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Inherited Thrombophilia and the Risk of Vascular Events

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1. Introduction

The definition of thrombophilia includes the impact of hereditary base in the tendency to develop thrombosis and its clinical manifestations. Prothrombotic phenotype results from the interaction of inherited disorders of coagulation and various "clinical" risk factors such as obesity, immobility, major and minor surgery, hormone therapy, malignancy, etc. According to the accepted multicausal model, inherited thrombophilia is a manifestation of mutual influence of gene-gene and gene-environmental factors. Inherited thrombophilia traits include a wide range of disorders. Deficits of some coagulation inhibitors are relatively rare, and their clinical significance is previously known. On the other hand, in genes which control the coagulation cascade, there are many variants that are widespread in the population. The practical significance of these variants, known as genetic polymorphisms, is different, and the subject of numerous epidemiological-genetic, clinical and health economic studies (Novaković et al, 2010; Krcunović et al., 2010; Pavlović et al., 2011).

In summary, the most common congenital disorders associated with thrombophilia are: a deficiency of antithrombin, protein C and protein S, variants of factor V Leiden and prothrombin 20210, and mild hyperhomocysteinemia (Table 1). Individually or in combination, these traits are present in about 40% of patients with venous thromboembolism (VTE), and in approximately the same percentage of women with disorders of pregnancy and puerperium, such as fetal loss, fetal growth restriction and preeclampsia (De Stefano et al, 2002).

1.1 Deficiency of natural coagulant inhibitors

Deficiency of antithrombin, protein C and protein S are rare disorders, the total present in about 1% of the general population. It is considered that holders of these properties have a 5-8 times greater risk of VTE, and VTE among patients with their representation is about 10-15%. They are usually associated with the action of environmental factors, and the first VTE event occurs before 45 years of age (De Stefano et al, 2002).

1.2 Factor V Leiden

It is determined that a variant of coagulation factor V, designated as factor V Leiden, is basically a genetic polymorphism. This is a SNP polymorphism in the 506th codon, which triplet CGA for arginine replaces the CAA triplet for glutamine. The prevalence of factor V

Leiden varies: is about 1-5% in North America, higher in North and Central Europe, while in the African and Asian population, this polymorphism is almost absent. It is estimated that this polymorphism originated 21- 24000 years ago. Under normal conditions, APC protein binds to factor V and cuts it into two inactive fragments. It is determined that the Leiden variant is resistant to APC protein, which prolongs the action of factor V. The result is a continuation of prothrombin activation and continuously maintain coagulation cascade (Robertson et al, 2005, Ho et al, 2006).

Hypercoagulable State	General Population (%)	Patients with Single VTE (%)	Thrombophilic Families (%)
Factor V Leiden	3-7	20	50
Prothrombin G20210A	1-3	6	18
Antithrombin deficiency	0.02	1	4-8
Protein C deficiency	0.2-0.4	3	6-8
Protein S deficiency	N/A	1-2	3-13
Hyperhomocysteinemia	5-10	10-25	N/A
Antiphospholipid antibodies	0-7	5-15	N/A

N/A, not readily available or unknown; VTE, venous thromboembolic event.

Table 1. Prevalence of major hypercoagulable states in different patient populations (after De Stefano et al, 2002).

Heterozygous for FVL have a 2-7 times greater risk of VTE event, while the homozygous risk increased even 40-80 times. The first event of VTE often occurs after 45 years of age. The risk is further multiplied in women during pregnancy, as well as due to the intake of oral contraceptives or hormone replacement therapy in menopause (Table 2). Thus, women heterozygous for factor V Leiden who take hormone preparations have 15.6 times higher risk of venous thrombosis, while pregnant women homozygous for this polymorphism show 34 times increased risk of thrombosis. The risk of spontaneous abortion is also increased (Robertson et al, 2005, Ho et al, 2006).

| Factor | Number of | | % with factor in women who | | Relative risk |
	Studies	Patients	Had VTE	Had no VTE	(95% CI)
Oral contraceptive use	7	2530	61	29	2.0 (1.8 to 2.2)
Factor V Leiden, no OC	6	1617	19	6	3.5 (2.5 to 4.9)
Oral contraceptive and Factor V Leiden	6	1612	27	2	12 (7.9 to 19)
Hormone replacement therapy	2	359	62	38	1.7 (1.4 to 2.1)
Factor V Leiden, no HRT	2	221	21	7	3.1 (1.4 to 6.7)
HRT and Factor V Leiden	2	218	27	3	9.2 (3.5 to 24)

Table 2. Results for association of venous thromboembolism with oral contraceptive or hormone replacement use, Factor V Leiden, or both (after Wu et al, 2006).

