

Association of venous thromboembolism and myocardial infarction with Factor V Leiden and Factor II gene mutations among Libyan patients

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ABSTRACT

Factor V Leiden G1691A (FVL) and Factor II prothrombin G20210A (PGM) mutations are the leading causes of thrombophilia. In this study, we have investigated the prevalence of the FVL G1691A and PGM G20210A single nucleotide polymorphisms (SNPs) among Libyan deep vein thrombosis (DVT) and myocardial infarction (MI) patients. SNP genotyping was performed using high-resolution melt analysis (HRM) and DNA sequencing. Biochemical parameters conducted on 112 males and 93 females showed no significant difference in means between the control group and the deep vein thrombosis and myocardial infarction groups. For Factor V Leiden, 40 samples were genotyped. Of the 40 samples, 6 (15.0%) of them were heterozygous and no one was homozygous. As for Factor II SNP, 59 samples were genotyped and only 2 (3.3%) were heterozygous. All the heterozygous samples showed 100% concordance between the HRM-PCR and DNA sequence analysis. Our study showed, for the first time, that both the FVL and PGM mutations are present among Libyan DVT and MI patients and that the FVL mutation is significantly associated with DVT but not with MI. However, our results do not support the association of PGM G20210A mutation with DVT or MI.

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1. Introduction

Cardiovascular disease (CVD) is a class of disorders that involve the heart, and/or blood vessels (arteries, capillaries, and veins). CVD involves diseases that affect the cardiovascular system, primarily cardiac diseases, peripheral arterial disease, and vascular diseases of the brain and kidney [1,2]. A number of candidate genes and genetic abnormalities may be involved in the pathophysiology of CVD. SNPs in the FVL and the prothrombin gene mutation G20210A (PGM) comprise the most common genetic associations with thrombosis, and thus comprise the most commonly requested genetic thrombophilia investigations. A recent report describes an audit of local test findings that suggests growing futility in testing for FVL and PGM. Test requests for FVL and PGM were assessed for 2.5 years (from the start of 2016 to the end of June 2018) from a large tertiary-level pathology provider. Out of more than 10,000 thrombophilia-related test requests over the analysis period, 2,700 and 2,135 were, respectively, for FVL and PGM (Emmanuel J Favaloro 2019).

The gene for Factor V is located on the first chromosome (1q21-q25). It is genomically related to the family of multi-copper oxidases, and is about 40% identical to coagulation factor VIII. The gene spans 70 kb and consists of 25 exons. The produced mRNA is 9179 nucleotides, which encode 2224 amino acids

including a 28-residue leader peptide. The resulting glycoprotein has a molecular mass of approximately 330 kDa [3].

Coagulation Factor V has a single point mutation at position 1691 in exon 10, replacing guanine with adenine (G1691A); it is called Factor V Leiden. This mutation leads to the replacement of the amino acid arginine (R) at codon 506 with the amino acid glutamine (Q), (R506Q). This mutation makes Factor V become resistant to cleavage by activated protein C (APC) [4]. The lack of FV inactivation by APC leads to a 5- to 10-fold increase in the likelihood of thrombus formation [5]. The gene for Factor II is located on chromosome 11p11-q12 [6]. Prothrombin is a vitamin K-dependent glycoprotein. It is synthesized in the liver as an inactive zymogen, and it is activated by Factor Xa to the serine protease thrombin. The prothrombin gene contains 14 exons and spans about 21 kb. It encodes a 622-residue prepropeptide with a molecular mass of about 70 kD [7]. The mature circulating protein has 579 residues. The active enzyme alpha thrombin is produced by several cleavage events of prothrombin protein. A point transition mutation G20,210A (rs1799963), which was found in the 3' untranslated region of the prothrombin gene in 1996, was associated with higher plasma prothrombin levels and an increased risk of venous thrombosis.

The G20210A mutation leads to increased prothrombin levels and thus to the activation of thrombin [8,9].

The prevalence of these two SNPs varies depending on the geographical location and the ethnic background of the population. In this study, we used HRM and DNA sequencing to investigate the frequency of these SNPs in DVT and acute MI patients and among healthy individuals in Libya. To the best of our knowledge, this is the first study which assesses the SNP frequency in the Libyan population.

2. Materials and methods

2.1. Study group

Our sample consisted of 205 subjects – 112 males and 93 females. The sample was divided into three different groups: 54 individuals aged 47.66 ± 7.14 years, had no documented history of cardiovascular diseases; 72 individuals, aged 49.09 ± 16.76 years, had evidence of DVT; and 69 individuals, aged 53.70 ± 10.68 years, had evidence of MI.

