Role of Polymorphisms in Factor V (FV Leiden), Prothrombin, Plasminogen Activator Inhibitor Type-1 (PAI-1), Methylenetetrahydrofolate Reductase (MTHFR) and Cystathionine β-Synthase (CBS) Genes as Risk Factors for Thrombophilias

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Abstract: Thrombophilias are defined as a predisposition to thrombosis due to hematological changes which induce blood hypercoagulability; they can be inherited or acquired. They are individually characterized by a large phenotypic variability, even when they occur within the same family. Hereditary thrombophilias are, in most cases, due to changes related to physiological coagulation inhibitors or mutations in the genes of coagulation factors. High levels of plasma homocysteine may also be responsible for vaso-occlusive episodes and may have acquired (nutritional deficiencies of folate and vitamins B6 and B12) and/or genetic causes (mutations in the genes responsible for expression of enzymes involved in the intracellular metabolism of homocysteine). Considering that: (1) thromboses are events of multigenic and multifactorial etiopathology; (2) the presence of mutations in several genes significantly increases the risk of their occurrence; (3) the vascular territory (venous and/or arterial) affected involves different pathophysiological mechanisms and treatments, knowledge of genetic variants that may contribute to the risk and variability of the phenotypic manifestations of these diseases is extremely important. This understanding may provide support for a more individualized and therefore more effective treatment for thrombophilia carriers. Thus, this mini-review aims to address a comprehensive summary of thrombophilias and thrombosis, and discuss the role of polymorphisms in Factor V (FV Leiden), Prothrombin, Plasminogen activator inhibitor type-1 (PAI-1), Methylenetetrahydrofolate reductase (MTHFR) and Cystathionine β-synthase (CBS) genes as risk factors for thrombophilias.

Keywords: Coagulation factors, thrombosis, thrombophilias, gene polymorphisms, hypercoagulability, fibrinolysis.

INTRODUCTION

Thrombophilias are defined as a predisposition to venous thrombosis, or occasionally arterial thrombosis, due to hematological changes which induce blood hypercoagulability [1,2]. They are individually characterized by a large phenotypic variability, even when they occur within the same family, varying from asymptomatic to severe clinical forms [1].

Hereditary thrombophilias arise, in most cases, from changes related to physiological inhibitors of coagulation (antithrombin, protein C and protein S) or mutations in the genes of coagulation factors [2-4]. High levels of plasma homocysteine (Hcy) may also be responsible for vaso-occlusive episodes. In this case, a thrombophilia may have acquired causes, such as nutritional deficiencies of folic acid (folate), vitamins B6 (pyridoxine) or B12 (cyanocobalamin), and/or genetic causes, involving mutations in genes responsible for expression of the enzyme methylene tetrahydrofolate reductase (MTHFR) and cystathionine β-synthase (CBS), which participate in the intracellular metabolism of Hcy [1, 3, 4]. Clinically, hereditary thrombophilia usually manifests itself as venous thromboembolism (VTE) with some particular characteristics: a) occurrence in young individuals (before 45 years old); b) increased susceptibility to recurrent thrombosis; c) family history of thrombotic events; d) migratory or diffuse thrombosis, or in an unusual location; e) thrombotic episode disproportionately severe in respect to the triggering stimulus [2, 4]. Thrombophilic defects can also cause several obstetric complications, such as difficulty conceiving, complicated pregnancies, fetal growth retardation, abortions and fetal loss. Laboratory investigations should be carried out in all the afore-mentioned situations [4].

Thrombophilia is considered to be acquired when it results from another clinical condition such as hypertension, dyslipidemia, diabetes, advanced age, surgery, malignancy, myeloproliferative disorders, antiphospholipid syndrome, nephrotic syndrome, prolonged immobilization or use of medications such as hormone replacement therapy and oral contraceptive use [3-6]. However, an important factor to be considered is the vascular (venous and/or arterial) region affected, since this involves different pathophysiological mechanisms, with correspondingly different laboratory investigations and treatments [4]. Risk factors for VTE differ from risk factors for arterial thrombosis: hypertension,

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1875-5607/12 $58.00+.00

smoking, dyslipidemia and diabetes, for example, are not associated with increased risk for VTE [3,7].

Until 1992, only three risk factors for venous thrombosis had been analyzed: deficiencies of antithrombin (AT), protein C (PC) and protein S (PS). However, while these deficiencies are independent risk factors for VTE, together these abnormalities account for only 5 to 15% of all cases of VTE in different populations, and showed no relationship with arterial thrombosis [3,6]. Currently, the list of new tests for thrombosis also includes resistance to activated protein C (APC)/G1691A mutation in the factor V gene (factor V Leiden), G20210A mutation in the prothrombin gene (Factor II), high levels of factor VIII, tissue plasminogen activator (t-PA), plasminogen activator inhibitor 1 (PAI-1), hyperhomocysteinemia (fasting Hcy check), detection of lupus anticoagulant and anticardiolipin antibodies (related to antiphospholipid antibody syndrome - APS), high sensitivity C-reactive protein (hs-CRP) and tests for platelet activity [1,3,6], the first three tests being mainly associated with venous thrombosis, high Hcy and APS with both venous and arterial thrombosis, and the other parameters particularly with arterial thrombosis, as will be shown below.

