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ORIGINAL RESEARCH

Risk factors for deep vein thrombosis of lower extremities in Sudanese women

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Nadir Ahmed Ibrahim[1,2]
Fathelrahman M Hassan[3]
Mahmoud Mohamed Elgari[2]
Sana Eltahir Abdalla[4]

[1]Department of Hematology and Immunohematology, College of Medical Laboratory Science, Sudan University of Science and Technology, Khartoum, Sudan; [2]Department of Clinical Laboratory Sciences, Faculty of Applied Medical Sciences, Taibah University, Medina, Saudi Arabia; [3]Department of Clinical Laboratory Science, College of Applied Medical Science, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia; [4]Neelain Medical Research Centre, Al Neelain University, Khartoum, Sudan

Aim: In this study, we aimed to analyze the genetic and acquired risk factors for deep vein thrombosis (DVT) of the lower extremities among Sudanese women.

Methods: A total of 136 women were enrolled in the study, including 75 DVT patients and 61 healthy controls. Demographic and clinical data were collected using a specific questionnaire. Citrated blood samples of patients and controls were used for coagulation assays, and DNAs isolated from EDTA-blood samples were used for the detection of Factor V Leiden and prothrombin G20210A mutations using multiplex polymerase chain reaction-restriction fragment length polymorphism analysis.

Results: Both gene mutations were found to be absent from all 136 subjects, and therefore did not account for the incidence of DVT in Sudanese women. Of the 75 DVTs, 70 (93.3%) were localized in the left leg and 5 (6.7%) in the right leg. Additionally, 84% of the DVTs were proximal and 16% were distal. Among the 75 patients, 22 (29.33%) were postpartum, 7 (9.33%) were pregnant, and 46 (61.33%) were nonpregnant. Levels of prothrombin fragment 1+2, prothrombin time, activated partial thromboplastin time, and D-dimer were significantly higher in DVT patients than in healthy controls ($P<0.0001$).

Conclusion: Risk factors that most significantly affected patients in the 18–45 years age group were pregnancy and oral contraceptive usage, whereas those that most significantly affected patients in the 66–90 years age group were immobility, heart disease, and history of DVT.

Keywords: pregnancy, oral contraceptive, DVT, Factor V (G1691A), prothrombin (G20210A), PCR-RFLP

Introduction

Deep vein thrombosis (DVT) occurs at an incidence ranging from ~45 to 117 in 100,000 adults per year.[1] African Americans have higher rates of DVT than Caucasians.[2,3] The prevalence of DVT in Africans varied between 380 and 448 per 100,000 births per year in pregnant and postpartum women, and between 2.4% and 9.6% in postoperative patients.[4] DVT is a major cause of morbidity and mortality in older adults and one of the leading causes of maternal mortality in the western world.[5] Venous thrombosis is a reproductive health risk for women. During pregnancy, the risk of venous thrombosis increases by up to 5-fold, and the risk further increases by 60-fold postpartum.[6] Furthermore, a large number of women worldwide are at a high risk of developing venous thrombosis because of the use of oral contraceptives or hormone replacement therapy (HRT). Moreover, women undergoing infertility treatment may be exposed to circumstances related with an increased risk of venous thrombosis.[7] DVT is uncommon among patients younger than 40 years of age, and more common in women than in men amid reproductive

Correspondence: Nadir Ahmed Ibrahim
Department of Clinical Laboratory Sciences, Faculty of Applied Medical Sciences, Taibah University, Medina 41411, Saudi Arabia
Tel +966 501 486 141
Email nadir.ibrahim@outlook.com

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age,[8] which supports the role of hormonal contraceptives and pregnancy in developing DVT during this period of life.[9] The incidence of venous thrombosis is ~1 in 1000 pregnancies.[10] Additionally, pregnant women are 6- to 10-fold more likely to develop DVT than nonpregnant women of the same age.[11,12] Among pregnant women, the frequency of thrombosis is similar in each of the three trimesters and is increased during the postpartum period.[13]