1.3 Prothrombin G20210A

The gene for prothrombin (coagulation factor II) also has a significant polymorphism 20210G> A. This polymorphism is located in the 3' untranslated region of gene, and is supposed to have a regulatory role. Its frequency in European populations is 1-5%, while it is very rare in people of African or Asiatic origin. 20210 allele leads to increased activity of prothrombin, and the hypercoagulation state (Robertson et al, 2005, Ho et al, 2006)

Meta analyzes show that the risk for the first thrombotic event increased 2-10 times in heterozygotes for factor V Leiden and 2-6 times in heterozygotes for allele 20210A. Combined heterozygous, possessing both polymorphisms, have as much as 20 times higher risk. These data are particularly important in situations that in themselves predispose thrombosis and thrombus-embolism, such as major orthopedic surgery or malignancy (Robertson et al, 2005, Ho et al, 2006).

1.4 Mild hyperhomocysteinemia

In relation to thrombophilia, the importance of some other polymorphisms should be emphasized, such as polymorphism in the gene for methylene-tetrahidrofolat-reductase, MTHFR 677C> T. This polymorphism leads to substitution of alanine to valine at position 222 in the polypeptide, which causes thermolable form of the enzyme with decreased activity. T allele frequency is highest in Asian populations, lower in white European and American, and the lowest in African populations. In Europe, the incidence of TT homozygosity is 5-15%. People with 677TT and 677CT genotype have only 30% and 50% MTHFR enzymatic activity, respectively. MTHFR catalyzes the development of methyl-tetrahidrofolate, which is the main donor of methyl group in the remethylation of homocysteine to methionine. That is why the MTHFR 677 polymorphism associated with hyperhomocysteinemia, which is considered to be an independent risk factor for thrombophilia, and other disorders, such as atherosclerosis, neural tube defects, etc (Robertson et al, 2005, Wu et al, 2006; Simić-Ogrizović et al. 2006; Todorović et al. 2006; Damnjanović et al. 2010).

- Patients with VTE, independently of the age of onset (</> 45 years), the circumstances of thrombosis (provoked /unprovoked), and the severity of the clinical manifestations.
- As a rule, patients with cancer may be excluded. Yet patients with hematologic neoplastic diseases and venous thromboembolism are potential candidates.
- Women with complications of a pregnancy other than VTE:
  - one or more episodes of late fetal loss
  - two or more episodes of early fetal loss
- Asymptomatic individuals who are first-degree relatives of a diagnosed carrier of a thrombophilic trait. This should be accompanied by accurate information and counseling.
- Potential candidates:
  - Women with pre-eclampsia, fetal growth retardation or abruptio placentae
  - Asymptomatic women with a family history of VTE, before use of oral contraceptives, hormone replacement therapy, or pregnancy.

Box 1. Candidates for screening for inherited thrombophilia (adapted from de Stefano et al, 2002 and Wu et al, 2006)

- Antithrombin heparin cofactor activity (amidolytic method)
- Protein C (clotting or amidolytic method)
- Protein S (total and free antigen fractions)
- APC-resistance plasma assay
- Factor V Leiden
- Prothrombin G20210A
- Homocysteine

Box 2. Screening for inherited thrombophilia (first line panel) (adapted from de Stefano et al, 2002 and Wu et al, 2006)

Oral anticoagulant prophylaxis for as long as in patients with a normal genotype:
- First unprovoked deep venous thrombosis with or without pulmonary embolism in patients with isolated heterozygosity for factor V Leiden or for the prothrombin G20210A or with moderate hyperhomocysteinemia.
- First provoked deep venous thrombosis with or without pulmonary embolism (all genotypes).

Oral anticoagulant prophylaxis for an indefinite duration:
- Two or more recurrent unprovoked episodes of deep venous thrombosis with or without pulmonary embolism (all genotypes).
- First unprovoked deep venous thrombosis with or without pulmonary embolism affecting individuals with severe thrombophilia (AT, PC, or PS deficiency, homozygosity for factor V Leiden, combined defects)
- First life-threatening thrombotic episode (massive pulmonary embolism, cerebral venous thrombosis, splanchnic venous thrombosis), in particular if unprovoked (all genotypes).