THROMBOSIS

Thromboses are events of multigenic and multifactorial etiopathogenesis, resulting from the interaction of genetic and environmental factors, today constituting one of the most common causes of morbidity and mortality [3,8]. In contrast to monogenic diseases, in which a single gene mutation results in disease, in multigene diseases different mutations in different genes interact to cause the event. In this case, the risk of disease manifestation associated with each single genetic alteration is relatively low, but the presence of mutations in several genes significantly increases this risk [2,3].

In general, it is known that arterial thrombosis is predominantly derived from the activation of platelets, lipid deposits and cell proliferation in the plaque, while venous thrombosis (VTE) is essentially dependent on the hemostatic factors [9]. VTE affects an average of one adult per 1000 inhabitants/year. In about 40% of affected individuals it is possible to identify a hereditary thrombophilia, often in combination with one or more risk factors [1,3]. The usual clinical presentations include deep vein thrombosis (DVT) of lower limbs and pulmonary embolism, although more rarely a thrombotic episode may occur at other sites (retinal, mesenteric, upper limbs, cerebral veins, and recurrent superficial thrombophlebitis) [3]. In the case of arterial thrombosis, the role of inherited thrombophilia is not well defined. Studies have failed to establish a consistent relationship between inherited thrombophilia and the risk of arterial thrombosis. However, hyperhomocysteinemia (abnormal elevation of plasma Hcy) and antiphospholipid antibody syndrome are documented risk factors [1]. Although plasma Hcy may be related to environmental and/or genetic causes, as we shall see in this mini-review, APS is an autoimmune disorder that alone provokes blood clots in arteries and veins as well as pregnancy-related complications [10]. It is therefore necessary to remove any association with environmental causes such as decreased serum folate and/or vitamin B12, as well as the PPS phenotype, before conducting association studies with genetic polymorphisms, to avoid spurious associations.

Some studies also indicate that hyperfibrinogenemia is associated with thromboembolic disease [8,11,12] and it has been proposed that hyperfibrinogenemia directly promotes thrombosis and thrombolysis resistance, *via* enhanced fibrin formation and stability [12]. The increase in blood levels of fibrinogen accelerates the formation of clots/thrombi, increases platelet aggregation, fibrin formation and plasma viscosity, besides being an acute-phase reactant that is increased in inflammatory states [11]. Some forms of dysfibrinogenemia are inherited conditions in which fibrinogen molecules are synthesized erroneously; they are manifested clinically by arterial and/or venous thrombosis in 5% of cases [9]. Thus, screening for thrombophilia may be of interest in arterial thrombosis in younger individuals (up to 50 years) in the absence of vascular risk factors (smoking, hypertension, dyslipidemia, diabetes mellitus) [1].

Given the wide range of genetic risk factors for thrombophilias, this mini-review intends to examine the role of polymorphisms in Factor V (FV Leiden), Prothrombin, Plasminogen activator inhibitor type-1 (PAI-1), Methylenetetrahydrofolate reductase (MTHFR) and Cystathionine β-synthase (CBS) genes as risk factors for thrombophilias.

POLYMORPHISM IN THE FACTOR V GENE

The blood clotting system consists of a cascade of proteolytic enzymes and co-factors that bind and stabilize their respective enzymes, where the inactive precursors are activated in series, each precursor giving rise to a higher amount of the next [13]. It involves the interaction of many plasma serine proteases known as blood clotting factors, which interact with calcium and phospholipid surface to produce a tough fibrin meshwork, which reinforces the friable platelet plug and stops bleeding until tissue repair can occur [14]. The final enzyme, thrombin, derived from prothrombin (factor II), converts soluble fibrinogen (factor I) into an insoluble network of fibrin, forming a clot. The two pathways (intrinsic and extrinsic) of the clotting cascade constantly interact with one other and both result in the activation of factor X, which then converts prothrombin to thrombin [13-16]. In both pathways, factor V (FV) is an essential cofactor for factor Xa, being a central regulator of hemostasis. When factor V is activated, it interacts with factor X, and the active forms of these two coagulation factors (factors Va and Xa, respectively) form the prothrombinase complex which, in the presence of calcium and phospholipid surface, converts prothrombin to active thrombin (Fig. **1**) [8].

Besides its role as procoagulant factor, FV is also involved in the physiologic anticoagulant pathway, through the activated protein C (APC) pathway [17]. During normal blood coagulation, thrombin binds to the membrane receptor of the endothelial cell, the thrombomodulin, being able to activate protein C, which in the presence of protein S, cleaves and inactivates factors VIIIa and Va (Fig. **1**) [8]. Downregulation of the procoagulant activity of FVa is