Thrombophilia increases the risk of pregnancy- and oral contraceptive-associated venous thrombosis, especially in women with combined or homozygous defects.[14] Factor V Leiden and prothrombin G20210A gene mutations are the most common forms of heritable thrombophilia. Both these mutations have a distinctive worldwide distribution; both mutations are the most prevalent among Caucasians and Middle Easterners, and have a moderate to rare prevalence among other populations, including Africans. Both mutations cause venous thrombosis with high mortality and morbidity.[2,15,16] Factor V Leiden mutation renders Factor Va resistant to cleavage by activated protein C (APC), which results in increased thrombin generation and higher levels of prothrombin fragment 1+2.[17–19] Mutant proteins confer a 5- to 10-fold greater risk of developing DVT in heterozygous individuals and a 50- to 100-fold higher risk in homozygotes compared with normal individuals.[20] The second most common genetic risk factor for hereditary thrombophilia is the prothrombin gene mutation. Substitution of G to A at nucleotide at position 20,210 of the prothrombin gene leads to an increased prothrombin production.[21] This defect is associated with a 3-fold greater risk of venous thrombosis.[22] The prevalence of the G20210A prothrombin variant is between 1% and 4%, and it appears to be highly rare in African individuals.[23]

In addition to genetic risk factors, there are several acquired risk factors for DVT, which are mostly identified in young and middle-aged women; these include prolonged immobility, surgery, malignant diseases, heart disease, pregnancy, HRT, and oral contraceptives.[7,24–26] In this study, we focused on identifying the acquired and genetic risk factors for DVT in Sudanese women and the interaction between them. We investigated risk factors for DVT in nonpregnant, pregnant, and postpartum women. Knowledge of these risk factors will enhance our understanding of the factors that increase the risk of developing DVT, which would further help medical practitioners to determine the optimal time and method of thromboprophylaxis and other treatments.

Materials and methods

Study group

This case control study was conducted from July 2014 to July 2017 in Khartoum State, the capital of Sudan. A total of 136 Sudanese women, including 75 female patients with confirmed DVT diagnosis using duplex ultrasound and 61 nonpregnant healthy females (controls), were recruited for this study. The control subjects had no history of venous thromboembolism or coagulation disorders. Demographic information and clinical data were collected from all subjects using a standardized questionnaire (Figure 1). EDTA-blood and citrated blood samples were collected for each individual for DNA extraction and coagulation tests, respectively.

DNA extraction

Total genomic DNA was extracted from peripheral blood using a G-spin DNA Extraction Kit (iNtRON Biotechnology Inc, Jungwon-gu, South Korea) according to the manufacturer's instructions.

Coagulation assays

Coagulation tests including prothrombin time (PT) and activated partial thromboplastin time (APTT) were performed using a STart 4 Hemostasis Analyzer (Diagnostica Stago, France). Fibrinogen assays and D-dimer tests were performed using Multifibren U and Innovance D-dimer reagents, respectively, with a BCS XP System (Siemens Healthcare GmbH, Erlangen, Germany). The APC resistance test was performed using STA-STACLOT (Diagnostica Stago). Sandwich enzyme-linked immunosorbent assay (ELISA) was performed for the quantitative detection of prothrombin fragment 1+2 using Human Prothrombin Fragment 1+2 ELISA Kit (Abbexa, Cambridge, UK).

Primer-engineered multiplex polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) detection of Factor V Leiden and prothrombin G20210A

Simultaneous detection methods were used for Factor V Leiden and prothrombin G20210A mutations in which the *Hin*dIII digested amplification products were analyzed via agarose gel electrophoresis in a single gel lane and visualized by ethidium bromide staining.[27] A 241-bp region of exon 10 of Factor V gene, and 506 bp of the 3′ untranslated region of the prothrombin gene was PCR amplified using gene-specific primers as previously described,[27] with some modifications. Briefly, each 25 µL PCR reaction comprised 100 ng genomic DNA, 12.5 µL of 2X Go*Taq* Green Master Mix (Promega Corporation, Fitchburg, WI, USA), 0.6 µL of 10 pM each of the forward primers, 0.4 µL of 10

Figure 1 Questionnaire designed to collect demographics: age, sex, ethnicity (tribe), and patient and family history.