Uncertain indications for oral anticoagulant prophylaxis of indefinite duration to be given on an individual basis (all genotypes):
- Two or more recurrent unprovoked episodes of superficial thrombophlebitis
- Two or more recurrent provoked episodes of deep venous thrombosis with or without pulmonary embolism
- Two or more recurrent episodes of deep venous thrombosis with or without pulmonary embolism, of which only one unprovoked
- Diagnosis of severe thrombophilia in individuals with not recent occurrence of unprovoked deep venous thrombosis with or without pulmonary embolism.

Primary antithrombotic prophylaxis in asymptomatic relatives of proband patients with inherited thrombophilia:
- Contraindication to oral contraception or hormone replacement therapy (in par ticular for women with severe thrombophilia, i.e. AT, PC, or PS deficiency, homozygosity for factor V Leiden, combined defects)
- Prophylaxis:
  in the case of: surgery, bed immobilization, plastering of the arms or of the legs, long air journeys (more than 4 hours)
  in the puerperium (all genotypes)
  throughout the whole pregnancy (severe thrombophilia; the indication for primary antithrombotic prophylaxis with low molecular weight heparin throughout the pregnancy in women with isolated heterozygosity for factor V Leiden or for the prothrombin G20210A is not yet certain).

Box 3. Secondary prophylaxis with oral anticoagulants after VTE in patients with inherited thrombophilia (adapted from de Stefano et al, 2002 and Wu et al, 2006)

Results of extensive cost-benefit studies do not support universal polymorphism screening prior to the introduction of hormone therapy, during pregnancy or after major orthopedic surgery. Instead, selective testing of patients with a history previous thrombo-embolism is recommended. To prevent spontaneous abortions in women with thrombophilia, low-dose aspirin and heparin is recommended. There is no universal agreement on the need for preventive therapy in elective orthopedic surgery (Robertson et al, 2005, Ho et al, 2006, Wu et al, 2006).

2. New approaches and research strategies in inherited thrombophilia

Until recently, research aimed to elucidate the genetic component of the susceptibility to VTE was based on the traditional approach and employed primarily candidate gene studies; by this approach, the most significant and well-known polymorhisms of coagulation factors V and II (factor V Leiden and prothrombin 20210) were identified. Candidate genes are generally selected with respect to their known role in the traits and processes in interest. In relation to VTE, majority of studies have dealt with genes of coagulation/fibrinolysis cascade.

Thus, based on the known biology of clotting, Smith et al. (2007) investigated polymorphisms of 24 candidate genes coding for proteins affecting coagulation (factors II, V, VII, VIII, IX, X, XI, XII, XIIIa1, and XIIIb; fibrinogen α, β, and γ; and tissue factor), anticoagulation (antithrombin, proteins C and S, endothelial protein C receptor, thrombomodulin, and tissue factor pathway inhibitor [TFPI]), fibrinolysis (plasminogen and tissue-type plasminogen activator), and antifibrinolysis (PAI-1 and thrombin activatable fibrinolysis inhibitor [TAFI]).

Although candidate gene studies have yielded some significant results, selection of appropriate candidates may be difficult, and new approaches had to be employed. One of these modern, widely used and successful strategies for identification of new genetic variants associated with susceptibility to multifactorial diseases is GWAS (genome wide association studies). GWAS is based on testing the association of a complex phenotype with large numbers of SNPs in large samples of patients. Recent use of this and other modern strategies resulted in detection of a number of new genetic variants potentially associated with VTE, but further research is needed to elucidate their significance as general risk factors.

ABO locus variants were previously found to be associated with susceptibility to VTE: non-O blood group carriers were at higher risk, probably through higher levels of VWF and factor VIII, known risk factors for VTE (O'Donnell et al, 2002). This was confirmed by a recent study – Tregouet et al. (2009) conducted a GWAS on 317 000 SNPs in 453 VTE cases and 1327 controls, and found that 2 SNPs in ABO locus (rs505922 and rs657152) were significantly associated with VTE; carriers of O and A2 blood group were at lower risk.

Within the *FGB/FGA/FGG* gene cluster (coding for fibrinogen beta, alpha and gamma), several SNPs were associated with VTE: rs867186 located between FGB and FGA (Tregouet et al, 2009), rs6050 (Thr312Ala) in FGA (Gohil et al, 2009) and rs2066865 located in the 3'UTR of the FGG gene (Uitte de Willige et al, 2005).