Diagnosis of MI was based on electrocardiogram and clinical and laboratory data according to the WHO definition of MI. DVT patients were diagnosed according to Well's scores [10]. The patients and controls were matched for age and sex. They were recruited from the Tripoli University Hospital and Alwareed private clinic in Tripoli, Libya. Every participant has filled in a questionnaire and signed an informed consent form to take part in the study. The study was conducted in accordance with the Helsinki Declaration, and it was approved by the Libyan National Committee for Biosafety and Bioethics.

Blood samples for genetic analysis were collected into vacutainer tubes containing ethylene diamine tetra acetic acid (EDTA) as an anticoagulant. In addition, 5 ml of fasting whole blood was drawn into plain tubes, and the serum was stored at -20°C .

2.2. Biochemical analysis

Fasting blood samples were collected for the biochemical analysis of lipid profile (total cholesterol, triglycerides (TAG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL)); electrolytes (Na^+ , K^+ , Cl^-); creatinine; urea; cardiac enzymes (GOT, GPT, CK, CKMB, ALP, LDH); proteins; uric acid (UA); and TSH. Five milliliters of fasting whole blood was drawn into plain tubes, and the serum was stored at -20°C . The biochemical parameters were estimated using the fully automated Cobas Integra 400 Plus Analyzer from Roche-Diagnostics, and TSH was estimated using AIA-360 Automated Immunoassay Analyzer from Tosoh Corporation Tokyo, Japan, according to the manufacturer's instructions at Alwareed clinic.

2.3. Genetic analysis

The genetic analysis was carried out in the laboratory of the National Center for Disease Control (NCDC) in Tripoli and the Department of Biochemistry and Molecular Biology at the University of Tripoli.

2.4. Genomic DNA extraction

Genomic DNA was extracted from blood using QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. The extracted genomic DNA was run on a 1% agarose gel to check its quality, while the quantity and purity were assessed using the NanoDrop 1000 Spectrophotometer from Thermo Fisher Scientific Inc.

The extracted DNA was subjected to High-Resolution Melt (HRM) Analysis for mutation screening.

2.5. High-Resolution Melting (HRM)

In this study, we developed an HRM protocol to screen for the presence of G1691A polymorphism in exon 10 of factor V gene and G20210A mutation in the 3'-untranslated region of the prothrombin gene in the subjects.

We used the Genbank entry as a source to design FVL primer (Accession no. NG_011806.1) and to design prothrombin (factor II) primer (Accession no. NG_008953.1). The HRM primers were designed using the free online software Primer3 [11].

HRM analysis was performed on a Rotor-Gene Q real-time rotary analyzer (QIAGEN GmbH) at the National Center for Disease Control (NCDC) in Tripoli. The primers used for G1691A polymorphism detection were FVLF 5'-GCCAGTGCTTAACAAGACC-3' as a forward and FVLR.

5'-TTGAAGGAAATGCCCCATTA-3' as a reverse primer. The primers used for prothrombin G20210A mutation were 5'-GAACCAATCCCGTGAAAGAA-3' as a forward and 5'-CGAGTGCTCGGACTACCAG-3' as a reverse primer.

Reaction mixes of 25 μl contained 12.5 μl 2x mi-real-time EvaGreen[®] Master (Taq Polymerase: 0.05 u/ μl , dNTPs (dATP, dCTP, dGTP, dUTP) (200 μM), reaction buffer with KCl and MgCl_2 (3 mM), EvaGreen[®], stabilizers) from Metabion, Martinsried- Germany, 0.75 μl of 10pmol/ μl of each primer, and 10–60ng genomic DNA. The DNA fragments were amplified using the following parameters: activation of Taq polymerase and initial denaturation at 95°C for 2 min followed by 40 cycles of 95°C for 15 sec, 54°C for 30 sec, and 72°C for 20 sec. After amplification, HRM analysis data were collected from 75°C to 85°C raised by increments of 0.05°C with 1 sec rest in between.

2.6. DNA sequencing

The HRM genotyping results were checked and confirmed by automated sequencing of the region of

interest. Exon 10 of the FVL gene was amplified by conventional PCR using the aforementioned primers – FVLF and FVLR. An amplicon of 218 bp is produced.

