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# Study of thrombophilic gene polymorphisms in Egyptian patients with deep venous thrombosis using in situ hybridization technique

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## Abstract

Up to 70% of thrombotic patients with no identifiable risk factors were termed idiopathic. Now, molecular diagnostics combined with existing laboratory techniques allow accurate classification of at least half of patients with inherited thrombotic disorders. However, the previously studied genetic variants explain only a fraction of all thromboembolic events, so the aim of the present study was to expand the genetic prevalence determination to include also more extensive thrombophilic gene polymorphisms in patients with DVT compared to healthy subjects in Egyptian population. This study was conducted on 75 patients with DVT and 45 age and sex matched healthy subjects as control group. The diagnosis of DVT was based on patient's history, clinical findings, D-dimer test, and confirmed by Doppler ultrasonography. Both groups were assessed for thrombophilic gene polymorphisms using multiplex polymerase chain reaction and reverse-hybridization technique through CVD strip assay. It was found that FV Leiden G1691A (P=0.001), Factor V H1299R (P=0.02), MTHFR A1298C (P=0.02), β-fibrinogen−455 G/A (P=0.01), PAI-1 4G/5G (P=0.03) and ACE (P=0.03) polymorphisms were all significantly associated with an increased risk of DVT. Our data are of extreme importance in clinical practice for introducing the extended CVD panel into routine molecular diagnostic tests to allow management of thrombotic patients and screening, thromboprophylaxis and genetic counselling for high-risk individuals.

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## INTRODUCTION

Venous thromboembolism (VTE) represents a major health problem worldwide and has a high incidence in several populations across the world [1]. It is considered the third most frequent cardiovascular disorder after myocardial infarction and stroke. VTE is manifested as either deep venous thrombosis (DVT) or pulmonary embolism (PE). DVT is the thrombotic obstruction of deep veins in the lower extremities, and is a significant cause of morbidity and mortality [2,3].

VTE results from the interaction of environmental / acquired risk factors and inherited risk factors, and thus can be considered a typical example of complicated multifactorial disease [4]. Acquired risk factors include age, surgery, major trauma, hormone therapy, pregnancy, puerperium, prolonged immobilization, plaster cast, antiphospholipid syndrome and some cancer types that lead to predisposition to an increased DVT risk [5,6]. Family and twin studies indicated that genetic factors account for about 60% of DVT risk [7].

Three major pathologic factors which effectively contribute to venous thrombosis are hypercoagulable states, venous stasis and endothelial damage [8,9]. However, thrombosis is the end product of an imbalance of procoagulant, anticoagulant and fibrinolytic factors [1].

Inherited thrombophilia is a blood clotting disorder caused by one or more genetic risk factors or mutations that make a person susceptible to DVT/PE [1]. To date, several thrombophilic gene polymorphisms have been recognized that are involved in the pathogenesis of thromboembolic disorders [10]. Of these, factor V G1691A (FV G1691A) polymorphism; also called FV Leiden, in which an Arg to Gln change at amino acid 506 [11], renders factor V resistant to activated protein C (APC) degradation retaining its procoagulant activity favoring thrombosis [12], and the G20210A polymorphism in the 3-untranslated region of the prothrombin (PTH) gene, which results in higher prothrombin levels [13]. Moreover, two common polymorphisms in methylene tetrahydrofolate reductase (MTHFR) gene including C677T and A1298C, lead to diminished enzyme activity and therefore rise of homocysteine level. Several studies have shown that these two polymorphisms might be associated with DVT due to hyperhomocysteinemia [14–17].

The situation of FV at the crossroads of the procoagulant and anticoagulant pathways [18,19], has prompted the scientists to search for new candidate determinants of VTE in the FV gene. FV allele (FV H1299R, also known as HR2), marked by an A→G transition at position 4070 so called FV A4070G [20] and an amino acid substitution His to Arg at position 1299 [21], has been described and observed to be connected to a number of other FV gene polymorphisms (the HR2 haplotype), which encode several amino acid changes [22]. Carrier ship of the FV H1299R allele is associated with mild APC resistance [22] and with a relative excess [23] of the more thrombogenic [24] FV isoforms [25] ($FV_1$) in plasma.

Another polymorphism predisposing to thrombotic tendency is Plasminogen activator inhibitor-1 (PAI-1) 4G/5G in which there is a single guanosine insertion/ deletion at nucleotide-675 (4G/5G) in the upstream regulatory region of

PAI-1 gene [26]. The-675 4G/5G polymorphism affects the binding of nuclear proteins involved in the regulation of PAI-1 gene transcription [27] and is being associated with increased transcription of the PAI-1 gene [28,29]. The 5G allele is slightly less transcriptionally active than the 4G [30]. Therefore, the 4G allele is associated with a higher level of PAI-1 in plasma, which in turn results in a diminished fibrinolysis activity and accordingly a higher tendency to thrombus formation [16,17,31].

