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## APC Resistance

Gerry A.F. Nicolaes

*Cardiovascular Research Institute Maastricht, Maastricht University*

*The Netherlands*

### 1. Introduction

In order to secure the continued supply of oxygen to the tissues, the integrity of the vascular system is of prime importance for survival. Several systems therefore exist that safeguard the circulatory system and that include emergency- and repair systems. As a protective system, primary haemostasis and blood coagulation function in concert in order to prevent extensive loss of blood from the organism in the event of vascular damage (Dahlbäck, 2000). Not only humoral and cellular factors limit the loss of blood, a first-line-of-defense is formed by the physiological process of vasoconstriction which is initiated upon a breach of vascular integrity.

Being capable of converting the fluid-like medium blood into a gel-like blood clot within a very short span of time implies that the haemostatic system incorporates the intrinsic dangerous capacity of damaging the very system it is intended to protect. This is the reason why the haemostatic system is subject to strict regulation by several anticoagulant mechanisms, which together prevent the excessive or inappropriate deposition of blood clots within the vascular system.

Rather than being a dormant system that only responds to changes in the vasculature, it is generally accepted that the haemostatic response, as it occurs in healthy individuals, is instead the result of the upregulation of ongoing coagulation reactions. Under normal conditions, these coagulation reactions, as well as anticoagulation reactions proceed continuously at a low level and pro- and anticoagulant reactions balance each other in a dynamic equilibrium. The temporal upregulation of only procoagulant reactions will shift the balance to favor a procoagulant response, (Dahlbäck & Stenflo, 1994).

The maintenance of both pro- and anticoagulant reactions at a low but vigilant level, ensures that the haemostatic system is able to generate a swift response when needed, which can be achieved through up-regulation of either the pro- or anticoagulant processes. The initiation of procoagulant processes implicitly instigates the initiation of anticoagulation responses, as will be detailed below, by the activation of the proteolytic protein C anticoagulant pathway. The coupling of pro- and anticoagulant responses is of vital importance since whenever procoagulant reactions are not controlled by anticoagulant mechanisms, or when anticoagulant processes are defective, the formation of thrombin will become excessive and the risk for thrombosis will increase.

Despite considerable progress in their diagnosis and treatment, ischemic heart disease and cerebrovascular disease, remain to be the major cause of mortality worldwide, with over 80% of cardiovascular disease deaths now taking place in low- and middle-income countries

and occurring almost equally in men and women (Global Health Observatory (GHO), World Health Organisation [WHO], 2008).

Thromboembolic disease contributes to this overall mortality, as it is implicated in many manifestations of cardiovascular disease. The clinical manifestation of a thrombus is that of the pathological presence of an occlusion in a blood vessel or in the heart, which causes an obstruction of the physiological flow of blood through the circulatory system. Such occlusions can occur in either the venous or arterial part of the vessel tree and the condition is consequently classified as either venous or arterial thrombosis. Despite the fact that in both venous and arterial thrombosis the normal haemostatic balance is disturbed, the two types of thrombosis are considered distinct disease states that each have their own particular molecular pathology and underlying risk factors (Rosendaal, 1999, 2005).

The proper functioning of the anticoagulant protein C pathway is undisputedly required to prevent the occurrence of thrombosis, and in particular venous thromboses, as is most strikingly illustrated for protein C deficiency. Newborns who are homozygous protein C deficient can develop the life-threatening condition of acute onset "purpura fulminans" (Dreyfus et al, 1991, 1995) which can be treated by replacement therapy employing purified protein C concentrates.

Resistance to activated protein C (APC), or APC resistance, the subject of this chapter, has been implicated mostly in pathogenesis of venous thrombosis, despite the fact that the causative FV<sub>Leiden</sub> mutation, which is present in a majority of cases, has been related to myocardial infarction and/or overall coronary disease (Rallidis et al.2003; Segev et al., 2005; Ye et al., 2006). Various clinical studies have been performed to identify the contribution of the anticoagulant protein C pathway to arterial thromboses and stroke and these have not been conclusive in providing clear evidence for the contribution of the protein C pathway to arterial thrombosis (e.g. Folsom, 1999; Hankey, 2001; Boekholdt, 2007). That there is a link between the hemostatic system and arterial thrombosis or atherosclerosis appears clear as it has been established that many complex diseases, such as atherosclerosis, show an extensive crosstalk between inflammation and coagulation, as was recently reviewed by Borisoff and coworkers (Borisoff et al, 2011).

