



DOI: 10.1515/rrlm-2015-0005

Prothrombotic risk mutations and polymorphisms in patients with hemophilia A – a preliminary study

Polimorfismele și mutațiile cu risc protrombotic la pacienții cu hemofilie A - studiu preliminar

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Abstract

Introduction. In search for explanations of the clinical heterogeneity in patients with haemophilia (PwH) with the same mutation or degree of factor VIII deficiency, the coexistence of single or associated prothrombotic risk mutations has been widely evaluated. **Objective.** The evaluation of the frequency of prothrombotic risk mutations and polymorphisms in PwH in comparison with the general population. **Method.** The study was performed on 113 consecutive PwH consisting of PCR technology aiming to detect: factor V Leiden - G 1691A (FVL) and prothrombin (PT) - G 20210 A mutations, methylentetrahydrofolat - reductase (MTHFR) and plasminogen activator inhibitor type 1 (PAI-1) polymorphisms. **Results.** Within the whole study group, 52.21% patients have been identified with associated prothrombotic risk mutations or polymorphisms, 40.70% with one and 7.08% without any such alterations. The global frequency was characterized by the predominance of PAI-1 polymorphism present in 82.29% and MTHFR in 52.21% of patients. Heterozygous variants of PT G20210A, FV G1691A, MTHFR and PAI-1 were found in 7.96%, 9.73%, 39.82% and 53.98% cases, respectively. According to the disease severity, in 89 patients with severe hemophilia, the following frequencies of polymorphisms were found: for MTHFR 52.80%, for FV G1691A 5.61%, for PT G20210A 8.99% and for PAI-1 polymorphism 79.77%. **Conclusions.** The frequency of FV, PT and PAI-1 genes alterations in our group of hemophilia patients is higher than in the normal population. Nevertheless, considering their uneven distribution in different ethnic groups and geographical regions, more studies on a larger age- and sex-matched patient population are needed.

Keywords: haemophilia A, prothrombotic risk mutations, polymorphism

Rezumat

Introducere. Necesitatea explicării heterogenității clinice la pacienții cu hemofilie (PcH), cu același grad de severitate sau cu aceeași mutație, a determinat preocupările de evaluare a coexistenței polimorfismelor și mutațiilor

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cu risc protrombotic, unice sau asociate. **Obiectiv.** Determinarea frecvenței polimorfismelor și mutațiilor cu risc prothrombotic la PcH în comparație cu populația generală. **Metoda.** Studiul a fost efectuat la 113 PcH consecutivi; s-a utilizat tehnologia PCR (reacția de polimerizare în lanț) cu scopul de a detecta: mutațiile factorului V Leiden - G 1691A (FVL) și al protrombinei (PT) - G 20210 A, polimorfismele metilentetrahidrofolat - reductazei (MTHFR) și ale inhibitorului activatorului de plasminogen tip1 (PAI-1). **Rezultate.** În lotul de studiu, 52,21% dintre pacienți au prezentat asocieri de polimorfisme sau mutații cu risc protrombotic, 40,70% un defect genic, iar la 7,08% nu s-a identificat modificări. Frecvența globală a fost caracterizată prin predominanța polimorfismelor PAI-1, prezente la 82,29% și MTHFR la 52,21% din pacienți. Variante heterozigote ale PT G20210A, FV G1691A, MTHFR și PAI-1 au fost găsite în 7,96%, 9,73%, 39,82% și respectiv 53.98% din cazuri. În funcție de severitatea bolii, la 89 de pacienți cu hemofilie severă, frecvența polimorfismelor investigate a fost: 52,80%, pentru MTHFR, 5,61% pentru FVL G1691A, 8,99% pentru PT G20210A și 79,77% pentru PAI-1. **Concluzii.** Frecvența modificărilor genice FV, PT și PAI-1 în grupul nostru de studiu este mai mare decât la populația generală. Cu toate acestea, având în vedere distribuirea lor inegală în funcție de diferite grupuri etnice și regiuni geografice, devin necesare studii mai ample și mai cuprinzătoare la o populație de pacienți reprezentativă pentru diferite grupe de vârstă și de sex.