Reaction mix of 25 μ l contained 5 μ l of 5X Red Load Taq Mix (Taq Polymerase: 0.05 u/ μ l dNTPs (dATP, dCTP, dGTP, dTTP) (200 μ M), reaction buffer with KCl and MgCl₂ (1.5 mM), red dye, gel loading buffer, stabilizers from Metabion, Martinsried- Germany, 0.75 μ l of 10pmol/ μ l of each primer, and 10–60 ng genomic DNA. The thermal profile for contained one cycle of Initial denaturation at 95°C for 2 min followed by 40 cycles of 95°C for 15 sec, 54°C for 30 sec, and 72°C for 20 sec. The PCR reaction was run using a TC-412 thermocycler from Techne, Duxford, Cambridge.

Five microliters of the PCR amplification products were electrophoresed in 2% agarose (BIOSPA, Milano, Italy) containing ethidium bromide (*Sigma*, St. Louis, MO, USA) at 80 Volt in 1X TAE buffer. The amplified PCR products were visualized under UV light and electronically documented using the gel documentation system (MultiDoc-It Digital Imaging System UVP, Cambridge, UK). DNA of 50 bp ladder (Metabion, Martinsried- Germany) was used as a molecular size marker.

The remainder of the PCR reaction was purified by the QIAquick PCR purification kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions and was sequenced by the dideoxy chain termination method using a BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA) with the ABI Prism 3130 Sequencer (PE Applied Biosystems, Foster City, CA). Sequence analysis was performed by BLAST search of the GenBank database. Forward and reverse DNA sequencing reactions were assembled separately by mixing the following: 4 μ l BDT v3.1 reaction mix, 4 μ l 5X sequencing buffer, 1 μ l 3.2pmol/ μ l primer, 1 μ l purified PCR product, and 10 μ l deionised H₂O. The mixture was mixed by flicking, and the cycle sequencing was performed by the TC-412 thermocycler (from Techne, Duxford, Cambridge) under the following parameters: initial denaturation at 96°C for 1 min followed by 25 cycles of denaturation at 96°C for 10 sec, followed by annealing and extension at 60°C for 4 min.

Sequencing reaction products were purified by manual Ethanol/EDTA- precipitation method. The resultant pellet was re-suspended in 20 μ l of Hi-Di formamide, loaded in a 96-well plate, and analyzed with an ABI Prism 3130 genetic analyzer (PE Applied Biosystems, Foster City, CA). The resultant sequences were edited and the database analysis was done using BLAST <http://www.ncbi.nlm.nih.gov/BLAST>.

2.7. Statistical analysis

Allelic frequencies were calculated by a gene-counting method. Odds Ratio and confidence

intervals (CI) were calculated using the online software MedCalc at https://www.medcalc.org/calc/odds_ratio.php.

3. Results

3.1. Biochemical analysis

Analysis shows that there is no significant difference in means between the control group and both DVT and MI groups. Even though the total cholesterol and LDL levels were in the normal range, the DVT and MI groups showed lower numbers. This may be attributed to the cholesterol-lowering drug, statin, given to DVT and MI patients.

In this study, we developed and employed high resolution melting analysis-based protocols to identify the genotypes of FVL (G1691A) and prothrombin (G20210) polymorphisms. The results of HRM for FVL (G1691A) and prothrombin (G20210) polymorphisms are presented in two forms: normalized melting curves and temperature-shifted difference plots. In the former, the temperature is plotted against the normalized fluorescence emitted by each sample (data not shown). The latter were generated from normalized and temperature-shifted data by selecting a control (in our case heterozygous) for comparison and subtracting the fluorescence of the control from all other melting curves. The fluorescence difference between all other curves and the comparison curve was then plotted against temperature (-dF/dT).

Figure 1 HRM analysis presented different graph curves showing HRM profiles of 1691 GG, 1691 GA genotypes of G1691A Polymorphism, respectively. A Factor V SNP decreased the melting temperature from 79°C to 78°C (Figure 1), and the Factor II SNP will decrease the melting temperature from 83°C to 80°C

Figure 2 shows the HRM analysis presented as different graph curves showing HRM profiles of GG20210 and GA20210 genotypes of G20210A Polymorphism, respectively.