Variation in gene encoding protein C (*PROC*) responsible for protein C deficiency was initially supposed to be strongly related to thrombosis, but this was not confirmed later (Dahlback, 2008). However, two polymorphisms within the promoter region (C/T at

position -2405 and A/G at position -2418) were recently associated with increased risk of VT (Pomp et al. 2009). Carriers of CC/GG genotype, who showed the lowest protein C levels, were at higher risk of VT compared to carriers of the TT/AA genotype. Polymorphism at *CADM1*, encoding cell adhesion molecule 1, also expressed in endothelial cells, was recently identified as a probable risk factor for VTE in protein C-deficient kindreds (Hasstedt et al, 2009), but it is not clear whether it is a general risk factor also. In gene coding for protein C receptor (*PROCR* or *EPCR*) haplotype A3 (Gly219 allele of the Ser219Gly substitution) was associated with increased VTE risk (Qu et al, 2006; Tregouet et al, 2009), especially in high-risk groups of individuals, carriers of other mutations (Morange & Tregouet, 2010).

The association of gene *F12* (encoding coagulation factor FXII) variants with VTE has been subject to a long debate. Common *F12* variant has attracted much research interest, but the results were not unequivocal. *F12* C-46T variant (rs1801020) was reported to be associated with increased VTE risk (homozygous carriers of T allele compared to noncarriers; Tirado et al, 2004). This association was also observed in a number of other studies, e.g. in patients with cerebral VT (Reuner et al, 2008). However, some studies failed to confirm these findings, e.g. Grunbacher et al. (2005) found that F12 46-TT genotype was not associated with thrombosis risk, nor with age at first thrombosis. A metaanalysis by Johnson et al. (2011) led to conclusion that evidence for association of this *F12* variant with VTE was weak. By genomewide linkage analysis, Calafell et al. (2010) demonstrated that the F12 gene represents a quantitative trait locus (QTL) that influences factor XII levels, and showed that only the promoter -46C>T variant (rs1801020) accounted for the variance attributed to this QTL. The authors concluded that this variation is evolutionary neutral and that T allele appeared approximately 100,000 years ago, reaching high frequencies by genetic drift.

Buil et al. (2010) reported that a new locus, *C4BPB/C4BPA* (coding for C4-binding protein), is involved in susceptibility to VT through a still unknown, but protein S–independent mechanism. Bezemer et al. (2008) found SNPs significantly associated with VT in *CYP4V2/KLKB1/F11* gene cluster, as well as in the *GP6* and *SERPINC1* genes. Three SNPs were strongly associated with VT: rs13146272 in CYP4V2, rs2227589 in SERPINC1 and rs1613662 in GP6; 4 additional SNPs (in CYP4V2, KLKB1, and F11) were also associated with VT. The effect of CYP4V2 and GP6 loci polymorphisms (SNPs rs1613662 and rs13146272) was further confirmed by Tregouet et al. (2009), and the effect of F11 polymorphisms (SNPs rs2289252 and rs2036914) by Li et al. (2009). Recently, in a multi-stage multi-design study, Antoni et al. (2010) found evidence that *BAI3* locus (encoding brain specific angiogenesis inhibitor 3) is associated with early-onset VTE; rs9363864-AA genotype was associated with a lower risk for VTE and low levels of FVIII and VWF. Three variants were recently found to be associated with activated partial thromboplastin time: *F12* (rs2731672), *KNG1* (rs710446), and *HRG* (rs9898) (Houlihan et al. 2010); *KNG1* Ile581Thr was confirmed as a risk factor for VT (Morange et al., 2011).

Morange et al. (2010) in a follow-up study found another new locus involved in susceptibility to VT that was not a part of commonly studied coagulation/fibrinolysis pathway: *HIVEP1* (coding for human immunodeficiency virus type 1 enhancer-binding protein). Allele rs169713C was associated with an increased risk for VT.

Future research in relation to genetic basis of VTE is aimed to explore other possibilities as well (Morange & Tregouet 2010), such as the effects of CNVs (copy number variations) or changes in epigenetic mechanisms (e.g. DNA methylation patterns).