Cuvinte cheie: hemofilie A, mutații cu risc protrombotic, polimorfism

Received: 10th September 2014; Accepted: 31st January 2015; Published: 3rd March 2015

Introduction

Recent decades have witnessed an impetuous development of exploratory technology and an unprecedented enrichment of the clinical laboratory work-up. A gratifying aspect is the successful identification of the intimate nature of the bio-molecular substrate of a huge number of diseases, which allows to answer questions related to risk: significance of various mutations, genotype-phenotype correlation or heterogeneous clinical and biological expression of the same mutation (1-4). These are issues which have largely been debated in the frame of hemophilia A. It is a genetic disorder defined by factor 8 gene abnormalities responsible for the occurrence of the disease, hemorrhagic pathology per excellence. Interestingly, in search of explanations for the diversity of clinical expression of the same mutation or of the same degree of factor VIII activity the coexistence of single or associated mutations, recognized for their potential risk of thrombophilia, has been widely assessed. The question to be raised: is it a singular accidental situation or is it an evolutionary protective reaction, occurred over the centuries with the purpose of mitigating the hemorrhagic risk? (1-5)

In order to try a reply to this question, special attention is focused on the main alterations, polymorphisms and mutations, more evidently connected to thromboembolic events:

- factor V with the substitution of adenine (A) for guanine (G) at nucleotide position 1691 (G1691A) guanine: GG - wild type without harming effects; GA – heterozygous and AA homozygous factor V Leiden (FVL) (6,7)
- prothrombin with the mutation of guanine (G) to adenine (A) transition at nucleotide position 20210 generating: GG - wild type (normal), GA - heterozygous and AA - homozygous type (7)
- MTHFR structural changes with nucleotide at position 677 in the gene, generating two possibilities, cytosine (C) or thymine (T): 677CC represents the “normal” or “wild type” genotype, 677TT - the homozygous with mild MTHFR deficiency and 677CT - the heterozygote pattern with almost a normal activity (8,9)
- PAI-1 gene mapped on chromosome 7 (q21.3-q22) with 4G/5G polymorphism (deletion/insertion of guanine in position 675 of the PAI-1 gene promoter) and three possible outcomes: homozygous genotype

with normal or wild type - 5G/5G and heterozygous genotype - 4G/5G or homozygous genotype - 4G/4G, both with abnormal activity (10, 11).

Aiming at evaluating the frequency of these mutations in persons with hemophilia (PwH), we started this single-center, analytical cross-sectional study supported by the Laboratory of Molecular Biology, Munster (Germany).

Objective

The objective of the study was the estimation of the frequency of isolated and associated prothrombotic risk mutations and polymorphisms in PwH, in comparison with general population.

Patient characteristics

The study was performed on 113 consecutive PwH A: 89 - severe hemophilia A (SHA) (Factor VIII <1%), 16 - moderate hemophilia A (MHA) (Factor VIII -1-5%) and 8 - mild hemophilia A (MiHA) (Factor VIII >5%). The mean age of the study group was 21.48 years (SD = 12.45); 52 (46.02%) patients were aged under 18, and 61 (53.98%) were adults. Bleeding history was documented in 91.15% of patients, dominated by joint bleeds in 76.99% of them; those without hemorrhagic expression have been diagnosed on randomly performed hemostasis investigation; none of patients has been confronted with arterial or venous thrombo-embolic complications.

Method

Ethics. The present study was performed in accordance with the ethical standards laid down in the updated version of the Declaration of Helsinki and was approved by the medical ethics committee of the Children Emergency Hospital "Louis Turcanu" Timisoara.