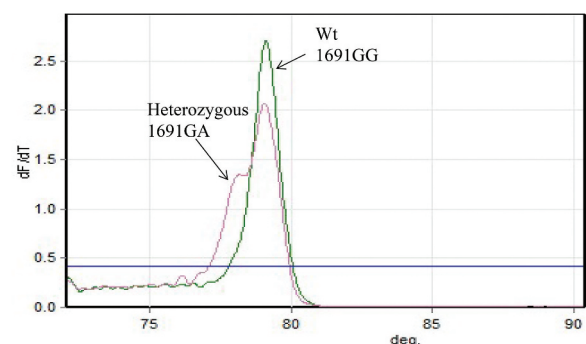


Figure 1. Analysis of temperature-shifted difference curves showing HRM profiles of 1691GG, 1691 GA genotypes of G1691A Polymorphism of FVL. Wt = Wildtype.

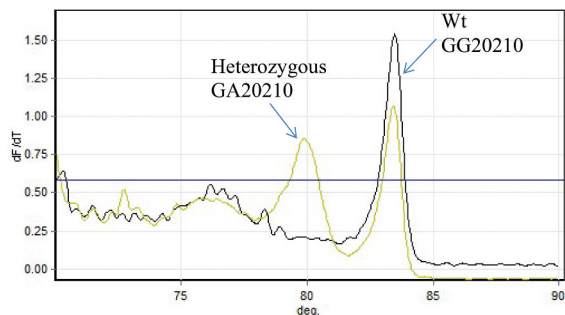


Figure 2. Analysis of temperature-shifted difference graph curves showing HRM profiles of 20210GG, 20210 GA genotypes of G20210A Polymorphism. Wt = Wildtype.

The HRM protocol designed in this study was able to clearly discriminate between different genotypes showing different homozygous wild type and heterogeneous mutant melting profiles of G1691A of FVL gene and prothrombin G20210A. The fact that HRM uses a double-stranded DNA saturating dye makes it able to detect both known and unknown mutations, and it is much cheaper in comparison with the hydrolysis probe real-time PCR; as it needs only two primers but not an allele-specific probe which is expensive. shows that in the control group, FVL HRM analysis revealed that all samples worked and all are wild type, while prothrombin HRM analysis revealed that three samples are heterozygous, 48 samples are wild type.

Regarding the deep vein thrombosis group, FVL HRM analysis revealed that six samples are heterozygous and 40 samples are wild type, while prothrombin HRM analysis revealed that 5 samples are heterozygous and 55 samples are wild type (Table 1 and 2).

Regarding myocardial infarction group, FVL HRM analysis revealed that two samples are heterozygous, 59 samples are wild type, while prothrombin HRM

analysis revealed that 1 sample is heterozygous and 40 samples are wild type (Table 1 and 2).

4. The results of HRM were confirmed using DNA direct sequencing

4.1. Direct sequencing

In order to confirm the HRM results, some samples were subjected to DNA sequencing. The primers used for HRM were used for the sequencing of the regions of interest in FVL and prothrombin genes. For sequencing of FVL (G1691A) and prothrombin G20210 polymorphisms, primers FVLA + FVLB and F2F + F2R were used. Sequencing results of FVL G1691A mutation revealed wild type (1691GG) heterozygous (1691 GA) and no homozygous (1691AA). Sequencing results of prothrombin (G20210A) mutation revealed wild type (20210GG) and heterozygous (20210 GA) (Data not shown) but no homozygous (20210AA) genotypes. Table 3 Figure 3 shows an example of sequence analysis of heterozygous type1691GA.

4.2. Discussion

Our study was carried out to investigate the prevalence of G1691A polymorphism in clotting Factor V gene and G20210A polymorphism in clotting factor II gene and to investigate the association of these two polymorphisms with deep vein thrombosis and/or myocardial infarction in the western region of Libya.

The control group included 54 individuals free of cardiovascular diseases. The myocardial infarction group included 69 individuals that were diagnosed based on electrocardiogram and clinical and laboratory data according to the American College of Cardiology and European Society of Cardiology [12].

Table 1. Biochemical analysis between healthy controls, deep vein thrombosis and myocardial infarction patients.