3. Molecular testing of inherited thrombophilia

Nowadays, direct molecular detection of thrombophilia risk factors including F2 G20210A, factor V Leiden and MTHFR C677T mutations is offered by many clinical diagnostic laboratories. It is possible to discriminate between individuals heterozygous and homozygous for factor V Leiden on the basis of the degree of APCR in functional clotting-based assays. Testing for APC resistance (using an accurate assay) can be helpful in assessing for the presence of FVL, whether used initially as a ''screen'' or if used in conjunction with molecular testing. Therefore the APCR test serves as a phenotypic marker for FVL. A normal modified APCR test excludes the presence of FVL, but if abnormal, the FVL genotype should be confirmed by genetic analysis. Although the presence of PT G20210A is associated with increased levels of prothrombin in plasma, there is no phenotypic screening test for PT G20210A, and detection is always by genotyping. In contrast, there are no analogous functional assays for the detection of the PT G20210A mutation because there is no simple distinction between measurable PT levels in those with and without the mutation. Similarly, although the MTHFR thermolabile variant may be associated with increased levels of homocysteine, there is no other direct functional measure of any effect upon in vitro clotting assays. Detection of thrombophilias by functional testing is associated with a degree of uncertainty because interpretation of results can be influenced by the accuracy of normal ranges and the influence of other factors (both genetic and environmental). Hence repeat testing is often required.

Many different DNA-based methods have been described. In every laboratory method is chosen based on required sensitivity, necessary equipment, number of samples and economical capacity. In contrast to functional analyses results obtained by DNA based methods are essentially absolute, provided the requisite controls are in place, such that repeat testing is not required. Most of the methods described rely on DNA amplification by polymerase chain reaction (PCR). Genomic DNA is required for genetic analysis, but quantity and quality of DNA preparation may vary, depending on the requirements of the assay. Most laboratories routinely purify DNA for the analysis, but where only one or two single nucleotide polymorphisms (SNP) are to be evaluated, avoidance of time consuming DNA preparation can be of benefit. PCR-based methods rely on different tools to detect the genotype of the amplified alleles, such as RED, amplification-refractory mutation system (ARMS), enzyme-linked immunosorbent assay (ELISA)-based primer extension assay and fluorescence resonance energy transfer (FRET) assay. Multiplexing allows single procedure to detect more than one mutation. However, when electrophoresis is used, there may be many fragments present and care must be taken to ensure that interpretation of genotypes is clear-cut and not prone to error. Rare mutations at, or very close to the SNP site of interest may suggest different genotypes, depending on methodology. For example, rare silent mutations within the F5 gene (A1692C, G1689A and A1696G) and rare sequence variations at or near the prothrombin (F2) G20210A mutation (C20209T, A20207C, A20218G and C20221T). These will influence genetic analysis in a manner dependent upon the test system used. However, when these sequence variations are rare in the population their influence on the assay may be considered negligible.

3.1 Restriction enzyme digestion

Original methods for detection of mutations are based on restriction fragment length polymorphism (RFLP). DNA fragment of interest is amplified by polymerase chain reaction

(PCR) and than subjected to digestion by specific restriction enzymes whose restriction site is either created or abolished by the presence of mutation. Fragments of different sizes, generated after digestion, are separated by agarose or polyacrylamide gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide. The number of bands allows the discrimination of possible genotypes. For the FVL mutation restriction enzyme MnlI is used. The fragment generated by PCR contains one restriction enzyme site which serves as a positive internal control. It ensures that the restriction enzyme has been added, and that the restriction digestion has occurred. The second restriction site for MnlI is created by FVL mutation (Bertina, 1994). In the method described for detection of the PT G20210A in the presence of the mutant allele an artificial HindIII restriction site had to be created by the mutagenic amplification primer (Poort et al, 1996). Improvement of the original method provides additional restriction site which serves as a control as in the case of FVL detection (Pecheniuk, 2000). The MTHFR C677T mutation is also easily detected by PCR and RFLP analysis since the mutation creates restriction enzyme site for HinfI enzyme (Frosst, 1995). The use of multiplex PCR prior to RFLP and the use of whole blood instead of isolated DNA largely improved the original RFLP analyses (Gomez, 1998).
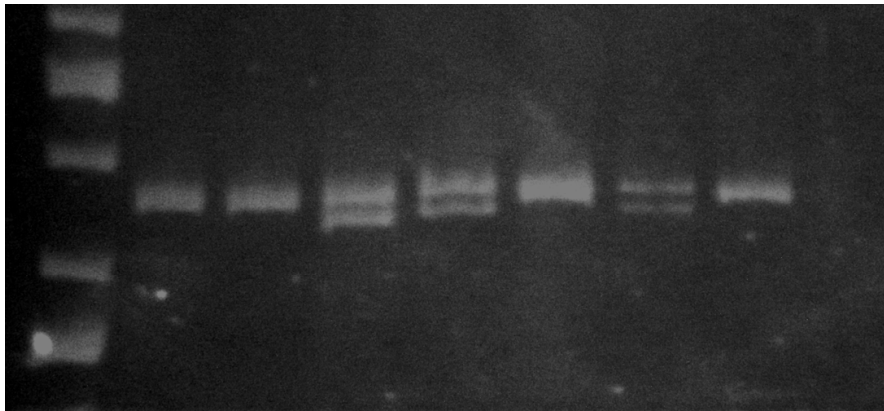