Laboratory testing. For genetic analysis, venous blood was collected in EDTA-treated sample tubes (Sarstedt) from which cells were sepa-

rated by centrifugation at 3,000g for 15 minutes. The buffy-coat layer was removed and DNA extraction was performed by Invisorb® Blood Universal Kit 11/2006, Invitex GmbH, Germany, then stored at -70 °C.

In all subjects, the presence of the C677T methylenetetrahydrofolate reductase (MTHFR) gene mutation was investigated by amplification with polymerase chain reaction (PCR) and digestion of the fragment by endonuclease *HinfI*. The G20210A substitution in the prothrombin (PT) gene was detected by PCR amplification and *HindIII* digestion, and the G1691A mutation in the factor V gene was amplified and digested with *Mnl I*. The relevant region of the plasminogen activator inhibitor type 1 (PAI-1) promoter genotype was amplified by PCR using a mutated upstream primer that inserted a *BsiI* restriction site in the 5G allele. (6, 7, 12, 13)

Statistical analysis. Continuous variables were expressed as median and categorical data as percentage. Differences between groups were assessed using χ^2 test and 2-tailed *P* value < .05 was used to indicate statistical significance. All data were analyzed using SPSS statistical package (PASW Statistics Version 17 for Windows).

Results

Within the study group, 59 (52.21%) patients have been identified with associated prothrombotic risk mutations or polymorphisms, 46 (40.70%) with only one and 8 (7.08%) without any such alterations (Figure 1). Homozygous genotypes were found in an isolated pattern in 15 (13.27%) cases for PAI-1 and in one (0.88%) for the MTHFR, whereas in an associated formula we assessed them in 32 (28.31%) for PAI-1 and in 14 (12.39%) for MTHFR. Heterozygous variants were detected in isolated form in 23 (20.35%) for PAI-1, 5 (4.42%) for MTHFR and 2 (1.76%) for FVL (Figure no.2). Combined (isolated and associated) heterozygous variants

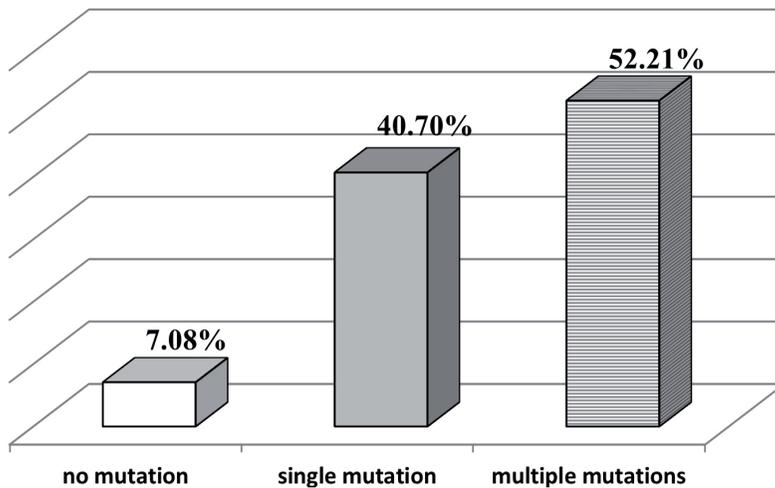


Figure 1. General distribution of prothrombotic risk mutations and polymorphisms in our study group

of PT G20210A, FV G1691A and MTHFR were found in 9 (7.96%), 11 (9.73%) and 45 (39.82%) cases, respectively. We have noticed a specially increased frequency of PAI-1 and MTHFR polymorphisms (Figure 2).