The above stated genetic variants only explain a fraction of all thromboembolic events. Therefore, the aim of the present study was to expand the genetic prevalence determination to include not only the above stated genetic variants but also other thrombophilic gene polymorphisms in patients with DVT compared to healthy subjects in Egyptian population. This appears to be the first study to use these extensive thrombophilic gene polymorphisms in diagnosis of thromboembolism in Egyptian population.

## MATERIAL AND METHODS

A total of 75 individuals with DVT clinical symptoms were enrolled in this study as a patient group, and 45 healthy subjects without both personal and family histories of thromboembolic disorders were included as a control group. All case subjects were referred to cardiology department of Benha University hospitals, Benha, Egypt. The diagnosis of venous thromboembolic disease was based on patient's history, clinical findings and D-dimer test. Finally DVT was confirmed by Doppler ultrasonography and CT pulmonary angiography was done for detection of pulmonary embolism. Patients with idiopathic DVT were only included in this study. Patients of DVT with identifiable risk factor such as trauma in the past 6 months, smoking, malignancy, immobility, surgery, pregnancy, use of contraceptive pills or hormone replacement therapy, and renal or liver disease were excluded from this study. None of the healthy control had any evidence of thrombotic events. The present study was conducted according to World Medical Association (WMA) Declaration of Helniski (2008) and approved by the Ethics Committee of Benha Faculty of medicine, Benha, Egypt. Written informed consents were filled out by all participants.

- ***Sample collection and polymorphism detection:***

Three ml of peripheral blood samples from all patients and the control subjects were withdrawn from the antecubital vein and placed immediately into sterile EDTA-containing tubes. Collected whole blood samples were stored at −80 °C for further genetic analysis. The following polymorphisms of genes encoding proteins involved in thrombophilia were analyzed: FV G1691A (Leiden), FV H1299R (R2), PTH G20210A, Factor XIII V34L, ß-Fibrinogen − 455 G/A, PAI-1 4G/5G, Human platelet antigen-1 (HPA-1); also called Glycoprotein IIIa L33P (GPIIIa L33P), MTHFR C677T, MTHFR A1298C , Angiotensin converting enzyme insertion/deletion (ACE I/D), Apolipoprotein B R3500Q (Apo B R3500Q) and Apolipoprotein E2/E3/E4 (Apo E2/E3/E4).

The CVD Strip Assay (ViennaLab, Labordiagnostika GmbH, Vienna, Austria) was used to detect the previously described polymorphisms of genes according to the manufacturer's protocol.

Briefly, the procedure included three steps: DNA isolation, PCR amplification, and hybridization of amplification products as follows:

- ***DNA Isolation***

Genomic DNA was extracted from patients' whole blood. The quantity and quality of the DNA were determined on the NanoDrop ND-2000 spectrophotometer (Thermofisher scientific, USA)

- ***Polymerase Chain Reaction (PCR)***

The different gene sequences were simultaneously amplified in vitro and biotin labeled in two parallel PCR reactions (Multiplexing). T100™ Thermal Cycler, Bio-Rad**,** Singapore, was used for amplification according to the following program: an initial denaturation step of 94 ° C for 2 min, followed by 35 cycles of 94 ° C for 15 s, 58 ° C for 30 s, 72° C for 30 s, and a final extension step of 72 ° C for 3 min.

- ***Reverse Hybridization***

The amplification products were specifically hybridized to a test strip which contained allele-specific oligonucleotide (corresponding to wild type or mutant) probes immobilized as an array of parallel lines. Hybridization was done for 30 min at 45 ° C in a shaking water bath. After 3 stringent washes at 45 ° C, bound biotinylated PCR fragments were detected utilizing a streptavidin-alkaline phosphatase conjugate and color substrates. Upon positive reaction, a purple staining was noticeable after 15 min [32].

**Interpretation of genotyping results**

For each polymorphic position, one of three possible patterns could be obtained: Homozygous normal, heterozygous, or homozygous mutant genotype. For the Apo E isoforms E2, E3 and E4, six possible homozygous and heterozygous Apo E genotypes (E2/2, E3/3, E4/4, E2/3, E2/4, and E3/4) could be obtained.

❖ ***Statistical analysis***

Statistical analysis was done using SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA). Quantitative variables were reported as mean ± standard deviation (SD), whereas categorical variables were reported as number and percentage. The Pearson's chi-square ($\chi^2$) and Fisher's exact tests were used for assessment of the significance of genotype and allele frequencies in the DVT patients compared to the control group. Odds ratio and 95% confidence interval were calculated for each gene polymorphism. The homozygote mutant and heterozygote genotypes of each group were brought together as a new group and afterward odds ratios and 95% confidence intervals using logistic regression analysis were calculated. *P* values less than0.05 were considered statistically significant.

**RESULTS**

The demographic data of the study subjects are presented in Table (1). An example of the result of CVD strip assay is presented in figure (1). The genotype frequency of the different gene polymorphisms in the patients and control groups are displayed in table (2).