Fig. 1. Polyacrylamide gel electrophoresis of PCR-RFLP products for the FII polymorphism. Lane 1-molecular size marker; lane 2,3,6-normal patient; lane 4,5- heterozygous patient; lane7-heterozygous control; lane 8-normal control; lane 9-blank control
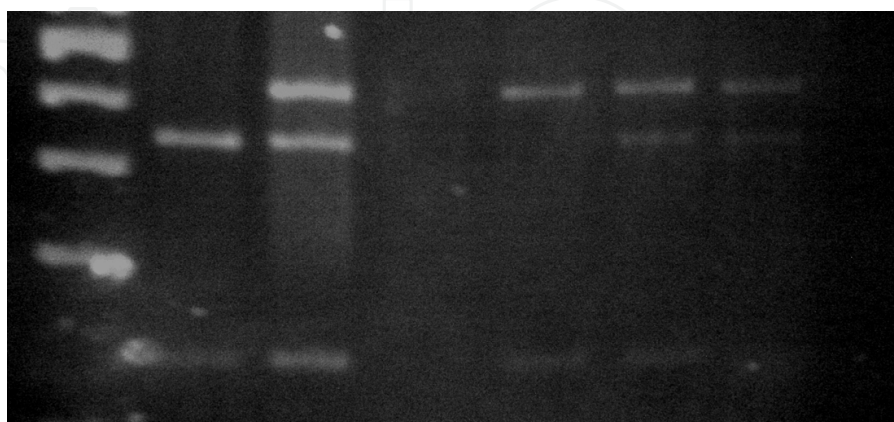

Fig. 2. Polyacrilamide gel elelctrophoresis of PCR-RFLP products for the FVL polymorphism Lane 1-molecular size marker; lane 2-normal control; lane 3-hetrozygous control; lane 5-homozygous mutant control, lane 6,7-heterozygous patients, lane 8-blank control
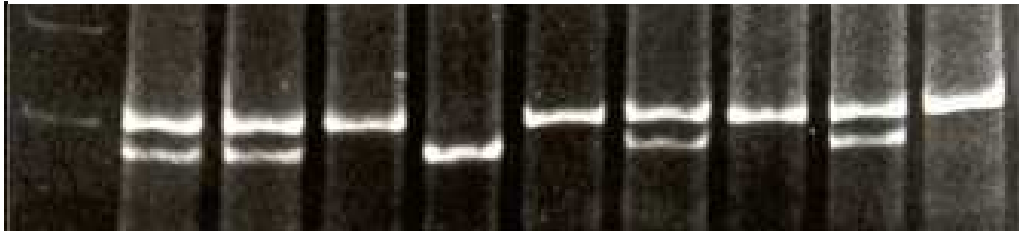
Fig. 3. Polyacrilamide gel electrophoresis of PCR-RFLP products for the MTHFR polymorphism Lane 1- molecular size marker; lane2,3,7,9-heterozygous patients; lane4,6,8,10-normal homozygous patients; lane 5-mutant homozygous patient

3.2 Amplification refractory mutation system (ARMS)

In order to simplify the procedure, increase the efficiency and reduce the costs by eliminating the use of restriction enzymes new methods have been developed. One of them is ARMS based on allele specific amplification of DNA sample. Standard procedure is performed in two separate reactions and with three different primers. In each reaction one of the primers is common (used in both reactions) and the other one is allele specific with 3'end complementary either to wild type allele or mutant allele. Results are obtained by analysis of PCR products on agarose or polyacrylamide gels. The presence or absence of PCR products represents the presence or absence of target alleles. Usually control primers are amplified in the same reaction with allele specific primers to ensure that the PCR reaction is working properly. Multiplex ARMS assay is also widely in use and it allows the analysis of two or more mutations. The Stagen kits for FVL and PT G20210A (Stago, Asnieres, France) amplify ß-2 microglobulin and an unconnected region of F5 (or F2) genes as controls, to ensure efficiency of the PCR reactions. Although this method is simple, efficient and cost effective the specificity of amplification relies highly on careful primer design, careful titration of primer concentrations, and stringent temperature cycling parameters (McGlennen, 2002).