The global frequency was characterized by the predominance of PAI-1 mutations present in 82.29% and of MTHFR in 52.21% of patients (Figure 2, 3). Only 17.71% and 47.79% of PwH were without PAI-1 and MTHFR deleterious

polymorphism, respectively. Additionally, homozygous 4G/4G PAI-1 genotype was associated with one or two prothrombotic risk mutations in 16 patients (50%) and one patient (3.13%), respectively. The association of the heterozygous form of PAI-1 with one prothrombotic risk mutation was ascertained in 31 patients (50.82%), and two mutations in 7 patients (11.48%). (Figure 4)

According to the level of deficiency, in 89 patients with severe hemophilia, the frequencies of

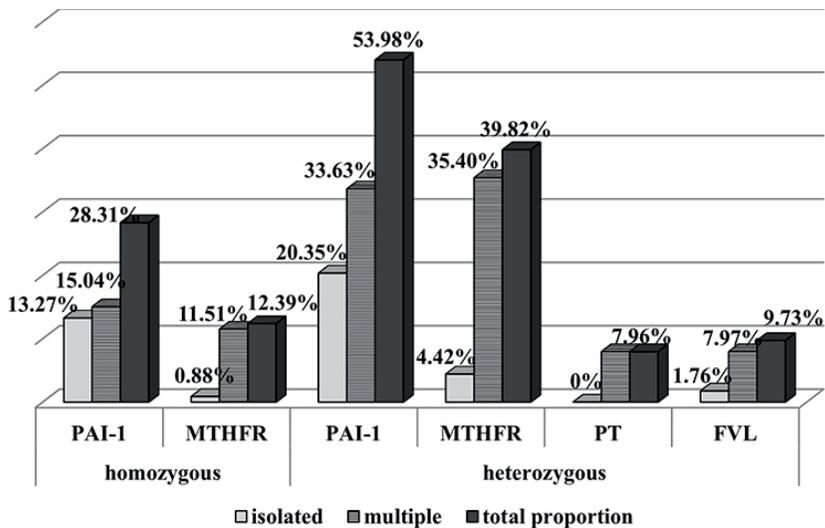


Figure 2. Distribution of heterozygous and homozygous variants in patients with haemophilia

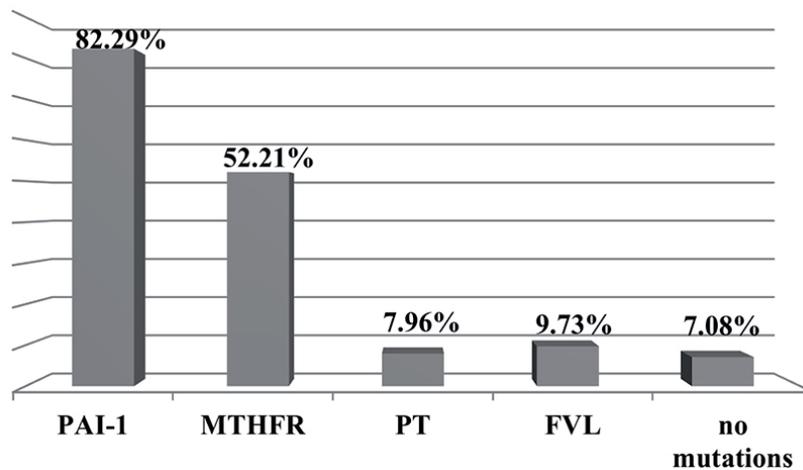


Figure 3. Global situation of prothrombotic risk mutations and polymorphisms in patients with haemophilia

mutations were: for MTHFR - 52.80% (47), for FVG1691A - 5.61% (5 cases), for PTG20210A - 8.99% (8 cases) and for PAI-1 polymorphisms - 79.77% (71 cases). In patients with moderate hemophilia, we identified 5 cases (31.25%) with heterozygous FVLG1691A mutation, 9 cases (56.25%) with MTHFR mutation and 14 cases (87.5%) with PAI-1 mutation. Finally, in the group with mild hemophilia 3 (37.5%) patients with MTHFR, 8 patients (100%) with PAI-1 and one (12.5%) case with PT and FVL mutation were assessed, respectively (Figure 5). We

have noticed that the proportion of heterozygous FVL mutation was significantly increased in patients with moderate hemophilia, compared with the severe form ($p = 0.006$). Regarding the other homozygous or heterozygous variants analyzed according to hemophilia severity, we did not find statistically significant differences.