It appears that there was absence of homozygous mutant genotypes of FV Leiden, FV H1299R, PTH G20210A, Factor XIII V34L, ß-Fibrinogen − 455 G/A, MTHFR C677T, Apo B R3500Q andHPA-1 in the sample of Egyptian population studied; either normal subjects or DVT patients.

**Table 1:** Demographic data of the study subjects

| Parameter | | Control group (Number=45) | Patients group (Number=75) | P value |
|---|---|---|---|---|
| **Age** | Mean±SD<br>Range | 40.5±13.6<br>20-60 | 39.6±12<br>23-59 | 0.7 |
| **Sex:**<br>**Male**<br>**Female** | <br>Number [%]<br>Number [%] | <br>17 [37.8]<br>28 [62.2] | <br>27 [36%]<br>48 [64%] | <br>0.8 |
| **Positive Family history of VTE** | Number [%] | 0 [0%] | 15 [20%] | - |
| **Hypertension** | Number [%] | 0[0%] | 14 [18.7%] | - |
| **Ischemic heart disease** | Number [%] | 0 [0%] | 10 [13.3%] | - |
| **Diabetes** | Number [%] | 0 [0%] | 11 [14.7%] | - |
| **Pulmonary embolism** | Number [%] | 0 [0%] | 21 [28%] | - |
| **INR** | Mean±SD<br>Range | ----- | 1.1±0.15<br>1-1.5 | - |
| **Protein C (IU/dl)** | Mean±SD<br>Range | ----- | 100.2±14.4<br>59-121 | - |
| **Protein S (U/dl)** | Mean±SD<br>Range | ----- | 96.9±20.8<br>62-119 | - |

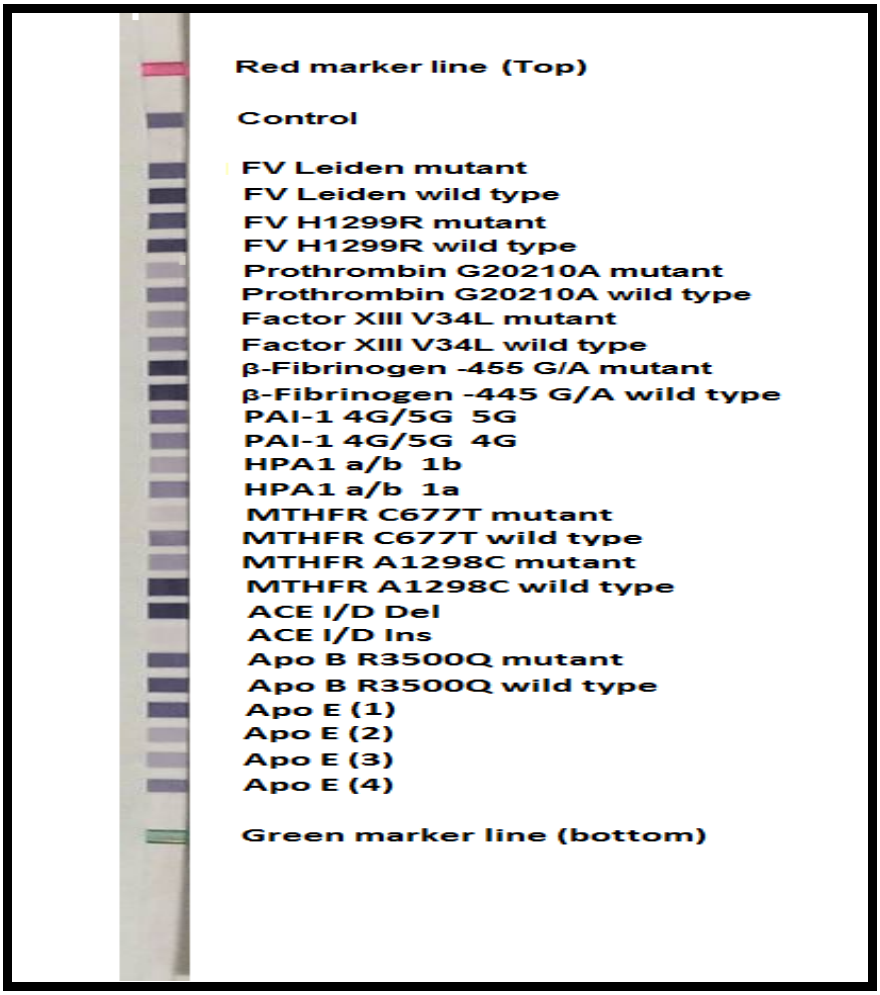


**Figure 1:** The result of CVD strip showing different thrombophilia gene polymorphisms. Heterozygous genotypes of FV Leiden, FV H1299R, Factor XIII V34L, ß-Fibrinogen-455 G/A, PAI-1 4G/5G, Apo B R3500Q are shown on the strip. In addition, the homozygous normal genotypes of Prothrombin G20210A, HPA-1, MTHFR C677T and MTHFR A1298C were revealed. Homozygous mutant genotype of ACE I/D was also revealed. Moreover, ApoE3/3 genotype demarcated by Positive Apo E lines 1 and 4 was revealed.