Date of collection: ----------	Research ID number: ----------
Name of patient: ----------	Age (in years) ----------
Gender:	Male ☐ Female ☐
Tribe: ----------	Occupation----------
Initial diagnosis:	Proximal ☐ Distal ☐
Affected leg:	Right ☐ Left ☐
Duration of illness:	----------
Pregnant:	Yes ☐ No ☐
Postpartum:	Yes ☐ No ☐
Under surgery:	Yes ☐ No ☐
Immobility status:	Yes ☐ No ☐
Bone fracture:	Yes ☐ No ☐
Clotting disorder:	Yes ☐ No ☐
Hormone therapy:	Yes ☐ No ☐
Cancer:	Yes ☐ No ☐
Oral contraceptive:	Yes ☐ No ☐
Cigarette smoking:	Yes ☐ No ☐
Renal failure:	Yes ☐ No ☐
Heart disease:	Yes ☐ No ☐
On warfarin anticoagulant:	Yes ☐ No ☐
On heparin anticoagulant:	Yes ☐ No ☐
Family history of thromboembolism:	Yes ☐ No ☐
How many members of your family have thrombosis?	.....
Sig.....	Date----------

pM each of the reverse primers, and 6.5 µL of not diethyl pyrocarbonate-treated nuclease-free water (Thermo Fisher Scientific, Waltham, MA, USA). The PCR was performed as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. Subsequently, 10 µL of each PCR product was digested with 1.0 µL of 10 U *Hin*dIII (Thermo Fisher Scientific) with 2 µL of 10X enzyme buffer and 18 µL of nuclease-free water at 37°C for at least 3 hours. The digestion products were separated by gel electrophoresis on a 2% agarose gel (Sigma-Aldrich Co, St Louis, MO, USA) and stained with ethidium bromide. Undigested products resulted in two products of 241 and 506 bp, representing Factor V and prothrombin amplicons, respectively. The digestion of wild-type alleles resulted in fragments of sizes 241 bp for Factor V and 407 bp + 99 bp for prothrombin. The digestion of Factor V Leiden homozygous mutants produced 209 and 32 bp fragments, whereas digestion of prothrombin G20210A homozygous mutants produced three fragments of sizes 384, 99, and 23 bp. The digestion of heterozygous individuals carrying Factor V Leiden mutation yielded fragments of sizes 241, 209, and 32 bp, whereas heterozygous prothrombin G20210A mutants yielded fragments of 407, 384, and 99 bp.

Statistical analysis

Statistical Package for Social Sciences (SPSS Statistics version 20; IBM Corporation, Armonk, NY, USA) was used for statistical analysis. Results were expressed as mean ± SD. Continuous variables were analyzed by Student's *t*-test.

Ethical approval

This research related to human use complied with all the relevant national regulations, institutional policies, and tenets of the Helsinki Declaration, and has been approved by the Research Ethical Committee of Sudan University of Science and Technology.

Written informed consent was obtained from all individual participants included in the study.

Results

A total of 136 Sudanese women, including 75 DVT patients and 61 normal controls, were evaluated. There was a significant difference between the mean age of DVT patients (40.75±16.630 years) and controls (31.31±8.698 years)

(*P*=0.000). Among the 75 patients, 29.33% were postpartum (n=22), 9.33% were pregnant (n=7), and 61.33% were nonpregnant (n=46). Of the 7 pregnant women, the number of women in their first, second, and third trimesters were 3 (42.9%), 2 (28.6%), and 2 (28.6%), respectively. Of the 75 DVTs, 93.3% were present in the left leg and 6.7% in the right leg; moreover, 84% were proximal and 16% were distal (Table 1). Among pregnant women, DVTs were always located on the proximal end of the left leg. Among postpartum women, however, DVTs were present in the right leg in 4 women and in the left leg in 8 women; all cases of DVTs were proximal among postpartum women.

We also compared the results of blood coagulation assays between DVT patients and controls. Data showed that levels of prothrombin fragment 1+2, PT, APTT, and D-dimer were significantly higher in DVT patients than in healthy controls (*P*=0.000) (Table 2). Table 3 categorizes the 75 DVT patients according to various risk factors. Additionally, we examined the risk factors for DVT according to the age of patients (Table 4). The 75 DVT patients were divided into three age groups (18–45, 46–65, and 66–90 years) and assessed for the environmental risk factors for DVT. Among the younger patients (18–45 years), postpartum and oral contraceptives usage were the most significant risk factors for DVT. By contrast, immobility status, heart disease, and history of DVT were the most significant risk factors affecting older patients (66–90 years). Notably, Factor V Leiden and prothrombin G20210A mutations were absent from all 136 individuals (Figure 2). As a result, no correlation was observed between DVT and the genetic risk factors for DVT among Sudanese women.