Parameter (unit)	Control (n = 54)		DVT (n = 59)		P value	MI (n = 63)		P value
	Mean	SD	Mean	SD		Mean	SD	
Age (year)	47.66	7.14	49.09	16.76	0.66	53.70	10.68	0.13
Urea (g/24 h)	30.56	11.55	29.27	16.83	0.92	30.35	13.69	0.86
Creatinine (g/24 h)	0.86	0.23	1.01	0.57	0.09	1.00	0.32	0.08
Na ⁺ (mmol/l)	139.98	3.60	136.27	3.01	0.32	135.00	15.83	0.20
K ⁺ (mmole/l)	4.35	0.57	4.06	0.42	0.61	4.61	0.75	0.57
Cl ⁻ (mmole/l)	105.70	4.00	101.27	4.15	0.51	100.18	4.19	0.52
Ca ⁺² (mg/dl)	8.62	0.92	8.01	1.13	0.45	11.22	1.45	0.52
TC (mg/dl)	173.51	52.27	132.32	61.54	0.81	114.22	47.34	0.88
LDL (md/dl)	121.30	45.86	110.03	44.83	0.33	80.88	47.16	0.65
HDL (mg/dl)	45.46	21.83	50.70	29.31	0.43	47.08	17.67	0.43
TAG (mg/dl)	143.81	62.14	123.57	82.32	0.36	124.42	78.90	0.26
LDH (U/l)	253.76	73.12	267.04	139.39	0.81	258.20	92.53	0.77
GPT (U/l)	14.73	7.80	23.35	36.74	0.09	18.92	13.62	0.14
GOT (U/l)	19.91	11.84	14.99	5.67	0.71	17.03	7.93	0.80
ALP (U/l)	170.81	74.75	177.07	91.59	0.97	168.24	72.83	0.81
CK (U/l)	58.91	40.51	62.74	58.08	0.40	67.88	46.77	0.60
CK MB (U/l)	9.58	6.02	8.97	14.16	0.74	9.68	8.49	0.90
Protein (g/dl)	6.89	0.91	7.92	10.05	0.35	6.14	0.86	0.22
Uric Acid (mg/dl)	4.57	1.42	4.63	1.80	0.44	5.23	1.64	0.55
TSH (pg/ml)	2.07	3.74	2.28	1.96	0.31	3.81	9.40	0.20

Table 2. Distribution of FVL genotypes in the control, DVT, and MI samples.* indicates a significant p-value.

	Control	DVT	MI
1691GG Wild type	54 (100%)	40 (86.95%)	59 (96.72%)
1691 GA Mutated	0	6.0 (13.00%)	2.0 (3.27%)
Fisher's test (p value)		0.03*	0.5
prevalence of G allele	108 (100%)	86 (93.47%)	120 (98.36%)
Prevalence of A Allele	0	6.0 (6.52%)	2.0 (1.63%)

Table 3. Distribution of prothrombin G20210A genotypes in the control, DVT and MI samples.

	Control	DVT	MI
20210GG Wild type	45 (100%)	55 (89.83%)	40 (96.83%)
20210 GA Mutated	3.0 (6.6)	5.0 (9.0%)	1.0 (2.5%)
Fisher's test (p value)		1.0	0.36
Prevalence of G allele	93 (97.06%)	115 (96.21%)	81 (99.06%)
Prevalence A Allele	3.0 (2.94%)	5.0 (3.82%)	1.0 (0.94%)

The deep vein thrombosis group included 72 individuals that were diagnosed according to Well's scores [10].

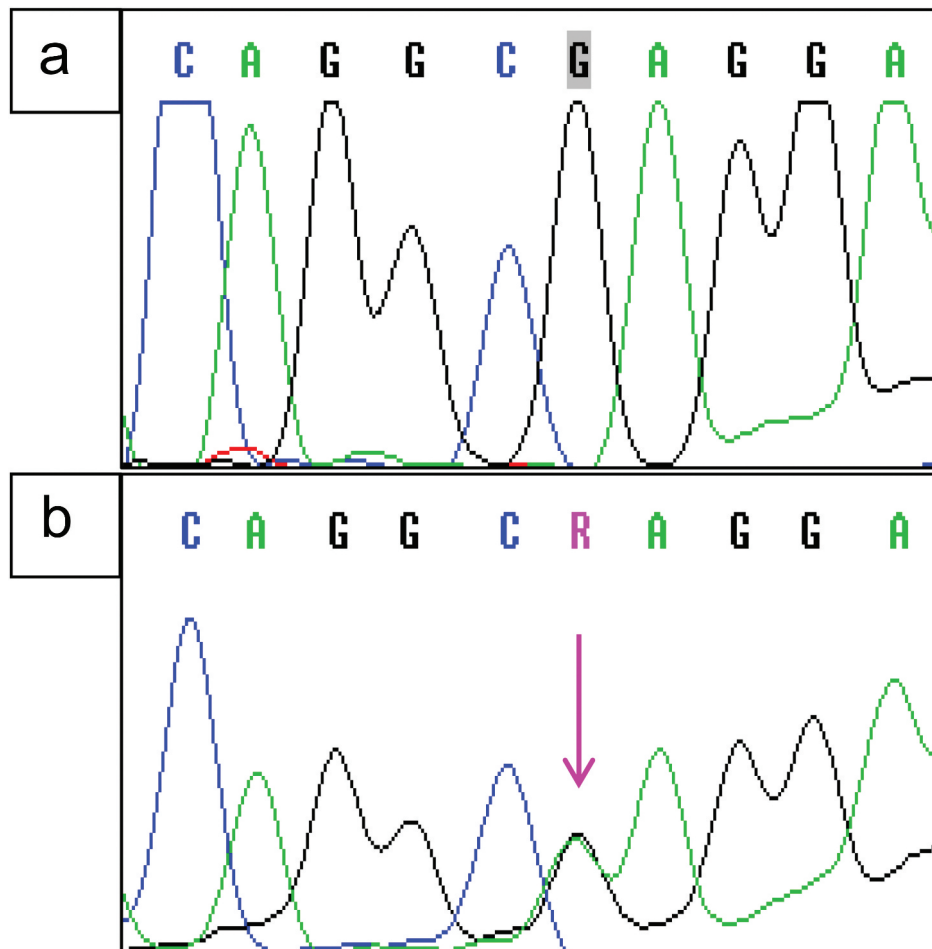
Instead of using PCR-RFLP for polymorphism detection as stated in our primary proposal, we developed simple new protocols based on high resolution melting analysis (HRM) to detect the polymorphisms.