**Table (2):** Genotype frequency of the thrombophilia gene polymorphisms in the patients with DVT compared with the control group.

| **Groups** | **Controls (number=45)** | | | **Patients (number=75)** | | | **Risk of DVT** | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Genotype / Gene | Homo Zygous normal | Hetero zygous | Homo Zygous Mutant | Homo Zygous normal | Hetero zygous | Homo zygous mutant | P value | Odds ratio (95% confidence interval) **P value** | | |
| | Number % | | | | | | | Homo Zygous normal | Hetero Zygous | Homo zygous mutant |
| factor V Leiden G1691A | GG genotype | GA genotype | AA Genotype | GG genotype | GA genotype | AA genotype | 0.001 | 1.00 | 4.75 (1.77-14.06) | - |
| | 38 84.4% | 7 15.6% | 0 0% | 40 53.3% | 35 46.7% | 0 0% | | | **P=0.001** | |
| FV H1299R | AA genotype | AG genotype | GG genotype | AA genotype | AG genotype | GG genotype | 0.02 | 1.00 | 2.58 (1.09 to 6.08) | - |
| | 21 46.7% | 24 53.3% | 0 0% | 19 25.3% | 56 74.7% | 0 0% | | | **P=0.02** | |
| PTH G20210A | GG genotype | GA genotype | AA genotype | GG genotype | GA genotype | AA genotype | 0.45 | 1.00 | 0.71 (0.26-1.94) | - |
| | 34 75.6% | 11 24.4% | 0 0% | 61 81.3% | 14 18.7% | 0 0% | | | **P=0.45** | |
| Factor XIII V34L | GG genotype | GT genotype | TT genotype | GG genotype | GT genotype | TT genotype | 0.16 | 1.00 | 2.09 (0.65- 6.79) | - |
| | 9 20.0% | 36 80.0% | 0 0% | 8 10.7% | 67 89.3% | 0 0% | | | **P=0.16** | |
| ß-Fibrinogen − 455 G/A | GG genotype | GA genotype | AA genotype | GG genotype | GA genotype | AA genotype | 0.01 | 1.00 | 3.23 (1.20-9.67) | - |
| | 38 84.4% | 7 15.6% | 0 0% | 47 62.7% | 28 37.3% | 0 0% | | | **P=0.01** | |
| MTHFR C677T | CC genotype | CT genotype | TT genotype | CC genotype | CT genotype | TT genotype | 0.33 | 1.00 | 0.61 (0.20-1.89) | - |
| | 36 80.0% | 9 20.0% | 0 0% | 65 86.7% | 10 13.3% | 0 0% | | | **P=0.33** | |
| MTHFR A1298C | AA genotype | AC genotype | CC genotype | AA genotype | AC genotype | CC genotype | 0.02 | 1.00 | 1.43 (0.62-3.31) | - |
| | 23 51.1% | 22 48.9% | 0 0% | 27 36% | 37 49.3% | 11 14.7% | | | **P= 0.36** | **P=0.004** |
| Apo B R3500Q | GG genotype | GA genotype | AA genotype | GG genotype | GA genotype | AA genotype | 0.89 | 1.00 | 1.07 (0.32-3.33) | - |
| | 7 15.6% | 38 84.4% | 0 0% | 11 14.7% | 64 85.3% | 0 0% | | | **P=0.89** | |
| PAI-1 4G/5G | 5G/5G genotype | 4G/5G genotype | 4G/4G genotype | 5G/5G genotype | 4G/5G genotype | 4G/4G genotype | 0.03 | 1.00 | 1.33 (0.58- 3.06) | - |
| | 24 53.3% | 21 46.7% | 0 0% | 30 40.0% | 35 46.7% | 10 13.3% | | | **P=0.46** | **P=0.008** |
| HPA-1 (GPIIIa L33P) (GPIIIa 1565 C→T) | 1a/1a (CC) genotype | 1a/1b (CT) genotype | 1b/1b (TT) genotype | 1a/1a (CC) genotype | 1a/1b (CT) genotype | 1b/1b (TT) genotype | 0.09 | 1.00 | 5.25 (0.66-238.29) | - |
| | 44 97.8% | 1 2.2% | 0 0% | 67 93.3% | 8 6.7% | 0 0% | | | **P=0.09** | |
| ACE I/D | I/I genotype | I/D genotype | D/D genotype | I/I genotype | I/D genotype | D/D genotype | 0.03 | 1.00 | 1.50 (0.38 to 6.49) | 3.06 (1.23 to 7.89) |
| | 29 64.4% | 5 11.1% | 11 24.4% | 31 41.3% | 8 10.7% | 36 48% | | | **P= 0.52** | **P=0.008** |
| Apo E2/E3/E4. | E3/3 genotype | E2/3 genotype | E3/4 genotype | E3/3 genotype | E2/3 genotype | E3/4 genotype | P value | Odds ratio [95% confidence interval] **P value** | | |
| | Number % | | | | | | | E3/3 genotype | E2/3 genotype | E3/4 genotype |
| | 45 100.0% | 0 0% | 0 0% | 62 82.7% | 7 9.3% | 6 8.0% | 0.01 | 1.00 | 1.50 (0.38-6.49) **P= 0.52** | 3.06 (1.23-7.89) **P=0.008** |