3.3 Single strand conformational polymorphism

The other simple and fast method which avoids the need for restriction enzyme digestion is a single-strand conformation polymorphism (SSCP). It relies on the fact that denatured DNA molecules show different migration patterns on electrophoresis gels even when they differ in only one base. After PCR amplification DNA fragment of interest is denatured to single strands and analyzed by gel electrophoresis. Different alleles will form different 3D conformation and different migration patterns will be observed on gels. The advantage of this method is that it may identify rare mutations, such as the silent A1692C transition in the F5 gene, which is falsely identified as FVL when MnlI restriction enzyme is used (Keeney et al., 1999). These authors also described an SSCP multiplex assay to identify FVL and PT G20210A mutations in whole blood samples. Care must be taken on the interpretation of SSCP, as mutations may cause relatively small changes in electrophoretic mobility Cooper & Rezende (2007).

3.4 Enzyme-linked immunosorbent assays

ELISA-based methods generally hybridize a biotinylated PCR product to oligonucleotide bound to microtitre plate wells. Streptavidin–horseradish peroxidase conjugate then binds

to the bound amplicon and finally, buffered hydrogen peroxide with chromophore detects the bound amplicon. Comparison of colour density generated by reactions with wild-type and mutant oligonucleotide probes determines the genotype. A variation of this test is a multiplex assay, where two separate PCR reactions utilize a common biotinylated primer and reverse primers specific for wild-type or the mutant allele, and PCR products hybridize onto an oligonucleotide probe in microtitre plate wells. The former assay design may be more robust, as a single amplification reaction amplifies DNA irrespective of genotype. An ELISA assay for FVL using a reverse allele-specific oligonucleotide hybridization was described by Kowalski et al. (2000). ELISA-based methods require the use of a thermocycler, but other specialist genotyping apparatus is not required. In comparison to gel technology, chemicals used are less hazardous. The StripA Assay (ViennaLab, Vienna, Austria) is an ELISA, which utilizes the reverse hybridization principle, binding reactants to a membrane strip rather than to microtitre plate wells. The target sequence of DNA is amplified in a single reaction which produces a biotin-labelled amplicon. The amplicon is selectively hybridized to wild-type and mutant oligonucleotide detector bands on an individual membrane strip, and detected by means of streptavidin–alkaline phosphatase with colour-forming substrate. Washing and hybridization steps can be automated. Control and test bands are examined by eye or scanned to determine genotype. StripA kits are available for the detection of FVL and PT G20210A mutations and as a multiplex assay.

3.5 Real-time PCR

Real time high resolution melting curve analysis (HRM) employs a new class of fluorescent dyes that intercalate with double-stranded DNA. The intercalating dye is incorporated in PCR reaction and the products are then heated to separate the two strands. Fluorescence levels decrease as the DNA strands dissociate and this melting profile depends on the PCR product size and sequence. HRM appears to be very sensitive and can be used for high throughput mutation screening. Melting temperatures of wild type and mutant allele differ and genotype can be easily interpreted. Whilst Tm values can be extremely useful and reliable in detecting wild-type and common mutations, they cannot be relied upon to characterize unusual mutations. Instrument is relatively expensive and prone to contamination. A multiplex assay for FVL and PT was developed by Van den Bergh et al. (2000). The authors considered their assay to be robust, reliable with high discrimination power. Interpretation of results is based on the presence or absence of peaks with a Tm that is specific for the wild-type or mutant allele. It helps to identify additional sequence variations. This approach is simple allowing the result to be obtained in approximately two hours. Although the instrument is relatively expensive and the method is prone to contamination it is robust and reliable for identification of mutation of interest.