The results have been compared with the data, revealing the prevalence of these mutations in the general population (Table I). Odds ratio (OR) for expressing heterozygous FVL and PT mutations in our study group was 2.34 (test χ^2 ,

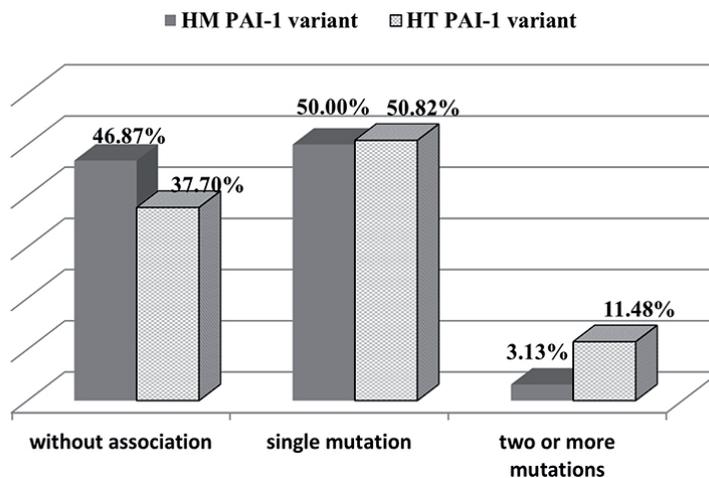


Figure 4. Association of homozygous and heterozygous PAI-1 variants with one or two prothrombotic risk mutations

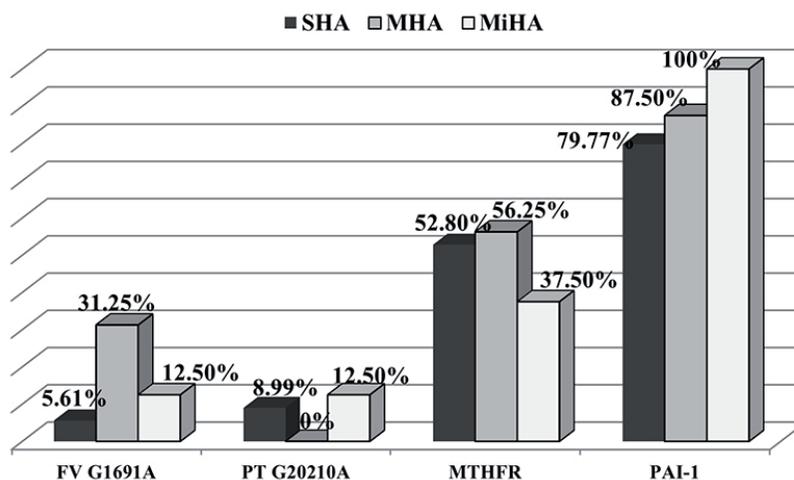


Figure 5. Prothrombotic risk mutations and polymorphisms distribution according to the disease severity

Table I. Prothrombotic risk mutations and polymorphisms frequency in the general population versus hemophilia study group

Genetic polymorphism	General population	Hemophilia study group	p value
Factor V Leiden mutation (G1691A)	2-4.4-15%	9.73%	0.017
Prothrombin G20210A mutation	0.7- 2 - 4%	7.96%	<0.001
MTHFR gene polymorphism - C677T			
TT	5-8-15%	12.39%	0.114
CT	28-38-44%	39.82%	0.763
PAI-1 gene polymorphism			
4G/4G	26.3%	28.31%	0.704
4G/5G	50.5%	53.98%	0.518
total	76%	82.29%	0.146

(Mean value – bold)

$p=0.017$) and 4.24 (test χ^2 , $p<0.001$) respectively; the 95 % confidence interval (CI) for OR was (1.26 - 4.37) and (2.14 - 8.39), respectively.

