent thrombosis	√	√	1	F	6Y
DVT	Diagnosed as End-Stage Renal Disease (ESRD)	-	-	1	M	12Y
DVT	systemic lupus erythematosus (SLE)	-	-	1	F	11Y
DVT	Nephrotic syndrome ESRD	-	-	1	M	10Y
DVT	Diagnosed SLE	-	-	1	F	9Y
	Steroid resistant nephrotic syndrome					
DVT	Brain stroke on 12Year old	√	-	1	M	14Y
CVST	CVA on 2-year-old	√	-	1	F	5Y
	Case of arteriovenous Malformation					
IJVT	Case of Acute myeloid leukemia (AML)	-	-	1	F	12y
Thrombosis at AV graft	ESRD	-	-	1	M	13Y
	Dialysis was through AV. Fistula develop thrombosis					
CVA	recurrent stroke complicated by hemiparesis and epilepsy (primary angiitis of the CNS)	√	-	1	M	6Y

3.2.

3.3. Genotyping results of children with thrombotic events vs. control group

Results of this study showed that 85% of the children diagnosed with thrombotic events had at least 2 of the studied thrombophilic genetic variants. The most common thrombophilic mutations in children with thrombotic events were as follows: PAI-1(4G/5G), MTHFR A1298C, MTHFR C677T and FV Leiden (G1691A) constituting 63.3%, 56.7%, 48.3% and 41.7%, respectively. None of the children with any thrombotic event or in the control group carried the FV Y1702C mutation (Figure 1). The prevalence of thrombophilic mutations among the control group was as follows: 22 (44%) had MTHFR C677T mutation, 16 (32%) had MTHFR A1298C mutation, 7 (14%) had PAI-1(4G/4G), 5 (10%) had FV H1299R, 4 (8%) had FV Leiden (G1691A) mutation, 4 (8%) had PAI-1(5G/5G), 39 out of 50 (78%) had PAI-1(4G/5G), 2 (4%) had FII G20210A.

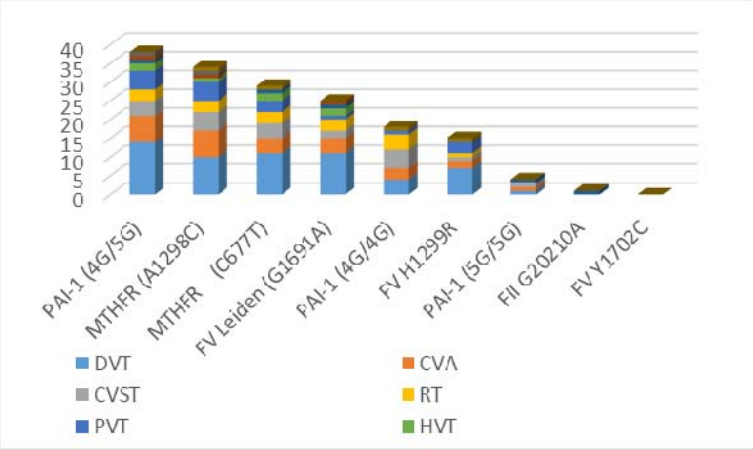

Figure 1: Thrombotic events associated with the studied mutations

The incidence of FV Leiden (G1691A), FV H1299R (HR2), FV Y1702C, MTHFR C677T, MTHFR A1298C, FII G20210A, PAI-1(4G/5G) polymorphism among children diagnosed with thrombotic events compared to the control group is illustrated in Table 3. No statistically significant difference was found between males and females for the presence of any of the seven studied mutations in both control group and children with thrombotic events. A significant difference in the incidence of mutations in the control group and children with thrombotic events was found only in the incidence of FV Leiden (G1691A), MTHFR A1298C and FV H1299R (Table 3). On the other hand, no significant difference in the homozygous/heterozygous ratio was found between the control group and the thrombotic group for the mutations MTHFR A1298C, MTHFR C677T and PAI-1 (Table 3).

Mutation	Frequency in patients	Genotype status HET vs HOMO	Male: female	* P-Value	Frequency in control group	Genotype status HET vs HOMO	Male: female	£P-Value	#P-Value	Ω P-Value
FV Leiden (G1691A)	25 (41.7%)	Heterozygous (GA): 23 Homozygous (AA): 2	11: 14	.295	4 (8%)	Heterozygous	2:2	1.000	**.0001**	-
FV H1299R (HR2)	15 (25%)	Heterozygous (GA): 14 Homozygous (GG): 1	8: 7	1.000	5 (10%)	Heterozygous	2:3	1.000	**.049**	-
FV Y1702C	0	-	-	-	0	-	-	-	-	-
MTHFR C677T	29 (48.3%)	Heterozygous (CT): 25 Homozygous (TT): 4	13: 16	.300	22 (44%)	Heterozygous (CT): 20 Homozygous (TT): 2	10: 12	.776	.703	0.687
MTHFR A1298C	34 (56.7%)	Heterozygous (AC): 31 Homozygous (CC): 3	18: 16	1.000	16 (32%)	Heterozygous (AC): 15 Homozygous (CC): 1	6: 10	.363	**.012**	1.000
PAI-1	56 (93.3%)	Heterozygous 4G/5G:38 Homozygous 4G/4G: 18	29: 27	.615	46 (92%)	Heterozygous 4G/5G:39 Homozygous 4G/4G: 7	24:22	.609	1.000	0.064
FII G20210A	1 (1.7%)	Heterozygous	0: 1	.466	2 (4%)	Heterozygous	1: 1	1.000	0.589	-

Table 3: Frequency, genotype status and sex ratio in the control and thrombotic children groups.