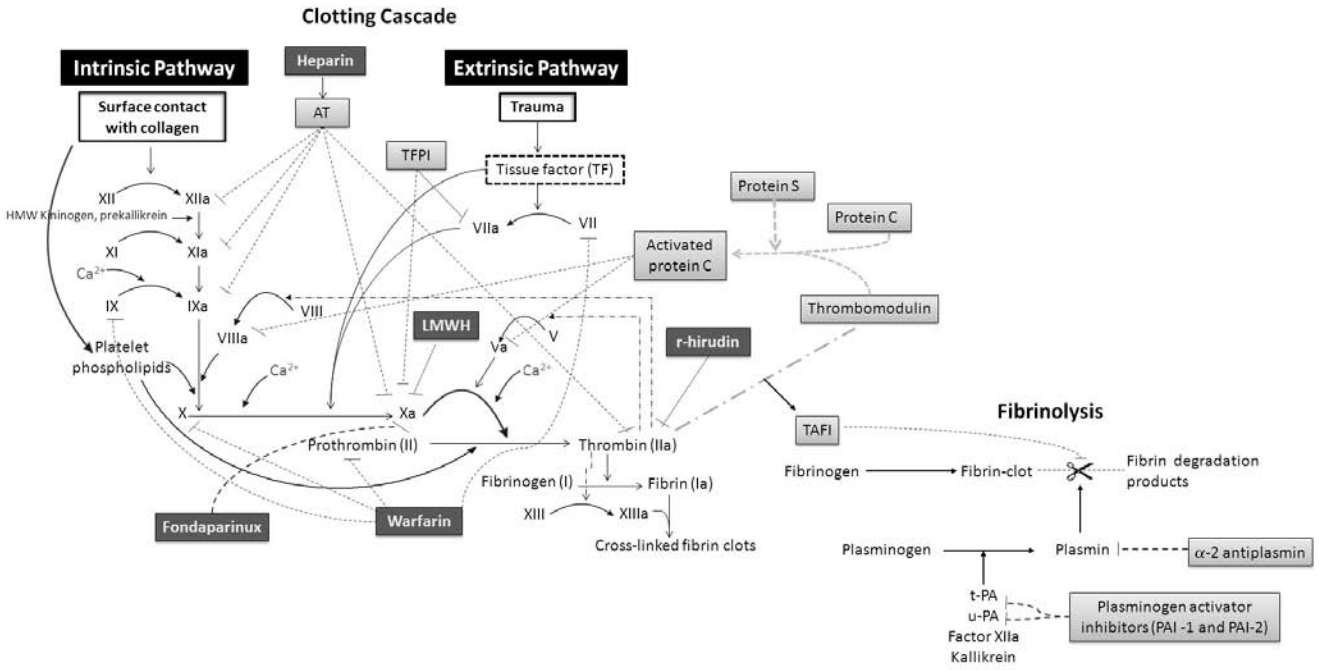

Fig. (1). The Clotting Cascade and fibrinolysis: intravascular anticoagulants, inhibitors of fibrinolysis, and site of action of pharmacologic anticoagulant agents.

Clotting Cascade: endogenous intravascular anticoagulants are represented in light gray squares, while pharmacologic anticoagulant agents are represented in dark gray squares. HMW Kininogen= High-molecular-weight Kininogen; AT= Antithrombin III; TFPI= Tissue factor pathway inhibitor. Continuous black arrows indicate activation; dash-dot black arrows indicate activation by thrombin; dashed gray arrows indicate the activation of protein C pathway; dashed gray lines interrupted by a dash at the end indicate inhibition.

Fibrinolysis: TAFI= Thrombin-activatable fibrinolysis inhibitor. Continuous black arrows indicate activation; dashed gray lines indicate inhibition.

achieved by APC-mediated proteolysis at Arg506, Arg306, and Arg679 residues, in this kinetic order. The first cleavage at Arg506 reduces both FVa cofactor activity (of 25–40%) and its affinity for FXa, resulting in partial inactivation. The subsequent cleavage at Arg306 leads to the complete inhibition of FVa, while Arg679 cleavage is likely to be less important. This inactivation slows down the clotting process and prevents clots from growing too large. Given the dual role of FV, genetic defects in FV gene may result in opposite hemorrhagic (FV deficiency) or thrombotic phenotypes [17].

Bertina *et al.* (1994) [18] demonstrated that the phenotype of resistance to the action of activated protein C (APC) was associated with heterozygosis or homozygosis to a point mutation in the factor V gene (locus 1q24.2). As the mutation was initially characterized in Leiden in the Netherlands, the defect is commonly referred to as factor V Leiden (FV Leiden or FVL) [19]. This mutation consists of the substitution of a guanine (G) for adenine (A) at nucleotide 1691 (G1691A), which causes the exchange of an arginine (R) for a glutamine (Q) at position 506 of the protein (FV R506Q or FV Q506 mutation; dbSNP rs6025) [18]. The Leiden mutation is responsible for the APC resistance phenotype because it affects one of the target sites for APC, impairing both the efficiency of APC-mediated degradation of FVa and the FV APC-cofactor activity in the inactivation of FVIIIa [17]. Thus, the FV mutant becomes less susceptible to cleavage by APC, remaining active for a longer time as a clotting factor in blood [2,3].

FVL mutation is the most common cause of inherited thrombophilias in Caucasian populations [16], being observed in 20% to 40% of patients with thrombosis [2]. Women with this variant allele are at increased risk of pregnancy complications [20]. It is present in a number of Caucasian populations and the prevalence of the gene in the general population ranges from 1% to 15%, depending on the ethnicity of those studied [19], being extremely rare among Africans, Chinese, Japanese, Amerindians and peoples of South Asia [2]. Heterozygous individuals have an approximately 3–8 times increased risk for an initial episode of VTE compared with the general population; the homozygous have this risk increased to 80 times [3,17,19,20] and usually require life-long anticoagulation with Warfarin, an inhibitor of vitamin K [19].