**P < 0.05 is significant**

The heterozygous mutant genotypes of FV Leiden, FV H1299R and ß-Fibrinogen-455 G/A were significantly higher in DVT patients than in normal subjects. According to the odds ratio calculated, individuals with heterozygous mutant genotypes of FV Leiden, FV H1299R or ß-Fibrinogen - 455 G/A were 4.75, 2.58 and 3.23 times more likely to have DVT respectively. On the other hand, PTH G20210A, Factor XIII V34L, MTHFR C677T, Apo B R3500Q and HPA-1 heterozygous mutant genotypes were not significantly higher in the DVT group compared to the control group.

Homozygous mutant genotypes of MTHFR A1298C, PAI-1 4G/5G and ACE I/D were significantly higher in the DVT group compared to the control group. However, heterozygous mutant genotypes did not show significant difference between both groups. According to the odds ratio calculated, individuals with homozygous mutant genotype of ACE I/D were 3.06 times more likely to have DVT. E3/3 genotype of Apo E was the only genotype present in the control group. However, E3/3, E2/3 and E3/4 genotypes were present at frequencies of 82.7%, 9.3% and 8.0% respectively in the DVT group. E3/4 genotype was significantly higher in the DVT group compared to the control group. Individuals with E3/4 genotype were 3.06 times more likely to have DVT.

The allele frequency of the different gene polymorphisms in the patients and control groups are displayed in table 3. It was found that A alleles of factor V Leiden and ß-Fibrinogen − 455 G-A, C allele of MTHFR A1298C, 4G allele of PAI-14G/5G, D allele of ACE I/D and E2 allele of Apo E were significantly associated with DVT, Leading to 3.77, 2.72, 2, 1.9, 2.67 higher risk of DVT respectively.

The mutant allele frequencies of most gene polymorphisms included in the study was compared with frequencies detected by Alkhiary et al. (2015) [33] and Gialeraki et al. (2008) [34] in different ethnic groups and presented in table 4.

A logistic regression was performed to ascertain the effects of mutant allele (homozygous mutant and heterozygous genotypes) on the likelihood that participants have risk of DVT. It revealed that FV Leiden G1691A, ß-Fibrinogen − 455 G/A, MTHFR A1298C, PAI-1 4G/5G and ACE I/D were significantly associated with DVT with higher risk for DVT of 8.49, 3.77, 2.78, 4.08 and 4.85 respectively as shown in table (5).

## DISCUSSION

Genetic screening for the most widely recognized genes in molecular thrombophilia profiling (Factor V, II, and MTHFR) is getting to be essential in most clinical practice. However, these common genes involved in thrombosis may not be sufficient alone to consider a patient “negative” for genetic predisposition [35]. Therefore, this study tried to expand molecular analysis to include a more extensive genetic thrombophilia profiling which was not evaluated before in Egyptian population with DVT, through using CVD StripAssay, which covers 12 polymorphisms.

According to our studied population, the incidence of DVT was about 2 fold higher in females than in males, a finding consistent with a previous study on Iranian population which reported that the prevalence of DVT was more in females [36].