3.6 Mutation detection using hydrolysis probes

Some systems use only a single fluorescently labeled oligonucleotide probe (TaqMan hydrolysis probes) that provide very sensitive and specific detection of DNA. For genotyping this technology uses a PCR primer pair and two allele specific hydrolysis probes, one designed to detect the mutated allele and the other wild-type allele. Both allele-specific hydrolysis probes have a quencher dye and different reporter dyes attached. During the extension phase of PCR amplification the probe is hydrolyzed by Taq polymerase, reporter and quencher dyes are separated which causes the increase in fluorescence. After

PCR amplification, an endpoint plate reading is performed using real time PCR system. Allelic discrimination is achieved by measuring of the fluorescence values based on the signals from each well (Spector, 2005).

3.7 Direct sequencing of DNA

The gold standard method of mutation detection (screening) is bidirectional sequencing. The advantage of this method is that unusual mutations which can cause analytical anomalies can be easily detected. Variation of the standard dideoxy sequencing is Pyrosequencing (Biotage AB, Uppsala, Sweden) which simplifies further the analysis and makes it possible to analyze a large number of PCR products quickly and with minimal effort.

3.8 DNA microarray technology

DNA microarray technology provides rapid simultaneous testing for large number of single point mutations. It is a method of choice for laboratories with large number of test requests (Schrijver, 2003). Custom designed oligonucleotide sequences complementary to the normal DNA sequence and known SNP are attached to a chip. The DNA sample screened for mutation is amplified by PCR, fluorescently labeled and hybridized with the oligonucleotides in the microarray. Computer analysis of the color pattern of the microarray generated after hybridization allows rapid automated mutation testing (Turnpeny & Ellard, 2007). Several commercial platforms which include factor V Leiden, PT G20210A and MTHFR C677T mutation are available. However, they are still expensive for most of the laboratories. Low-density arrays with 10 to 20 markers could be a good alternative. In a single test all thrombophilic markers identified through the human genome project could be analysed (McGlennen, 2002).

3.9 Invader assay

This assay, unlike most of the other DNA-based genotyping methods does not employ PCR. Analysis is performed with genomic DNA. The Invader technology is based on the generation of a fluorescent signal in the reaction solution following the cleavage of synthetic oligonucleotide probe assembled with a so called invader probe and the DNA template that contains either the normal or mutation nucleotide (McGlennen, 2002). The analysis is performed in microtiter well and the signal is detected by fluorescent plate reader.

The main advantages of the Invader assay are that it does not employ the PCR reaction, therefore, failures resulting from contamination are less likely to occur (Cooper & Rezende, 2007). There is only a requirement for heating block and a fluorometer, but no dedicated apparatus is required for this assay. Genotypes can be obtained reliably directly from genomic DNA. It suffers from two limitations. First it requires relatively large amounts of target DNA. Second, only one SNP can be genotyped per reaction (Olivier, 2005). Modifying of the technology to accommodate multiple SNPs and large numbers of samples simultaneously will significantly improve this assay .

4. Acknowledgement

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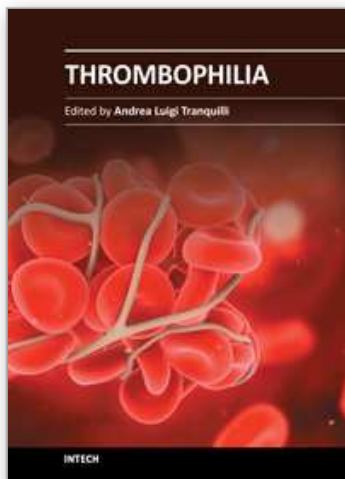
Thrombophilia
Edited by Prof. Prof. Andrea Tranquilli

Thrombophilia(s) is a condition of increased tendency to form blood clots. This condition may be inherited or acquired, and this is why the term is often used in plural. People who have thrombophilia are at greater risk of having thromboembolic complications, such as deep venous thrombosis, pulmonary embolism or cardiovascular complications, like stroke or myocardial infarction, nevertheless those complications are rare and it is possible that those individuals will never encounter clotting problems in their whole life. The enhanced blood coagulability is exacerbated under conditions of prolonged immobility, surgical interventions and most of all during pregnancy and puerperium, and the use of estrogen contraception. This is the reason why many obstetricians-gynecologysts became involved in this field aside the hematologists: women are more frequently at risk. The availability of new lab tests for hereditary thrombophilia(s) has opened a new era with reflections on epidemiology, primary healthcare, prevention and prophylaxis, so that thrombophilia is one of the hottest topics in contemporary medicine.

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