The clinical expression of Factor V Leiden is influenced by the number of FVL alleles, coexisting genetic and acquired thrombophilic disorders, and circumstantial risk factors such as travel, central venous catheters, pregnancy, oral contraceptive use, hormone replacement therapy, selective estrogen receptor modulators, organ transplantation, advancing age, and surgery. Diagnosis requires the activated Protein C resistance assay (a coagulation screening test) or DNA analysis of the FV gene,

which encodes the Factor V protein [19,21]. Cases of thrombosis in which a family member is known to have the factor V Leiden mutation, or the patient is known to have antiphospholipid antibodies, should be handled by proceeding directly to genetic testing [19].

In general, the diagnosis of thrombophilia does not modify the approach or the initial treatment of DVT. The first acute thrombosis is treated according to standard guidelines: an initial course of intravenous unfractionated heparin or low molecular-weight heparin (LMWH) and initiation of oral anticoagulation with Warfarin (except during pregnancy) (Fig. **1**) [1,21]. Long-term oral anticoagulation is considered in factor V Leiden homozygotes and those cases with recurrent VTE, multiple thrombophilic disorders or coexistent circumstantial risk factors [21].

A short course of prophylactic anticoagulation when circumstantial risk factors are present may prevent initial thrombosis in factor V Leiden heterozygotes. In women heterozygous for factor V Leiden who have a history of recurrent pregnancy loss, prophylaxis with Enoxaparin, a low molecular weight heparin, may increase the likelihood of a favorable pregnancy outcome. Oral contraceptives are also discouraged for homozygous women with or without prior VTE and also for asymptomatic heterozygous women [21].

POLYMORPHISM IN THE PROTHROMBIN GENE

Prothrombin (factor II) is another of the cascade steps in the blood clotting system, whose abnormal activity, like in factor V, is also associated with DVT [22]. It is the precursor of the serine protease thrombin, a key enzyme in the processes of hemostasis and thrombosis [22,23] that exhibits procoagulant, anticoagulant, and antifibrinolytic activities [23]. Prothrombin participates in the final stage of the clotting cascade when it is activated to thrombin by factor Xa in the presence of factor Va, calcium ions, and a phospholipid surface. Thrombin, in turn, cleaves fibrinopeptides A and B from the α and β chains of fibrinogen, respectively, to form the fibrin clot. After that, the activated thrombin enzyme converts fibrinogen to fibrin for blood clot formation, stimulates platelet aggregation, and activates coagulation factors V, VIII and XIII [24]. Thrombin is also involved in the physiologic anticoagulant pathway. While the clot is forming, approximately 85 to 90% of thrombin is adsorbed on the fibrin fibers, which helps to stop thrombin spreading to the remainder of the blood, preventing excessive growth of the clot. The thrombin that is not adsorbed on the fibrin fibers immediately combines with the AT, which further blocks the effect of thrombin on fibrinogen, and inactivates thrombin during the next 12 to 20 minutes [15]. By binding to the lysyl residues on AT, inducing in it a conformational change, heparin converts AT from a slow to a very rapid inhibitor of clotting enzymes [25], in such a way that the heparin-AT complex inhibits thrombin (IIa), factors XIIa, XIa, Xa and IXa [15,25]. In another way, the binding of thrombin to thrombomodulin, a protein linked to endothelial membrane, slows the process by removing thrombin, besides activating the plasma protein C, which acts as an anticoagulant by inactivating factors Va and VIIIa (Fig. **1**) [15]. Thus, like FV, given the dual role of thrombin, genetic defects in the prothrombin gene may also result in opposite hemorrhagic (congenital prothrombin deficiency or hypoprothrombinemia) [26] or thrombotic phenotypes [23].

The prothrombin gene, located on chromosome 11p11.2, is organized into 14 exons, separated by 13 introns with the 5' upstream untranslated (UT) region and the 3'-UT region [22]. The G to A transition at nucleotide 20210 in the 3'-UT region of the promoter of the gene (G20210A mutation; dbSNP rs1799963) increases the stability of messenger RNA, elevating plasma levels of prothrombin to 30% (hyperprothrombinemia) and resulting in increased risk for venous or arterial thrombosis due to its potential to form thrombin and an excessive growth of fibrin clots [2,3,6,9,27]. In fact, it is the second most common inherited thrombophilic tendency in patients with a personal and family history of DVT [6], with a prevalence within white populations of 1-3% [22,28]. It is found in 2-5% of the general population [19] and in 6-18% of patients with VTE [3]. It is often associated with other genetic (FV Leiden, deficiencies of proteins C and S, antithrombin III) or acquired (lupus anticoagulant, pregnancy, puerperium, trauma, immobilization, neoplasms) risk factors [9]. Heterozygous carriers of prothrombin G20210A face a 2- to 5-fold higher risk for first venous thrombosis than individuals without these mutations [29]. This risk is 20-fold higher in subjects who are heterozygous carriers of both factor V Leiden and prothrombin G20210A and 18-fold higher in homozygous factor V Leiden carriers [28]. As in the FVL mutation, a G20210A mutation in the prothrombin gene is extremely rare in non-Caucasian populations [2].