**Table (3):** Allele frequency of the thrombophilia gene polymorphisms in the patients with DVT compared

| **Groups** | **Controls (number=45)** | | **Patients (number=75)** | | **Risk of DVT** | | |
|---|---|---|---|---|---|---|---|
| Allele / Gene | Wild allele | Mutant allele | Wild allele | Mutant allele | P value | Odds ratio (95% confidence interval) | |
| | Number % | | | | | Wild allele | Mutant allele |
| factor V Leiden G1691A | G allele | A allele | G allele | A allele | 0.001 | 1.00 | 3.77 (1.54 to 10.52) |
| | 45 | 7 | 75 | 35 | | | |
| | 86.5% | 13.5% | 68.2% | 31.8% | | | |
| FV H1299R | A allele | G allele | A allele | G allele | 0.09 | 1.00 | 1.64 (0.89 to 3.05) |
| | 45 | 24 | 75 | 56 | | | |
| | 65.2% | 34.8% | 57.3% | 42.7% | | | |
| PTH G20210A | G allele | A allele | G allele | A allele | 0.48 | 1.00 | 0.74 (0.29 to 1.90) |
| | 45 | 11 | 75 | 14 | | | |
| | 80.4% | 19.6% | 84.3% | 15.7% | | | |
| Factor XIII V34L | G allele | T allele | G allele | T allele | 0.48 | 1.00 | 1.21 (0.69 to 2.13) |
| | 45 | 36 | 75 | 67 | | | |
| | 55.6% | 44.4% | 52.8% | 47.2% | | | |
| ß-Fibrinogen − 455 G/A | G allele | A allele | G allele | A allele | 0.02 | 1.00 | 2.72 (1.09 to 7.70) |
| | 45 | 7 | 75 | 28 | | | |
| | 86.5% | 13.5% | 72.8% | 27.2% | | | |
| MTHFR C677T | C allele | T allele | C allele | T allele | 0.35 | 1.00 | 0.64 (0.22 to 1.87) |
| | 45 | 9 | 75 | 10 | | | |
| | 83.3% | 16.7% | 88.2% | 11.8% | | | |
| MTHFR A1298C | A allele | C allele | A allele | C allele | 0.02 | 1.00 | 2.00 (1.08 to 3.77) |
| | 45 | 22 | 64 | 48 | | | |
| | 67.2% | 32.8% | 57.1% | 42.9% | | | |
| Apo B R3500Q | G allele | A allele | G allele | A allele | 0.95 | 1.00 | 1.02 (0.58 to 1.79) |
| | 45 | 38 | 75 | 64 | | | |
| | 54.2% | 45.8% | 54% | 46% | | | |
| PAI-1 4G/5G | 5G allele | 4G allele | 5G allele | 4G allele | 0.03 | 1.00 | 1.90 (1.02 to 3.62) |
| | 45 | 21 | 65 | 45 | | | |
| | 68.2% | 31.8% | 59.1% | 40.9% | | | |
| HPA-1 (GPIIIa L33P) (GPIIIa 1565 C→T) | C allele | T allele | C allele | T allele | 0.16 | 1.00 | 5.01 (0.65- 224.91) |
| | 45 | 1 | 75 | 8 | | | |
| | 97.8% | 2.2% | 90.4% | 9.6% | | | |
| ACE I/D | I allele | D allele | I allele | D allele | <0.001 | 1.00 | 2.67 (1.48 to 4.84) |
| | 34 | 16 | 39 | 44 | | | |
| | 68% | 32% | 47% | 53% | | | |
| Apo E2/E3/E4. | E3 allele | E2 allele | E3 allele | E2 allele | P value | Odds ratio [95% confidence interval] P value | |
| | Number % | | | | | E3 allele | E2 allele |
| | 45 | 0 | 69 | 13 | <0.001 | 1.00 | - |
| | 100.0% | 0% | 84.1% | 15.9% | | | |

**P < 0.05 is significant**

**Table (4):** Allele frequencies of some thrombophilia gene polymorphisms in healthy Egyptians included in this study compared to the frequencies detected by Alkhiary et al. (2015) in healthy Egyptians and to the frequencies in other ethnic origins detected by Gialeraki et al. (2008)**.**

| Polymorphism | Allele | This study | Alkhiary et al. | Gialeraki et al. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Egypt | Egypt | Turkey | Lebanon | Greece | Cyprus | Italy | Spain | Africa | China |
| | | % | | | | | | | | | |
| factor V Leiden G1691A | A | 13.5 | 7.5 | 9 | - | 5 | 3 | 14 | - | - | - |
| FV H1299R | G | 34.8 | 2.5 | 5 | 6 | 7 | 5 | - | - | - | - |
| PTH G20210A | A | 19.6 | 7.5 | 7 | 1 | 3 | 5 | 2 | 4 | 0.3 | 0.5 |
| Factor XIII V34L | T | 44.4 | 15 | 13 | 15 | 25 | 13 | 18 | 16 | 17 | 27 |
| ß-Fibrinogen − 455 G/A | A | 13.5 | 20 | 23 | 23 | 30 | 23 | 22 | - | 19 | 53 |
| MTHFR C677T | T | 16.7 | 37.5 | 13 | 19 | 4 | 36 | - | - | - | - |
| MTHFR A1298C | C | 32.8 | 42.5 | 34 | 49 | 35 | 41 | 40 | - | 45 | 55 |
| PAI-1 | 4G | 31.8 | 45 | 50 | 40 | 52 | 58 | 46 | 42 | 25 | - |
| HPA-1 | T | 2.2 | 17.5 | 14 | 19 | 20 | 12 | - | 15 | 8 | 0.5 |

**Table (5):** Risk of DVT determined by the presence of mutant allele of different thrombophilic gene polymorphisms as done by logistic regression analysis

| Homozygous mutant and heterozygous genotypes (Number =120) | Odds ratio (95% Confidence interval) | P value |
|---|---|---|
| FV Leiden G1691A | 8.49 (2.27-31.83) | 0.002 |
| ß-Fibrinogen − 455 G/A | 3.77 (1.04-13.66) | 0.04 |
| MTHFR A1298C | 2.78 (1.04-7.47) | 0.04 |
| PAI-1 4G/5G | 4.08 (1.47-11.27) | 0.007 |
| ACE I/D | 4.85 (1.59-14.77) | 0.005 |

**P < 0.05 is significant**

The factor V Leiden polymorphism is the most widely recognized inherited risk factor for venous thrombosis [37]. Our study showed that FV G1691A polymorphism prevalence among patients with DVT was 46.7%, as compared with healthy controls (15.6%) ($P < 0.05$). These results were previously supported by several studies [38-41]. Moreover, in a study on Croatian population FV Leiden was found to be the highest most frequent thrombophilia gene polymorphism out of 4 gene polymorphisms studied including factor II G20210A, FV Leiden, MTHFR C677T and PAI-1 5G/4G polymorphisms [42]. Conversely, there were previous studies, that failed to determine the contributory role of FV Leiden on the DVT occurrence in Iranian population [43, 44].