P≤ 0.05 was considered significant

* P- value for gender difference for the presence of the mutation within patients

£ P- value for gender difference for the presence of the mutation within control group

P- value for mutation presence between patients and control group

Ω P- value for homozygous/ heterozygous ratio between patient and control group

4. Discussion

Mutations in genes encoding proteins that activate coagulation process or inactivate anticoagulation play an essential role in predisposition and increase the risk of venous thrombosis (VT) (Nowak-Göttl et al., 2018). In the current investigation, a significant difference in the incidence of mutations was found in FV Leiden (G1691A), MTHFR A1298C and FV H1299R between the control group and children with thrombotic events.

FV Leiden results from the replacement of guanine by adenine at nucleotide 1691 in the FV gene. This leads to the replacement of glutamine by arginine at the activated protein C (APC)-cleavage site resulting in a lack of response to APC. APC is a naturally occurring anticoagulant protein that cleaves and inactivates the procoagulant FVa and FVIIIa, preventing thrombin production (Dahlbäck, 2008). Several studies highlighted the importance of FV Leiden (G1691A) genotype in the development of childhood and adult thrombosis. FV Leiden was found to be a risk factor for ischemic stroke in children (Masri and Al-Ammouri, 2016). In another study, factor V Leiden heterozygozygosity was linked to a seven-fold increased risk of arterial ischemic stroke, neonatal arterial ischemic stroke, and transient ischemic attack (Herak et al., 2009). In adults, the most frequent genetic risk factor for VTE was FV Leiden (G1691A), which was identified in 20- 25 % of VTE patients and 50 % of hereditary thrombophilia patients (Rosendaal et al., 1995). Similarly, FV G1691A mutation was linked to an increased risk of developing thrombosis, including ischemic stroke in children (Ozyurek et al., 2007).

Carrying FV H1299R (A4070G) allele is related to modest APC resistance and a relative excess of the more thrombogenic FV isoform FV1 in plasma (Castoldi et al., 2000a). The prevalence of FV H1299R variant in Jordanian children with thrombotic events was found to be 25% and only 10% in the control group with a significant difference between the two groups. A recent study conducted in Southern Italy on 282 patients (adults and children) with ischemic stroke and 87 patients with transient ischemic attacks, revealed that the prevalence of FV H1299R was 9.9% in ischemic stroke group, 11.5% in transient ischemic attacks group and 9.1% in general population (Cernera et al., 2021). Importantly, it has been reported that the FV H1299R mutation increases the incidence of VT in FV Leiden mutation carriers (Faioni et

al., 1999) and it was considered as a thrombotic risk factor itself (Alhenc-Gelas et al., 1999).

In the present investigation, none of the healthy children or children affected by any thrombotic event carried FV Y1702C mutation. Our study agrees with previous studies that reported a rare prevalence of FV Y1702C mutation in the general population (Castoldi et al., 2000b).

Prothrombin, also known as FII G20210A, is a vitamin K-dependent clotting factor made by the liver which is involved in the conversion of fibrinogen to fibrin. In patients with prothrombin gene mutation plasma, prothrombin is increased due to guanine replacement by adenine at location 20210 in the prothrombin gene (Dahlbäck, 2008). In patients with a family history of venous thrombophilia, FII G20210A mutation was considered to be a moderate risk factor for VT (Poort et al., 1996). In our study, the prevalence of FII G20210A in children diagnosed with thrombotic events was 1.7% with no statistically significant difference from the control group. Similar to our findings, this mutation was not found to be a significant predictor of cerebral thrombosis and arterial ischemic stroke risk in children (Herak et al., 2009; Ozyurek et al., 2007).

The enzyme MTHFR is involved in the methylation of homocysteine to methionine. Mutations in the MTHFR gene may lower enzyme activity, resulting in hyperhomocysteinemia which was associated with a variety of vascular problems, including coronary artery disease and DVT (Liew and Gupta, 2015). In the present investigation, MTHFR had a high prevalence among children diagnosed with thrombotic events. The MTHFR A1298C mutation was present in 56.7% while MTHFR C677T mutation was found in 48.3%. Twenty five percent of children with thrombotic events had both MTHFR A1298C and C677Tmutations. A significant difference in the occurrence of MTHFR A1298C, but not MTHFR C677T mutation, was found between the control group and children with thrombotic events. In an Italian study, the prevalence of MTHFR C677T was 52.1% in ischemic stroke group, 47.7% in transient ischemic attacks group and 43.8% in general population while the prevalence of MTHFR A1298C was 28.3% in ischemic stroke group, 28.2% in transient ischemic attacks group and 29% in the general population (Cernera et al., 2021). Another study showed a correlation between the presence of MTHFR C677T gene polymorphism and the onset of ischemic stroke in young age (Cernera et al., 2021). Moreover, a case-control study comparing 141 childhood patients with VT with 345 healthy controls from Germany revealed that MTHFR C677T mutation is a significant risk factor for venous vascular occlusion in children (Koch et al., 1999).