Factors that predispose an individual to thrombosis in prothrombin-related thrombophilia include: the number of G20210A alleles; presence of coexisting genetic abnormalities including factor V Leiden; and acquired thrombophilic disorders, such as antiphospholipid antibodies. Circumstantial risk factors for thrombosis include pregnancy and oral contraceptive use. Some evidence suggests that the risk for VTE in G20210A heterozygotes increases after travel [29]. Laboratory evaluation for the prothrombin gene mutation is best performed through direct genetic analysis to identify the G20210A transition. This type of testing is extremely reliable and can be performed under most conditions without interference from other medical treatments or conditions. Direct measurement of prothrombin levels, which is more subject to error and more difficult to interpret, is not recommended [19].

Treatment of manifestations depends on the clinical circumstances. The first acute venous thrombosis is treated according to standard guidelines with a course of low molecular-weight heparin (LMWH); subcutaneous Fondaparinux, a synthetic pentasaccharide chemically related to LMWH; or intravenous unfractionated heparin. Oral administration of Warfarin is started concurrently with heparin or Fondaparinux (except in pregnancy), and should be overlapped for at least five days. The international-normalized ratio (INR) is used to monitor Warfarin anticoagulation. The duration of anticoagulation therapy should be tailored to the individual, based on an assessment

of the risk for VTE recurrence and the risk for anticoagulant-related bleeding. In the cases of patients with heparin-induced thrombocytopenia complicated by thrombosis and for thrombosis prophylaxis after major orthopedic surgery, use of recombinant hirudin (r-hirudin), a direct thrombin inhibitor (Fig. **1**), has been approved for clinical use [30]. No consensus exists on the optimal management of prothrombin-related thrombophilia during pregnancy; guidelines for treatment of VTE are similar to those for individuals who are not pregnant [29].

4G/5G POLYMORPHISM IN THE PROMOTER REGION OF PAI-1 GENE (PLASMINOGEN ACTIVATOR INHIBITOR-1)

Fibrinolysis, the final stage of hemostasis, has the main function of removing the clot, restoring the blood flow, which is done by enzymatic degradation of fibrin. The fibrinolytic system consists of several components, of which the main ones are pro-enzyme plasminogen activators (t-PA and u-PA) and inhibitors (PAI) [9]. Under normal circumstances, plasminogen activator inhibitors PAI-1 and PAI-2 inhibit the activation of circulating plasminogen in blood [31], by blocking the activity of t-PA and u-PA (Fig. **1**) [9,16]. Of these, PAI-1 is the main inhibitor of fibrinolysis and when its rate is high, fibrinolytic activity is diminished, increasing the risk of arterial and venous thrombosis [9].

Epidemiological and population studies have identified an association between increased plasma levels of PAI-1 and increased risk of thrombotic vascular diseases, including ischemic heart disease, myocardial infarction and DVT [32,33], and plasma levels of PAI-1 are influenced by genetic factors, as well as metabolic, endocrine, dietary, and physical activity factors, being markedly increased in response to inflammation and injury [34].

The gene responsible for PAI-1 expression is located on chromosome 7q21.3-q22 and it has been demonstrated that plasma levels of PAI-1 are related to an insertion/deletion polymorphism of 1 base pair (bp) of guanine (4G/5G) in the promoter region of this gene, identified 675 bp upstream of the transcription start site [35,36]. This polymorphism affects gene expression and the presence of the 4G allele is associated with higher levels of PAI-1 [33,35-37]. The 4G allele binds only one transcription enhancer, while the 5G allele binds an enhancer and a repressor of transcription. Thus, the 4G/4G genotype has higher rates of transcription than the 5G/5G genotype, which is reflected in higher plasma levels of PAI-1 in individuals homozygous for the deletion [35,36,38]. Because increased plasma levels of PAI-1 have been considered as an independent risk factor for coronary artery disease, stroke and venous thrombosis [9], the presence of the 4G allele has been studied in association with increased risk of thrombosis [39] mainly in those patients with other thrombophilic defects [40], and of cardiovascular disease, including myocardial infarction (MI) [41], since it inhibits fibrinolysis and may exacerbate tissue damage, adversely affecting the prognosis. However, data regarding the effect of this genetic variant on the risk for thrombosis are contradictory. Thus, the nature of this polymorphism can be more accurately described as response polymorphism, since PAI-1 is considered a strong acute-phase reactant [38], while the transcriptional regulation of the PAI-1 4G allele appears, furthermore, to be regulated by triglyceride [42]. This means that different PAI-1 levels between 4G and 5G are more apparent in the presence of environmental and/or disease factors, which stimulate PAI-1 expression. In fact, clinical data more convincingly support the involvement of this polymorphism in VTE than in stroke or MI, particularly in subjects with other genetic thrombophilic defects [38]. Thus, testing for this polymorphism has been not recommended outside a research setting [19], although plasma levels of PAI-1 can be measured by immunoenzymatic assays (ELISA), while PAI-I activity is by chromogenic assay or ELISA [43-45].