In this study, there was an association between Factor V H1299R (also known as HR2) and DVT. Consistent with our data, Bouaziz-Borgi et al. (2007) [45] found that this polymorphism had a possible role in VTE. In addition, Gohil et al. (2009) [46] confirmed that there is existing evidence that support the association of Factor V H1299R polymorphism with DVT. Furthermore,

Castaman et al. (1997) [47] and Margaglione et al. (2002) [48] revealed the association of HR2 haplotype presence with increased VTE risk (2–3 fold) and that the coinheritance of HR2 and FV Leiden was associated with severe APC resistance phenotype [47]. Conversely, in contrast to our results, some previous studies demonstrated that there were no statistical differences in the prevalence of this polymorphism between the patient and control groups [49,50].

As regards PTH G20210A polymorphism, it was previously found that it leads to increased messenger RNA and protein expression [51]. High PTH plasma levels may act through an increase in endogenous thrombin potential, a key step in hemostasis and thrombosis [52]. Moreover, increased PTH levels may lead to an increase in the inhibitor of fibrinolysis; thrombin-activatable fibrinolysis inhibitor, that disturbs the fibrinolysis process and thus may add to the hypercoagulable status in these patients [53,54]. In accordance with these data, Alfirevic et al. (2010) [42] revealed that prothrombin G20210A polymorphism is the second most frequent thrombophilia gene polymorphism in Croatian population out of 4 gene polymorphisms studied including factor II G20210A, FV Leiden, MTHFR C677T and PAI-1 5G/4G polymorphisms. However, our data revealed a non significant association between prothrombin G20210A polymorphism and the increased DVT risk, which were in accordance with other studies that did not find any association between prothrombin G20210A polymorphism and DVT [55,56]. Moreover, Hosseini et al. (2015) [36] reported that Prothrombin G20210A polymorphism was a non-significant genetic factor for DVT in Iranian people.

This study showed that there was non-significant association between MTHFR C677T polymorphism and the increased DVT risk, a finding consistent with a meta-analysis study by Simone et al. (2013) [57] who found no significant association between DVT and homozygous pattern of MTHFR C677T. However, our study showed that MTHFR A1298C was more prevalent among patients with DVT than healthy controls and therefore MTHFR A1298C polymorphism is associated with an increased risk for DVT. This finding was supported by a previous study by Van der Put et al. (1998) [58] who confirmed that the prevalence of MTHFR A1298C reduces the activity of MTHFR enzyme to a lesser extent than in the case of the C677T polymorphism.

Factor XIII (FXIII), also known as fibrin stabilizing factor, is an enzyme of the blood coagulation system. Polymorphisms in factor XIII were observed to be associated with protection against some cardiovascular events. The most widely recognized polymorphism is V34L, which diminishes the clot stability by modifying the structure of the cross-linked fibrin, consequently causing spontaneous bleeding. This thusly can be viewed as a mode of protection against thrombotic conditions [59]. However, a higher prevalence of Factor XIII polymorphism was observed in DVT patients, but did not show a statistically significant difference. These findings could probably suggest that the protective role of this polymorphism had been abolished by other polymorphisms in other thrombophilic genes.

Another protein involved in the thrombotic events is β-fibrinogen in which - 455 G/A polymorphism is associated with an increased risk of VTE and atherothrombotic diseases [35]. Regarding our results, the prevalence of β-fibrinogen - 455 G/A polymorphism was higher in DVT patients (37.3%) as compared to healthy controls (15.6%) ($P < 0.05$) which suggest that β-fibrinogen- 455 G/A polymorphism has an increased risk of DVT. The frequency of G and A alleles were 86.5% and 13.5% in the control group and 72.8% and 27.2% in the DVT group with A allele being significantly associated with DVT. In a study on the Lebanese population, the frequencies of the G and A alleles were reported as 77% and 23% respectively and higher levels of plasma fibrinogen were found in individuals with homozygous or heterozygous for − 455A than those who were homozygous for − 455G [60].

The net fibrinolytic activity of cells and blood seems to reflect the balance between the plasminogen activators (PAs) on one hand and the plasminogen activator inhibitor PAI-1 on the other. Thus, changes both in the PAs or PAI-1 may alter this balance and prompt to thrombotic problems or a tendency to develop a bleeding diathesis [11].