Discussion and conclusions

There is a high diversity of the prevalence of polymorphisms and prothrombotic risk mutations in the general population. There are important discrepancies between the data reported in different centers, countries and ethnic groups, even within the Caucasian population. Is it main-

ly of ethnic origin, is it a consequence of population mixture or migration during history? Or, is it a founder effect depending on our search, study design and exploratory methods (14-16)? There are still many unanswered questions (Table II).

In fact, today the polymorphic allele frequency of the explored thrombophilic mutations varies significantly. Factor V Leiden has an average prevalence in the general population of about 4.4% (2–15%). A west to east increasing prevalence ($r = 0.479$, $p < 0.02$) of factor V Leiden

Table II. Frequency of prothrombotic risk mutations and polymorphisms in the world

	FV Leiden - HT	FII- HT	MTHFR - TT	MTHFR - CT	PAI-1 - 4G/4G	PAI-1 - 4G/5G
Croatians (Croatia)(21)	2.9%	6%	7%	57%	32%	52%
Czechs (R. Czech) (22)	6.5%	3.4%	-	-	29.7%	49.7%
Greeks (Cyprus)(14)	4%	2%	-	39%	-	-
Arabian (Saudi A)(15)	1.3%	0.7%	2.45%		10.1%	-
Arabian (Palestina)(16)	20.1%	9.1%	-	13.8%	-	-
Jewish (Israel)(26)			15.2%	44.1%	-	-
Hispanic (USA) (25)	1.2%	2.4%	-	41.5%	-	-
Afro Americans (USA) (25)	0.8%	0.3%	-	12.4%	-	-
Caucasians (USA) (25)	1.8%	1.3%	-	26.6%	-	-
Midwest (USA) (27)	16.7%	6.1%	-	-	-	-

(HT- heterozygous)

alleles was observed in Europe, with a decrease from south to north ($r = -0.801$, $p < 0.001$). (17) Factor V Leiden has a low incidence in French Basques, and is rarely detected in Afro-American, Asian and Australian population (17,18). FII G20210A mutation in Caucasian population varies from 0.7 to 4%, with an overall estimated frequency of 2%. The frequency in northern Europe ranges around 1.7% (95% CI = 1.3–2.2%), while in southern Europe it is two times higher (3.0%, 95% CI = 2.3–3.7%). The prothrombin variant is very rare in individuals from Asia and Africa. (19-21)

The frequency of the MTHFR polymorphisms is more common in Caucasians and Asians compared to African Americans. Previous studies among European populations have shown that the frequency of homozygotes (T/T)

ranges from 5–15% and the lowest rate of heterozygote variant (C/T) is in Norway (28%), as opposed to Italy, where it is 44%. (20, 22) The prevalence of the PAI-1 mutation carriers in Caucasian population is about 76%, of which 26.3% homozygotes (4G/4G) and 50.5% are heterozygotes (4G/5G); they are more frequently detected in white population compared to the Africans. (20, 21)

Concerning the PwH, the reported data on the coexistence of prothrombotic risk mutations and polymorphisms are more uniform and more similar, generally situated at the upper limit of the healthy population or even at higher rates. (20, 21)

In our study the higher proportion of patients with prothrombotic risk mutations (92.91%), the high frequency of multiple associated mutations

(52.21%) and the high percentage of PAI-1 mutation (82.29%) is obvious; we could not find important differences between the severe forms of the disease, moderate and mild hemophilia; they seem to be without statistical significance, possibly because of the small number of patients. Similar data were previously highlighted. (21-23)

The prevalence of prothrombotic risk mutations in German children with severe hemophilia A was rated for FV GA between 5.8 – 7.4%, for FII GA - between 3.2-3.9% and for MTHFR TT - 10.0%. (24, 25) There is raising evidence about the role of prothrombotic risk mutations or polymorphisms in modulating clinical phenotype of hemophilia, mitigating its expression and serving consequently as a marker for risk evaluation for bleeding, that could be important for a personalized therapy approach. (3-5)

The frequency of F V, F II and PAI-1 genes alterations in our group of hemophilia patients is significantly higher than in normal population. Therefore, considering the uneven distribution in different ethnic groups and geographical regions, more extended studies are worthwhile to be performed, with a better design, with the inclusion of a larger number of patients and finally with the use of a control group with age- and sex - matched persons from our geographical region and from our ethnic group; an impact analyze of the investigated polymorphisms and mutations on the clinical outcomes of hemophilia are pending.