Increased plasma levels of PAI-1 in patients with acute myocardial infarction have been treated according to the guidelines with 300 mg oral dose of acetylsalicylic acid (aspirin) prior to i.v. streptokinase (STK) infusion; although the latter treatment is considered outdated, it is still used in some countries. After STK, heparin is given for 48 h in doses adjusted to maintain activated partial thromboplastin time 2–2.5 times longer than normal values [45]. Because high concentrations of PAI-1 have been found in women with cause-unknown early abortion, since the impaired fibrinolysis promotes fibrin deposition in the circulation early placenta [46], treatment regimens used to manage thrombophilia may include heparin or Lovenox (low molecular weight heparin) injections, and baby aspirin or metformin (for insulin resistant patients with elevated PAI-1) [47].

HYPERHOMOCYSTEINEMIA AND POLYMORPHISMS IN THE GENES OF METHYLENE TETRAHYDROFOLATE REDUCTASE (MTHFR) AND CYSTATHIONINE B-SYNTHASE (CBS)

Hcy is a small sulfhydryl amino acid formed during methionine metabolism (Fig. **2**). It is initially derived from the demethylation of methionine; thereafter, the metabolism follows one of two pathways: remethylation or transsulfuration. The remethylation cycle is catalyzed by the enzymes methionine synthase (MS – EC 2.1.1.13) and methylene tetrahydrofolate reductase (MTHFR – EC 1.5.1.20). In this cycle, Hcy is recycled to methionine by acquiring a methyl group donated by methyltetrahydrofolate, a process that uses folate and vitamin B12 as cofactors. MTHFR catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate [48,49], serving as methyl donor in remethylation of Hcy to methionine, which in turn is converted to S-adenosylmethionine (SAM) that methylates specific cytosines in DNA, regulating gene transcription through methylation [48]. In the transsulfuration cycle, homocysteine condenses with serine to form cystathionine, in a reaction catalyzed by the enzyme cystathionine β-synthase (CBS – EC 4.2.1.22), which uses vitamin B6 as cofactor. The cystathionine is subsequently hydrolyzed to cysteine [50-52].

Hyperhomocysteinemia has been proposed as a risk factor for VTE and arterial thrombolytic disease. Its pathogenesis is complex, since genetic and environmental variables interact to determine plasma levels of Hcy [3,19,53]. The total fasting plasma Hcy ranges from 5 to 15