Table 1 Demographics, clinical characteristics, and treatment patterns of the study group (n=75)

Characteristics	(n=75)
Age (years) (mean ± SD)	40.75±16.630
Nonpregnant, n (%)	46.0 (61.33)
Pregnant, n (%)	7.0 (9.33)
Postpartum, n (%)	22.0 (29.33)
Duration of illness/days (mean ± SD)	9.59±5.731
Proximal DVT, n (%)	63.0 (84.0)
Distal DVT, n (%)	12.0 (16.0)
Right leg, n (%)	5 (6.7)
Left leg, n (%)	70 (93.3)
Anticoagulant therapy, n (%)	
Heparin	50 (66.7)
Warfarin	1 (1.3)
Heparin and warfarin	24 (32.0)

Abbreviation: DVT, deep vein thrombosis.

Table 2 Comparison of coagulation assay results between DVT patients (n=75) and healthy controls (n=61)

	Subject	Mean ± SD	*P*-value
Prothrombin time	Test	23.23±10.36	<0.0001
	Control	11.64±0.75	
Prothrombin fragment 1+2	Test	12.64±1.31	<0.0001
	Control	4.44±2.84	
D-dimer	Test	0.66±0.21	<0.0001
	Control	0.31±0.19	
Activated partial thromboplastin time	Test	40.55±14.82	<0.0001
	Control	31.57±7.03	
Activated protein C resistance	Test	2.69±0.32	0.508
	Control	2.73±0.39	
Fibrinogen	Test	2.96±0.47	0.878
	Control	2.94±0.54	

Abbreviation: DVT, deep vein thrombosis.

Table 3 Environmental risk factors among DVT patients (n=75)

	n	%
Age ≥40 years	35	46.7
Oral contraceptives	25	33.3
Postpartum	22	29.33
Under surgery	18	24.0
Cardiovascular disease	12	16.0
Previous history of DVT	8	10.7
Pregnancy	7	9.3
Immobility status	7	9.3
Obesity	6	8.0
Renal failure	2	2.7
Bone fracture	1	1.3
Hormone therapy	0	0.0
Cigarette smoking	0	0.0
Cancer	0	0.0
No risk factors	12	16.0
1 risk factor	32	42.7
2 risk factors	26	34.7
≥3 risk factors	5	6.7

Abbreviation: DVT, deep vein thrombosis.

Discussion

Although the awareness of DVT as an important health issue is growing,[28,29] our understanding of the DVT risk factors remains limited. Blood in women is more likely to coagulate than that in men because this property of blood in women protects against excessive bleeding during pregnancy, miscarriage, and childbirth. Both genetic and acquired risk factors can further increase the risk of thrombosis.[30] Knowledge of genetic and environmental risk factors is crucial for the effective application of diagnostic, prophylactic, and therapeutic interventions, especially among women in childbearing age who are at a higher risk of developing DVT.

In women of reproductive age, over half of all venous thrombotic events are related to pregnancy and puerperium.[6,31] However, we found that 29.3% of DVT women were postpartum and 9.3% were pregnant, indicating that both