Venous thrombosis (or venous thromboembolism, VTE) is a multifactorial disease (Seligsohn&Zivelin, 1997; Rosendaal, 1999) that affects one in 10,000 individuals under the age of 40 years annually and one in 1,000 individuals over 75 years of age, causing significant morbidity and mortality (Salzman & Hirsh, 1994; Anderson et al., 1991; Nordstrom et al. 1992). The multifactorial character of VTE implies that for the disease to occur often several circumstantial and genetic factors occur simultaneously. Together these factors are capable of tipping the natural haemostatic balance between pro- and anticoagulant forces. Important to note is that environmental and acquired factors are able to modulate the existing genetic risk factors for thrombosis in an individual and these acquired factors, which may be very diverse by nature, are consequently directly involved in the pathogenesis of thrombosis. Thrombosis will develop then when the combined risk factors interact such that the threshold for thrombosis is surpassed. Upon passing of the threshold, the anticoagulant mechanisms are no longer able to counteract the procoagulant forces. Consequently a thrombotic event can occur (Rosendaal, 1999).

Of the inherited risk factors for venous thrombotic disease, most are found in the protein C anticoagulant system which will be detailed below. Examples of these are resistance to activated protein C (APC) caused by the FV<sub>Leiden</sub> mutation (FV R506Q) and the deficiencies of the cofactor, protein S, and deficiency of protein C itself (Segers et al., 2007). Of the

acquired risk factors, use of oral contraceptives, pregnancy/puerperium, the presence of antiphospholipid antibodies, immobilization, surgery, malignancies and trauma are amongst the most studied. Furthermore, age and sex are recognized as independent contributing factors in the pathogenesis of venous thrombosis (Bertina, 2001).

In this chapter resistance to APC, the central protein of the protein C anticoagulant pathway, will be discussed, both for genetically determined forms of APC resistance and acquired types of APC resistance. Particular attention will be given to the molecular events that occur during the APC-catalyzed down-regulation of thrombin formation in normal individuals, since knowledge of these processes is pivotal in our understanding of the causative role of APC resistance in the occurrence of thromboembolic disease.

## 2. Protein C anticoagulant pathway

During homeostasis several anticoagulant systems balance the procoagulant forces that act in human blood, thereby preventing excessive platelet and fibrin depositions. A major inhibitory buffer is formed by the circulating inhibitors, which include antithrombin, alpha-2-macroglobulin, antitrypsin or tissue factor pathway inhibitor (TFPI) which provide the necessary negative feedback to procoagulant forces. These circulating inhibitors target the activated forms of the serine proteases from the coagulation cascade like thrombin and FXa and act by complex formation with their target proteases, resulting in a loss of proteolytic activity and clearance of the active protease from circulation.

Key regulators in the amplification of thrombin formation are however the non-enzymatic cofactor proteins factor V (FV) and factor VIII (FVIII) and by virtue of the absolute requirement of their activity for normal thrombogenesis, they are obvious targets for the attenuation of thrombin formation. Given that these molecules lack proteolytic activity, they cannot be inactivated via complex formation but instead they are targeted by proteolytic inactivation, resulting in disintegration of the protein structure and concomitant loss of cofactor activity. The main proteolytic enzyme responsible for the inactivation of activated FV (FVa) and FVIII (FVIIIa) is the activated form of protein C.

Low levels of activated protein C circulate, but for a sufficient anticoagulant response to occur, an upregulation of protein C is required through a specific set of molecular events, which together are described as the protein C anticoagulant pathway. The main player in this pathway is protein C. Protein C is a vitamin K dependent zymogen belonging to the class of chymotrypsin-like serine proteases (Stenflo, 1976). Being a zymogen, protein C circulates at a plasma concentration of ~65 nM in humans as an inactive pro-enzyme which requires processing in order to obtain its enzymatic activity. A pivotal role in the initiation of the protein C pathway is the formation of a complex between thrombin and thrombomodulin (Fuentes-Prior et al, 2000), see Fig. 1 below. Note that it is thrombin, the very enzyme that ultimately needs to be downregulated, that initiates its own attenuation. Thrombomodulin is a transmembrane protein that is present on the undamaged endothelium, in particular the endothelium of the smaller blood vessels. The membrane-bound thrombomodulin is able to capture thrombin by binding to the exosite I of thrombin upon which the thrombin-thrombomodulin complex is formed. The exosite I of thrombin is primarily involved in procoagulant interactions of the enzyme, namely in the recognition and activation of fibrinogen, FV and FVIII (Lane & Caso, 1989). After binding of thrombomodulin to the thrombin exosite I the procoagulant properties of any thrombin molecules that have migrated from a site of ongoing coagulation and are transported into