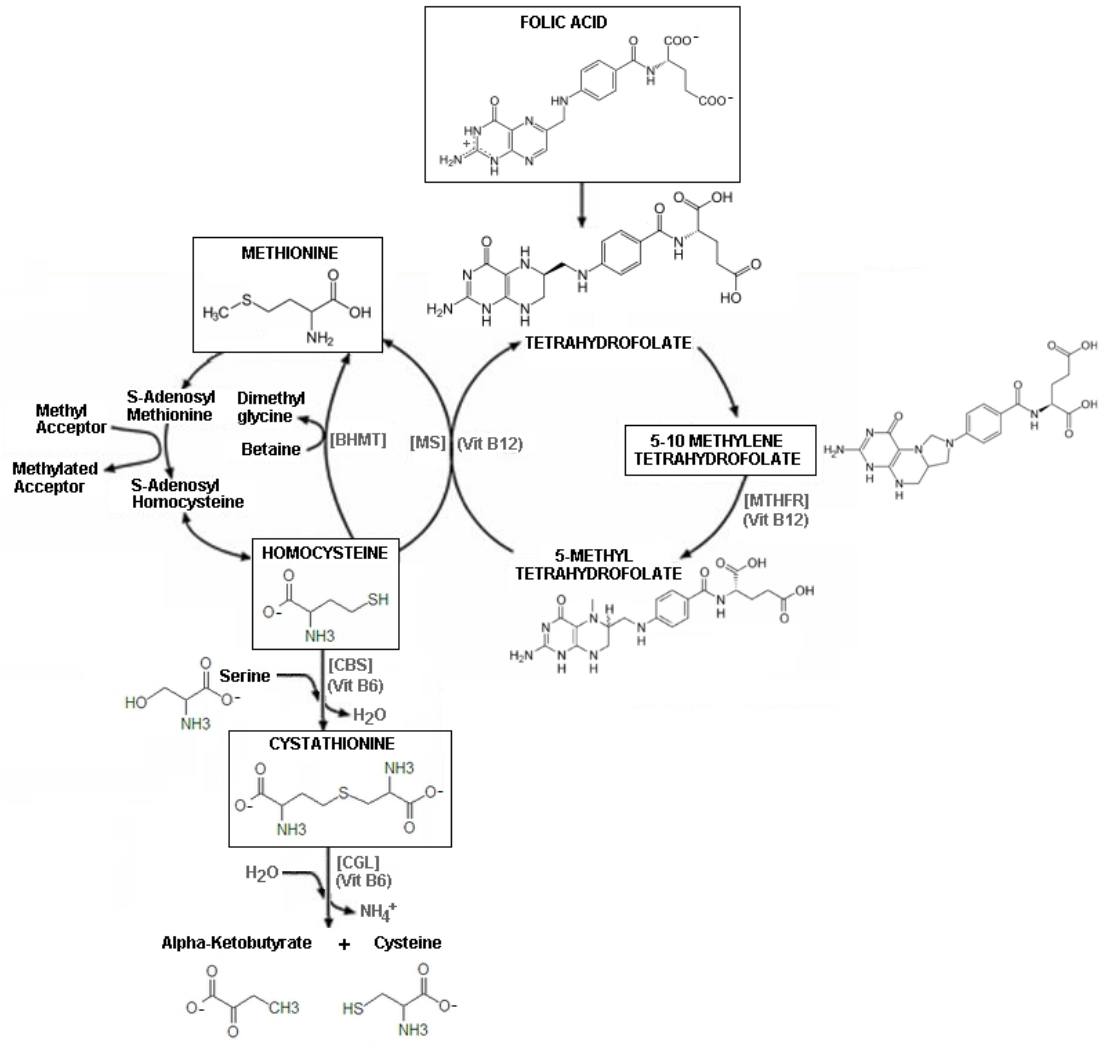

Fig. (2). Methionine-Homocysteine Metabolism.

Enzymes represented in gray inside the brackets: MS= Methionine synthase; MTHFR= Methylene tetrahydrofolate reductase; CBS= Cystathionine β-synthase; CGL= Cystathionine gamma-lyase; BMHT= Betaine homocysteine methyl transferase.

μM/L in a normal adult; above 100 μM/L is considered severe hyperhomocysteinemia. The genetic defect leads to clinical manifestations of homocystinuria which is associated with obstructive coronary and peripheral vascular events, including retinal ones [54].

Various forms of hyperhomocysteinemia are caused by genetic defects or non-genetic factors, or by a combination of both [51], and the exact extent of heritability of this trait remains questionable [19]. Approximately 5% of the population exhibit higher levels of Hcy than normal, and prevalence among patients with venous thrombosis is about 10% [19]. Acquired causes include nutritional deficiencies of vitamin B6, vitamin B12 or folate, advanced age, chronic renal failure and antifolate use [3,51]. Genetic defects involve deficiencies in MTHFR and CBS enzymes, of autosomal recessive inheritance, being, therefore, an innate error in the metabolism of homocysteine [3,9,50,51,53]. Thus, inherited or acquired deficiencies in both the remethylation pathway and the transsulfuration pathway of Hcy result in elevated plasma levels of this amino acid and in the clinical syndrome of hyperhomocysteinemia or homocystinuria [3,50,51,53].

Several mutations in the MTHFR and CBS have been identified, most of which are rare mutations whose clinical consequences are expressed only in homozygous, a situation in which patients present complex clinical signs characterized by diverse neurological deficits, psychomotor retardation, seizures, premature arterial disease and VTE [3]. In contrast to the rarity of these defects, two missense mutations in the MTHFR (C677T and A1298C) and CBS (G919A and T833C) genes and a 68 bp insertion in the CBS gene (844ins68) are prevalent and are therefore noteworthy for the increased risk of VTE and early arterial disease [3,53].

The most common genetic abnormality in Hcy metabolism is a substitution at nucleotide 677 (C677T; dbSNP rs1801133) in the gene encoding the enzyme

MTHFR, making it about 50% less active [52]. The MTHFR gene is located on chromosome 1p36.3 and the C677T polymorphism is found in approximately 12% of the general population, with variations from 5% to 18% [55]. The transition of a cytosine (C) for a thymine (T) at nucleotide 677 results in the substitution of an alanine (GCC) for a valine (GTC) at position 222 (Ala222Val), generating a thermolabile variant of the enzyme [56]. The 677TT homozygosity has been associated with reduced MTHFR activity and plasma folate levels as well as elevated plasma Hcy concentrations (mild to moderate hyperhomocysteinemia). However, its role as a risk factor for VTE and cardiovascular disease is still controversial [3,51,55,56].

The thermolabile genotype (677TT) has been associated with higher levels of Hcy, but not always with thrombolytic disease. Additionally, many homozygous patients have normal levels of plasma Hcy and it has been shown that the phenotypic expression of MTHFR is strongly dependent on the availability of folate, suggesting that the homozygous for the thermolabile genotype may have a higher requirement of folate than individuals with wild type genotype (677CC). One possible explanation is that many studies involved large numbers of patients with adequate levels of folate and, consequently, with normal concentrations of Hcy [57].

A second genetic polymorphism of the MTHFR gene – A1298C – results in the replacement of glutamate (Glu or E) by alanine (Ala or A) at position 429 of the enzyme (Glu429Ala or E429A; dbSNP rs1801131). The A1298C polymorphism lies in the S-adenosylmethionine-regulatory domain of the enzyme, and folate affects gene expression, regulating cellular SAM levels [48]. It is observed in approximately 10% of individuals and has been associated with reduced enzyme activity [55]. However, this polymorphism alone does not lead to a severe enzyme deficiency and does not seem to be associated with hyperhomocysteinemia or reduced plasma folate levels. On the other hand, the combination of heterozygosis of both polymorphisms of MTHFR (C677T and A1298C) leads to features similar to those observed in homozygotes 677TT, and has been associated with reduced enzyme activity, decreased plasma folate levels and hyperhomocysteinemia [3,58,59].