PAI-1, a member of the serine protease inhibitor (serpine) super family, is the main regulator for the endogenous fibrinolytic system and is the principle inhibitor of t-PA and u-PA in the fibrinolytic system [61]. It modulates the thrombosis progression [62]. Elevated levels of PAI-1 are associated with venous thrombosis [63].

This study showed that there was a significant association of the 4G/4G genotype and the 4G allele of the PAI-1 gene with the risk of DVT compared with healthy controls. On the other hand, this study found no differences between the 4G/5G genotype distribution in patients with DVT and healthy controls. Previous studies have reported higher PAI-1 levels in individuals with homozygous PAI-1 4G/4G gene polymorphism [64, 65]. Also, Segui et al. (2000) [66] confirmed that the presence of the 4G allele significantly increased the risk of thromboembolism in patients with other thrombophilic defects. Conversely, Hooper et al., (2000) [67] found no differences in the frequencies of heterozygous and homozygous PAI-I polymorphism when patients with DVT were compared with controls.

ACE not only transforms angiotensin I to active angiotensin II but also cleaves the vasodilator bradykinin, thus prompting to diminished generation of tissue plasminogen activator. The activity of ACE varies relying upon the carried genotype, with the highest activity being observed with the D/D genotype and the lowest with I/I [68]. The D/D genotype is highly associated with VTE and is expected to increase the risk of thrombosis about 11 times. Our results showed that the prevalence of the D/D, I/D and I/I genotypes among patients with DVT were 13.3%, 46.7% and 40.0% respectively , and respectively compared with that in the healthy controls (0%, 46.7% and 53.3%). A previous Lebanese study revealed that the D/D genotype is detected in about 39% of the population while the I/D and I/I are present in 45% and 16%, respectively [68].

A common polymorphism in the glycoprotein IIIa gene, leads to a substitution of proline for leucine at amino acid 33 (Leu33Pro); the wild-type allele is known as HPA-1a, and the 33 proline

substitution is known as HPA-1b [69]. Possible mechanistic associations between the HPA-1b polymorphism and thrombosis include increased sensitivity to platelet aggregation by various agonists [70] and altered sensitivity to aspirin [71]. Our study revealed that there was no significant association between HPA-1 polymorphism and venous thrombosis. However, a previous study revealed little association of the HPA-1b polymorphism with venous thrombosis [72]. In addition, previous studies suggested that the HPA-1b polymorphism may be associated with a risk for coronary thrombosis, but not atherosclerosis [72].

ApoB acts as a ligand of the low-density lipoprotein (LDL) receptor and any defect in the gene coding for ApoB, such as the R3500Q polymorphism, leads to diminished binding activity that in turns leads to a delayed clearance of LDL. This accordingly increases the risk of atherosclerosis and hypercholesterolemia [73]. ApoB-100 R3500Q polymorphism; a single nucleotide transition, CGG to CAG, in exon 26, reduces the affinity to the LDL receptor by at least 95% and is the major cause of familial defective ApoB-100 (FDB). Because the cholesterol concentration is often within the reference interval in FDB patients, the only reliable way of detecting FDB is by genotyping [74]. The frequency of *R3500Q* varies geographically, being the highest in Europe, which was consistent with our findings that revealed high prevalence of the *R3500Q* polymorphism among Egyptian patients with DVT and healthy controls. However, in Lebanon, the *R3500Q* polymorphism was not detected among healthy individuals [73].

ApoE is responsible for the regulation of the metabolism and the clearance of chylomicrons, intermediate density lipoprotein (IDL) and very low-density lipoprotein (VLDL). ApoE has several isoforms (E2, E3, or E4) which can contribute either to the protective role of this molecule (E2) or to increased risk of CVD (E4) [75]. In our study, the genotype frequencies of E3/E3, E2/E3 and E3/E4 were 82.7%, 9.3% and 8.0% in the DVT group compared to 100.0%, 0% and 0% in the control group respectively, and the E2/E4 and E4/E4 were not found in neither patients nor controls. However, Mahfouz et al. (2006) [76] reported that the genotype frequencies of E3/E3, E2/E3 and E3/E4 were 68.75%, 13.75% and 16.25% respectively, and the E2/E4 and E4/E4 were only found in 0.625% of the population.

Overall, according to our results, FV G1691A, Factor V H1299R, MTHFR A1298C, β-fibrinogen-455 G/A, PAI-1 4G/5G and ACE polymorphisms were all significantly associated with an increased risk of DVT and the contradicting data between this study and previous studies may reflect the different incidence of various thrombophilia polymorphisms among different populations or different population numbers of the different studies.

## CONCLUSION

To conclude, CVD strip assay allow a more extensive genetic thrombophilia profiling which is of extreme importance in clinical practice because it alerts to the potential need for (1) management of patients with a thrombotic episode presentation, (2) screening high-risk individuals, (3) use specific thromboprophylaxis in well-defined groups to prevent thrombotic diseases, and (4) family studies as well as genetic counseling for those identified

with high risk of inherited prothrombotic conditions

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