Acknowledgments

We gratefully acknowledge Prof .Dr .Ulrike Nowak-Göttl and Nadja Bogdanova Markov, University of Munster, Germany.

References

- van Dijk K, van der Bom JG, Fischer K, Grobbee DE, van den Berg HM. Do prothrombotic factors influence clinical phenotype of severe haemophilia? A review of the literature. *Thromb Haemost.* 2004 Aug;92(2):305-10.
- Franchini M, Lippi G. Factor V Leiden and hemophilia. *Thromb Res.* 2010 Feb;125(2):119-23. DOI: 10.1016/j.thromres.2009.11.003
- Sanna V, Zarrilli F, Nardiello P, D'Argenio V, Rocino A, Coppola A, et al. Mutational spectrum of F8 gene and prothrombotic gene variants in haemophilia A patients from Southern Italy. *Haemophilia.* 2008 Jul;14(4):796-803. DOI: 10.1111/j.1365-2516.2008.01705.x
- Dargaud Y, Meunier S, Negrier C. Haemophilia and thrombophilia: an unexpected association! *Haemophilia.* 2004 Jul;10(4):319-26. DOI: 10.1111/j.1365-2516.2004.00906.x
- Kurnik K, Kreuz W, Horneff S, Düring C, Schobess R, Bidlingmaier C, et al. Effects of the factor V G1691A mutation and the factor II G20210A variant on the clinical expression of severe hemophilia A in children--results of a multicenter study. *Haematologica.* 2007 Jul;92(7):982-5. DOI: 10.3324/haematol.11161
- Bertina RM, Koeleman BPC, Koster T, et al. Mutation in blood coagulation factor V - associated with resistance to activated protein C. *Nature.* 1994 May 5; 369(6475):64-7. DOI: 10.1038/369064a0
- Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood.* 1996 Nov 15;88(10):3698-703.
- Födinger M, Hörl WH, Sunder-Plassmann G. Molecular biology of 5, 10-methylenetetrahydrofolate reductase. *J Nephrol.* 2000 Jan-Feb;13(1):20-33.
- Schneider JA, Rees DC, Liu YT, Clegg JB. Worldwide distribution of a common Methylenetetrahydrofolate reductase mutation. *Am J Hum Genet.* 1998 May; 62(5):1258-60. DOI: 10.1086/301836
- Abeer M, Hesham A. Genetic polymorphisms of Plasminogen Activator Inhibitor-1, vascular endothelial growth factor and angiotensin converting enzyme among pre-eclamptic women. *Egypt J Med Lab Sci.* 2010 Mar; 19(1):39-44.
- Nordt TK, Lohrmann J, Bode C. Regulation of PAI-1 expression by genetic polymorphisms. Impact on atherogenesis. *Thromb Res.* 2001 Sep;103 Suppl 1:S1-5. DOI: 10.1016/S0049-3848(01)00292-4
- Falk G, Almquist A, Nordenhem A, Svensson H, Wiman