Table 4 Risk factors in DVT patients according to age

		Age in groups			
		18–45 years	46–65 years	66–90 years	
		n (%)	n (%)	n (%)	*P*-value
Previous history of DVT	Yes	0 (0.0)	1 (8.3)	7 (77.8)	<0.0001
	No	54 (100.0)	11 (91.7)	2 (22.2)	
Obesity	Yes	5 (9.3)	1 (8.3)	0 (0.0)	0.637
	No	49 (90.7)	11 (91.7)	9 (100.0)	
Hormonal therapy	No	54 (100.0)	12 (100.0)	9 (100.0)	–
Pregnancy	Yes	5 (9.3)	1 (8.3)	0 (0.0)	0.223
	No	49 (90.7)	11 (91.7)	9 (100.0)	
Postpartum	Yes	7 (13.0)	0 (0.0)	0 (0.0)	0.002
	No	47 (87.0)	12 (100.0)	9 (100.0)	
Oral contraceptives	Yes	19 (35.2)	2 (16.7)	0 (0.0)	0.059
	No	35 (64.8)	10 (83.3)	9 (100.0)	
Cigarette smoking	No	54 (100.0)	12 (100.0)	9 (100.0)	–
Under surgery	Yes	14 (25.9)	2 (16.7)	2 (22.2)	0.787
	No	40 (74.1)	10 (83.3)	7 (77.8)	
Bone fracture	Yes	1 (1.9)	0 (0.0)	0 (0.0)	0.821
	No	53 (98.1)	12 (100.0)	9 (100.0)	
Immobility status	Yes	2 (3.7)	0 (0.0)	5 (55.6)	<0.0001
	No	52 (96.3)	12 (100.0)	4 (44.4)	
Heart disease	Yes	2 (3.7)	3 (25.0)	7 (77.8)	<0.0001
	No	52 (96.3)	9 (75.0)	2 (22.2)	
Renal failure	Yes	1 (1.9)	1 (8.3)	0 (0.0)	0.393
	No	53 (98.1)	11 (91.7)	9 (100.0)	
Cancer	No	54 (100.0)	12 (100.0)	9 (100.0)	–

Abbreviation: DVT, deep vein thrombosis.

periods were associated with high risk of venous thrombosis. These data are in agreement with previous studies reporting approximately two and five times as many postpartum as antepartum DVT events, respectively.[6,32,33] Moreover, Gader et al previously reported that most of the DVT events among Sudanese women occurred in the postpartum period.[34] Consistent with our findings, James et al have reported that the risk of DVT was higher in pregnant women in their first trimester than those in their second or third trimesters. Thus, if prophylaxis is indicated, it should be initiated early in gestation.[35] However, DVT rates in the third trimester were higher according to a previous meta-analysis.[36] Here, we observed that in most of the cases, DVTs occurred in the lower left extremity and were proximal. This was particularly true among pregnant women; DVTs in all pregnant women occurred on the proximal end of the left leg. These observations were in complete agreement with previous studies.[34,36–38] However, why the left leg is the preferred site for DVTs is unknown.[39]

Fibrin-related markers, such as D-dimer, and prothrombin fragment 1+2 are considered to be useful for the diagnosis of thrombosis.[40] In this study, significant differences were observed between DVT patients and healthy controls in prothrombin fragment 1+2, PT, APTT, and D-dimer levels.

The development of DVT involves the interaction of multiple modifiable and non-modifiable risk factors.[41] The non-modifiable risks include Factor V Leiden and prothrombin G20210A mutations, which are the two most predominant DVT-associated mutations worldwide.[23,42] Available data suggest that Factor V Leiden carriers are at a 5- to 16-fold greater risk of developing DVT during pregnancy and puerperium than women homozygous for the wild-type Factor V, and are at a 35- to 100-fold greater risk among carriers using oral contraceptives.[14] The risk of pregnancy-associated venous thrombosis was 15-fold higher in women with the prothrombin gene mutation than those with the wild-type prothrombin gene. Moreover, the combination of oral contraceptives with the prothrombin gene mutation has