Several risk factors of cardiovascular diseases have been identified. The health scientists believe that genetic makeup of the individual is among the risk factors that are involved in the development of cardiovascular diseases. Mutations in clotting factors II and V genes are among the variations that are considered to be risk factors in cardiovascular diseases.

In the current study, we determined the prevalence of FVL (G1691A) and prothrombin (G20210A) polymorphisms for the first time among Libyans in Tripoli.

Results obtained using HRM analysis were confirmed by DNA sequencing. Previously, several epidemiological studies, in different ethnic groups, found association of these polymorphisms with deep vein thrombosis and/or myocardial infarction. However, these results were inconclusive.

Our results showed that FVL is associated with DVT; the two-tailed P value equals 0.03. However, it is not associated with MI; the two-tailed P value equals 0.5 in Libyans residing in Tripoli. These results are in agreement with other previously published studies in Netherlands [13,14], Pakistan [15], Tunisia and Lebanon [16], Saudi Arabia [17], US [18], Venezuela [19], and Denmark [20].

**Figure 3.** Sequence analysis shows a wild type 1691GG (a) and heterozygous (b) mutation type 1691 GA. Pink arrow shows the position of the double peaks that represent heterozygous type 1691 GA.

Regarding myocardial infarction, our results showed no association of FVL with MI. This is in agreement with other studies such as in Morocco [21], Tunisia [22], US [18], Venezuela [19], Denmark [20]. However, some previous studies reported the association of FVL with MI such as in Egypt [23], Italy [24], and India [25].

Our results showed that FII (20210) mutation is not associated with neither DVT ($P = 1.0$) nor with MI ($P = 0.36$) in Libyans residing in Tripoli region. This is in agreement with most of the previously published studies such as in Tunisia [16,22], Denmark [20], Netherlands [14], China [26], and Egypt [27]; and contradicts others such as in Lebanon [16], and Denmark [20], Netherlands [14,28].

These findings, despite of the small number of patients, are consistent with those reported in other populations, and they support the complex nature of cardiovascular disease and the many other associated risk factors. Finally, while screening for these two SNPs may be warranted for risk of thrombophilia in Libyans, it is not indicated for assessing risk of acute myocardial infarction in the Libyan population. Outcome variations may be attributed to differences in lifestyle; including diet, exercise, and habits among various populations; and most importantly to ethnic differences.

Recent data showed high prevalence of Factor V Leiden 1691G/A variation in cases like preeclamptic patients [29] and miscarriage in women [30] and in vitro fertilization [31]. Further studies with larger samples are required to test these findings in the Libyan population.

5. Conclusion

In conclusion, we have successfully developed new, simple, and economically efficient protocols to detect FVL and prothrombin (G20210A) mutations based on high-resolution melting analysis. We also were able, for the first time, to determine the prevalence of these two mutations in Libyans residing in Tripoli and to study their association with deep vein thrombosis and myocardial infarction.

Our research showed that the mutations are present among Libyans, and that FVL mutation is significantly associated with DVT but not with MI. However, prothrombin G20210A is neither associated with DVT nor with MI.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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