The CBS gene is located on chromosome 21q22.3 and more than 130 mutations in this gene have been identified. Most of them are missense mutations, leading to replacement of one amino acid [60,61]. Two of these mutations occur in exon 8 and deserve note for having been identified as prevalent in clinical cases of homocystinuria: G919A and T833C. The G919A transition results in replacement of a glycine (G) by a serine (S) protein at position 307 (G307S; dbSNP rs121964962), while the T833C transition results in a substitution of isoleucine (I) by a threonine (T) at codon 278 (I278T; dbSNP rs5742905) [62,63]. Additionally, another common mutation in exon 8, which involves an insertion of 68 bp (844ins68), was found co-segregating in Cis with the T833C CBS mutation. This mutation alone does not seem to influence Hcy levels and the risk of VTE, but in combination with MTHFR C677T mutation may lead to increased risk of thromboembolic events. Although common in several populations, CBS 844ins68 mutation has revealed ethnic and geographic variability [3,64-66].

Homozygous CBS deficiency is rare, with incidence estimated at 1 in 344,000 births (OMIM +236200). However, their heterozygous deficiency is relatively common and leads to moderate elevation of Hcy, being present in 1 in every 300 individuals [52]. Patients homozygous for CBS deficiency syndrome develop classical homocystinuria, including premature vascular disease and thromboembolism, mental retardation and skeletal abnormalities [50]. The expressivity is variable for all the clinical symptoms. Two phenotypic variants are recognized, homocystinuria responsive and non-responsive to vitamin B6, and these phenotypes are related, respectively, to the G919A (G307S) and T833C (I278T) mutations. Homocystinuria responsive to vitamin B6 is typically, but not always, milder than the non-responsive variation [67].

Although the laboratory diagnosis of hyperhomocysteinemia can be performed by immunoassay or high-pressure liquid chromatography (HPLC), changes in the CBS and MTFHR genes can only be analyzed by molecular methods [19,68].

Unlike many other thrombophilic disorders, hyperhomocysteinemia can be treated directly with vitamin supplementation, although there is a lot of controversy surrounding this. Supplementation with vitamin B6, B12 and folate can often reduce Hcy levels to normal, although there are no data at this time that address what impact this has on a person's risk of venous thrombotic problems [19]. Moreover, results from randomized placebo-controlled clinical trials testing the effect of vitamin therapy on outcome in these diseases have generally fallen short of expectations [69]. Therefore, treatments should be also done according to standard guidelines for thrombosis: initial course of intravenous unfractionated heparin or LMWH and initiation of oral anticoagulation with Warfarin. These medications are generally used for 3-6 months. Further treatment is generally not indicated in hyperhomocysteinemia after a single thromboembolic episode, given the risk of bleeding associated with anticoagulation. Patients that have had multiple thromboembolic episodes or are at high risk of further episodes (for example, multiple thrombophilic states) are usually started on long-term anticoagulation [1].

On the other hand, the identification of homocysteine as a risk factor for CAD and stroke carries important public health implications. If effective, the simplicity, availability, and presumably favorable side-effect profile of hyperhomocysteinemia treatment with combined folic acid and oral B6 and B12 vitamin supplementation make this an attractive addition to standard medical therapy for cardiovascular diseases [69].

CONCLUSIONS

The clinical supervision of patients with thrombosis or individuals with clinically suspected thrombophilia involves a complex evaluation of genetic and acquired risk factors, since thrombosis is a multifactorial disease. In general, treatments should be carried out according to standard guidelines for venous or arterial thrombosis. However, while

in light of current knowledge the data are not consensual, the laboratory identification of thrombophilia may potentially contribute to stratifying individual risk of recurrence and defining the strategy of secondary prevention. Moreover, considering that: (1) thromboses are events of multigenic and multifactorial etiopathology; (2) the presence of mutations in several genes significantly increases the risk of their occurrence; (3) the vascular territory (venous and/or arterial) affected involves different pathophysiological mechanisms and treatments, knowledge of genetic variants that may contribute to the risk and variability of the phenotypic manifestations of these diseases is extremely important. This understanding may provide support for a more individualized and therefore more effective treatment for thrombophilia carriers.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENT OF FUNDING

Declared none.

PATIENT CONSENT

Declared none.

ABBREVIATIONS

Hcy	=	homocysteine
MTHFR	=	methylene tetrahydrofolate reductase
CBS	=	cystathionine β-synthase
FVL	=	Factor V Leiden
VTE	=	venous thromboembolism
AT	=	antithrombin
PC	=	protein C
PS	=	protein S
APC	=	activated protein C
t-PA	=	tissue plasminogen activator
u-PA	=	urokinase-type plasminogen activator
PAI-1	=	plasminogen activator inhibitor type 1
DVT	=	deep vein thrombosis
LMWH	=	low molecular-weight heparin
UT	=	untranslated
MI	=	myocardial infarction
MS	=	methionine synthase
SAM	=	S-adenosylmethionine

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Received: October 19, 2011 Revised: February 04, 2012 Accepted: February 06, 2012