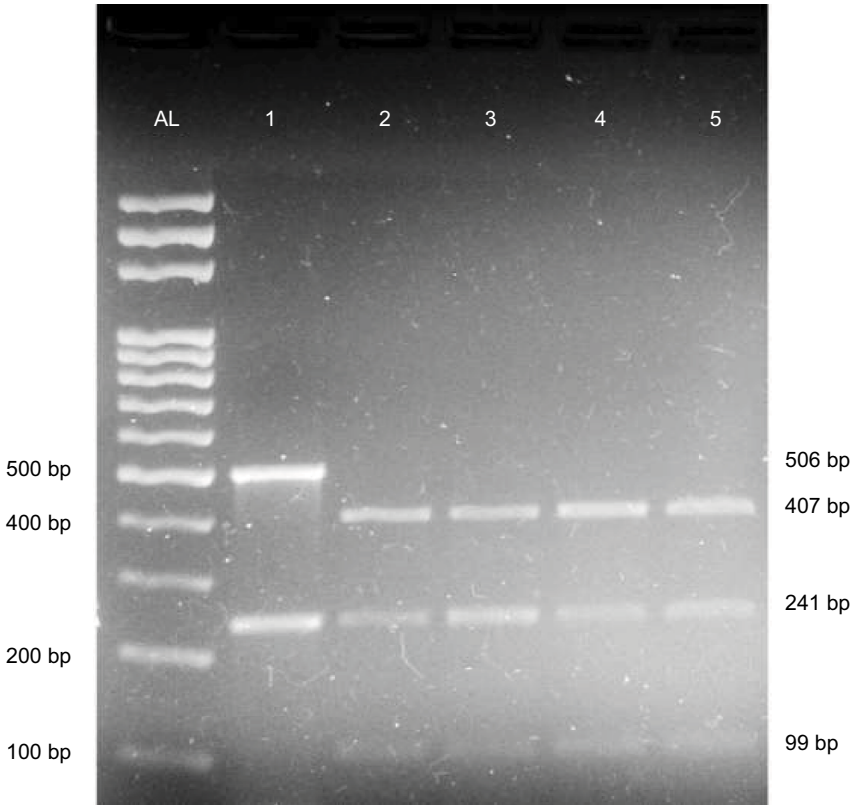

Figure 2 Multiplex PCR-RFLP for detecting Factor V Leiden and prothrombin G20210A mutations in DNA isolated from blood samples of Sudanese women. Photograph shows ethidium bromide-stained 2% agarose gel. AL denotes 100 bp DNA ladder. Lane 1: undigested wild-type 506 bp (prothrombin) and 241 bp (Factor V). Lanes 2–5: digested wild-type amplicons 407 and 99 bp (prothrombin) and 241 bp (Factor V).
Abbreviations: PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

a multiplicative effect on the overall thrombotic risk, with the odds ratio ranging from 16 to 59, as suggested previously.[14] However, our study demonstrated a total absence of Factor V Leiden and prothrombin G20210A mutations among Sudanese women. Therefore, no association was observed between these mutations and DVT in our study population, which is in accordance with the results of other studies.[28,43,44] By contrast, a very low prevalence of Factor V Leiden mutation has been shown among healthy individuals in Sudan.[29] Nonetheless, it is noteworthy that in Sudan, one of the economically struggling countries, it is a priority to adopt robust, cost-effective, and quick diagnostic tools for molecular analysis. The multiplex PCR-RFLP-based analysis used in this study for the simultaneous detection of both Factor V Leiden and prothrombin G20210A mutations was a stable and reproducible single-tube reaction requiring no special or expensive equipment and only a small amount of standard PCR reagents.

Venous thrombosis is commonly a disease of older adults.[26] Our study revealed a significant difference between the mean age of DVT patients and healthy controls. Approximately 47% of the DVT patients were >40 years old. However, this also means that 53% of the DVT patients were <40 years of age. This was expected, as the incidence rates of DVT are slightly higher in women in their childbearing age.[1] When DVT patients were divided into three groups according to age, we found that heart disease and history of venous thrombosis were the most predominant risk factors among elderly women, followed by immobility, which affected more than half of the elderly women. This was consistent with a previous study.[45] Among younger patients, the risk factors most significantly related to DVT were postpartum and oral contraceptive usage. These findings were in accordance with previous reports.[6,33,34,44]

Since 1996, a large number of observational studies have consistently shown a significant (2- to 4-fold) increase in the relative risk of developing venous thromboembolism in HRT users compared with nonusers.[14] Because none of the women involved in this study were undergoing HRT, its association with DVT in this study group was not investigated.

In summary, Factor V Leiden and prothrombin G20210A mutations were not associated with DVT in Sudanese women examined in this study. Further investigations into other hereditary causes of DVT among Sudanese women are needed. Multiplex PCR-RFLP analysis is a highly efficient and cost-effective method, and therefore, it is ideal for laboratories with limited budget. Women who are in their reproductive age or postpartum period and those using oral contraceptives are at a higher risk of developing DVT than other women. Additionally, older women with a history of DVT, heart disease, or prolonged immobility are also at a high risk of developing DVT. Therefore, special care should be given to both these groups to avoid the development of DVT.

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Disclosure

The authors report no conflicts of interest in this work.

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