- B. Allele specific PCR for detection of a sequence polymorphism in the promoter region of the plasminogen activator inhibitor-1 (PAI-1) gene. *Fibrinolysis*. 1995 May;9(3):170-4. DOI: 10.1016/S0268-9499(95)80007-7
13. Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet*. 1995 May;10(1):111-3. DOI: 10.1038/ng0595-111
14. Xenophontos SL, Hadjivassiliou M, Ayrton N, Karagrigoriou A, Pantzaris M, Nicolaidis AN, et al. Spectrum and prevalence of prothrombotic single nucleotide polymorphism profiles in the Greek Cypriot population. *Int Angiol*. 2002 Dec;21(4):322-9.
15. Saour JN, Shoukri MM, Mammo LA. The Saudi Thrombosis and Familial Thrombophilia Registry. Design, rationale, and preliminary results. *Saudi Med J*. 2009 Oct; 30(10):1286-90.
16. Hussein AS. High prevalence of three prothrombotic polymorphisms among Palestinians: factor V G1691A, factor II G20210A and methylenetetrahydrofolate reductase C677T. *J Thromb Thrombolysis*. 2012 Oct;34(3):383-7. DOI: 10.1007/s11239-012-0731-9
17. Rees DC, Cox M, Clegg JB. World distribution of factor V Leiden. *Lancet*. 1995 Oct;346(8983):1133-4. DOI: 10.1016/S0140-6736(95)91803-5
18. Lucotte G, Mercier G. Population genetics of factor V Leiden in Europe. *Blood Cells Mol Dis*. 2001 Mar-Apr;27(2):362-7. DOI: 10.1006/bcmd.2001.0388
19. Rosendaal FR, Doggen C, Zivelin A, Arruda V, Aiach M. Geographic distribution of the 20210G to A prothrombin variant. *Thromb Haemost*. 1998 Apr;79(4):706-8.
20. Alfirevic Z, Simundic A, Nikolac N, Sobocan N, Alfirevic I, Stefanovic M, et al. Frequency of factor II G20210A, factor V Leiden, MTHFR C677T and PAI-1 5G/4G polymorphism in patients with venous thromboembolism: Croatian case control study. *Biochemia Medica*. 2010 Mar;20(2):229-35. DOI: 10.11613/BM.2010.028
21. Kvasnička T, Hájková J, Bobčíková P, Cverhová V, Malíková I, Ulrych J, et al. The Frequencies of Six Important Thrombophilic Mutations in a Population of the Czech Republic. *Physiol Res*. 2014 Jan;63(2):245-53.
22. Pepe G, Camacho Vanegas O, Giusti B, Brunelli T, Marcucci R, Attanasio M, et al. Heterogeneity in world distribution of the thermolabile C677T mutation in 5,10-Methylenetetrahydrofolate reductase. *Am J Hum Genet*. 1998 Sep;63(3):917-20. DOI: 10.1086/302015
23. Hessner MJ, Luhm RA, Pearson SL, Edean DJ, Friedman KD, Montgomery RR. Prevalence of Prothrombin G20210A, Factor V G1691A (Leiden), and Methylenetetrahydrofolate Reductase (MTHFR) C677T in Seven Different Populations Determined by Multiplex Allele-specific PCR. *Thromb Haemost*. 1999 May;81(5):733-8.
24. Escuriola E, Ettingshausen C, Halimeh S, Kurnik K, Schobess R, Wermes C, Junker R, et al. Symptomatic onset of severe hemophilia A in childhood is dependent on the presence of prothrombotic risk factors. *Thromb Haemost*. 2001 Feb;85(2):218-20.
25. Nowak-Göttl U, Escuriola C, Kurnik K, Schobess R, Horneff S, Kosch A, et al. Haemophilia and thrombophilia. What do we learn about combined inheritance of both genetic variations? *Hämostaseologie*. 2003 Feb;23(1):36-40.
26. Friedman G, Goldschmidt N, Friedlander Y, Ben-Yehuda A, Selhub J, Babaey S et al. A common mutation A1298C in human methylenetetrahydrofolate reductase gene: association with plasma total homocysteine and folate concentrations. *J Nutr*. 1999 Sep;129(9):1656-61.
27. Bavikatty NR, Killeen AA, Akel N, Normolle D, Schmaier AH. Association of the prothrombin G20210A mutation with factor V Leiden in a mid-western American population. *Am J Clin Pathol*. 2000 Aug; 114(2):272-5. DOI: 10.1309/LQKG-F0D4-C6FF-FNEH