In our study, the most prevalent mutations among children diagnosed with thrombotic events was PAI-1 (93.3%: 68% 4G/5G and 32% 4G/4G). PAI-1 is the main inhibitor of plasminogen activation, blocking tissue plasminogen activator and urokinase plasminogen activator from converting plasminogen to the fibrinolytic enzyme plasmin (Wiman, 1995). Elevated PAI-I expression that leads to impaired fibrinolytic function is commonly observed in patients with thrombotic disease (Tang et al., 2018). However, the prevalence of PAI-1 mutations was not statistically significant from that of the control group. Endogamy among Jordanian society may explain the high prevalence of these mutations. In a previous study conducted on Jordanian general population, the prevalence of PAI-I 4G/5G polymorphism was 66% (Al-Zoubi et al., 2021). According to recent study, the presence of PAI-1 4G/4G polymorphism increased the risk of arterial and venous thrombosis of varied localization in infants by 48.8% and the likelihood of thrombosis by 9 folds (Filippova et al., 2020). On the other hand, PAI-1 4G/4G and PAI-1 4G/5G genotypes were linked to an elevated risk of VTE in Asian populations (Zhang et al., 2020). Also, the PAI-1 gene 4G/5G genotype was found to be a significant predictor of cerebral thrombosis risk in children in another investigation conducted in Turkey (Ozyurek et al., 2007).

In a previous study, 95% of children with VTE events had an underlying surgical or medical condition such as cardiac disease, cancer, infection or autoimmune disease that triggered thrombosis (Sadiq et al., 2021). In our study 21.7% of the studied children had thrombotic events associated with other diseases such as NS, SLE, acute myeloid leukemia and others. Three children with NS had DVT, and one had thrombosis at AV graft. According to another study NS was associated with a high incidence of VTE (Singhal and Brimble, 2006). Similarly, it was reported that a high prevalence of VTE (28%) is present in patients with primary NS in which 70% of VTE cases were unilateral RVT (Ismail et al., 2020). It has been suggested that the association of haemostatic imbalance secondary to NS (Ismail et al., 2014) with chronic inflammation associated with certain glomerulonephritis along with a genetic predisposition can trigger a VTE (Kerlin et al., 2012). Thus, inherited thrombophilia does not result in a spontaneous VTE, until an acquired hypercoagulable state in NS determines the clinical manifestation of the prothrombotic tendency due to loss of protein in urine that prevents thrombosis and increased synthesis of factors that promote thrombosis (Reich et al., 2003).

In our study, two children had SLE, and it was confirmed that they had DVT. It has been reported earlier that SLE was associated with three times- higher VTE rate compared to the general population (Lee and Pope, 2014). This acquired disease was considered as an independent risk factor for both arterial and venous thrombotic events (Bazzan et al., 2015).

None of the children who were diagnosed with thrombotic event died in this study. Advances in pediatric care may decrease fatality rate in cases of pediatric thrombosis. Long-term follow up of patients especially with recurrence may help in recording the mortality rate. The majority of cases in our cohort suffered from DVT. As reported earlier in literature, young age was a protective factor against developing VTE. Unlike in adults, pulmonary emboli in adolescents were rarely fatal (Spentzouris et al., 2012). In addition, pediatric strokes had better prognosis than adult strokes (Steinlin et al., 2004).

The control group in our study consisted of 20 healthy children and 30 adults. If the control group was composed of children only, one may argue that they may develop thrombotic event in the near future. Thus, including healthy adults in the control group would eliminate this possibility. This was evident in the case of PAI-1 mutations in which a high prevalence was present in both control and thrombotic children indicating that it may not represent a risk factor for thrombosis at later stages in life.

The main aim of the present work was to study the prevalence of thrombophilic mutations among children with thrombotic events. The main limitation of this study was the small number of cases. The reason for that is the rare occurrence of thrombotic events among children. As reported in other studies, thrombotic events in children are rare (Steinlin et al., 2004). Therefore, future longer-term studies are needed.

In conclusion, the present study showed that the most common thrombotic events among children were DVT, followed by CVA and CVST. Eighty five percent of children with thrombotic events had at least 2 mutations among the 7 studied mutations. The presence of FV Leiden (G1691A), MTHFR A1298C and FV H1299R mutations may present a risk factor for thrombosis in children. The findings of this study suggest that genetic testing for inherited thrombophilia in children with family history and/or associated disease such as NS and SLE may play an important role in detecting high-risk patients. The use of prophylactic interventions and life style modifications are recommended in these cases. The gain of knowledge with respect to the pathophysiology of VTE in children is important to correctly assess the need for genetic testing of children with thrombotic events and their family members as well.

Conflict of interest

The authors declare no conflict of interests.

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