the microvasculature, are lost, thereby thrombomodulin is anticoagulant in itself. However, not only are the procoagulant properties of thrombin inhibited, given the fact that the active site of thrombin is still available, a conformational change that is accompanied by the binding to thrombomodulin causes the local active site structure of thrombin to change. This structural change alters the substrate specificity of thrombin (Fuentes-Prior et al, 2000; Dahlbäck & Villoutreix, 2005) such that thrombin is transformed from a procoagulant into an anticoagulant protein. In the anticoagulant state, thrombin is able to efficiently activate protein C by removal of a 12 amino acid activation fragment from the serine protease domain of protein C. For optimal activation of protein C by thrombin furthermore the presence of the endothelial cell protein C receptor (EPCR) is required.

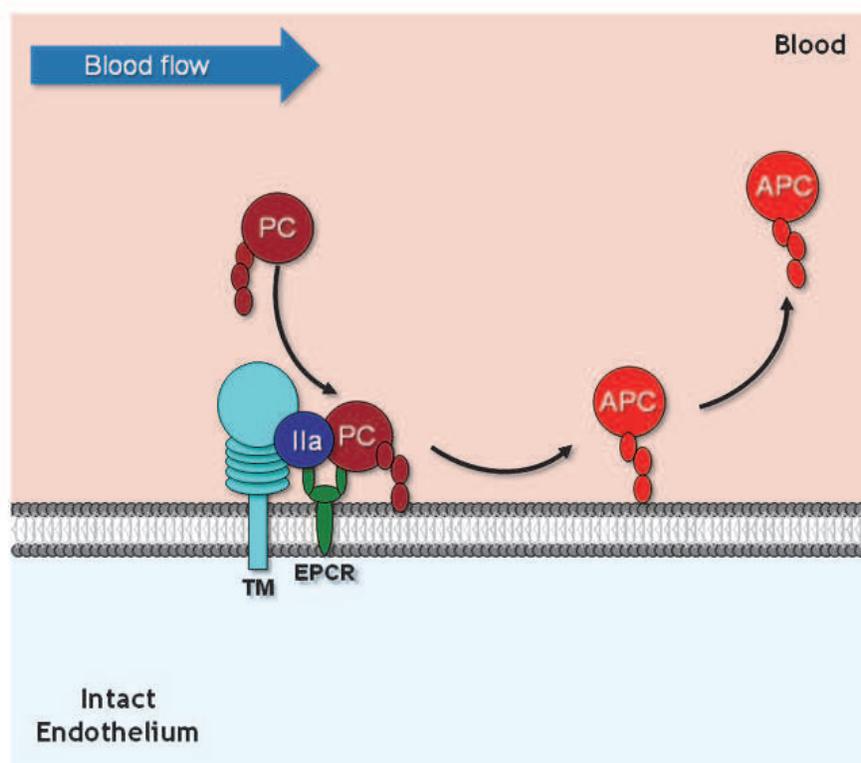


Fig. 1. Activation of protein C by thrombin. When bound to thrombomodulin (TM), in the presence of the endothelial cell protein C receptor (EPCR), thrombin (IIa) is able to activate protein C (PC) to activated protein C (APC).

The physiological requirement for the presence of both thrombomodulin and EPCR is most strikingly evidenced by the early embryonic lethal phenotypes being associated with deficiency of either of the two proteins in mice (Fukudome & Esmon, 1994; Esmon, 2001).

In the protein C pathway, a number of factors are important for the full expression of APC anticoagulant properties. These include the presence of the cofactor proteins, protein S and coagulation factor V (FV) and in addition a suitable membrane surface onto which both APC and its substrates can assemble in the presence of calcium.

Like protein C, protein S is also a vitamin K dependent coagulation factor, which however is devoid of enzymatic activity as it does not contain a catalytic domain. FV, the procofactor protein, has a dual function in coagulation and has been described as a “Janus faced” protein (Nicolaes & Dahlbäck, 2002), with properties in both the pro- and anticoagulant

pathways. In the APC-catalyzed inactivation of FVIIIa (see also below) FV acts in synergy with protein S as a cofactor to APC (Shen & Dahlbäck, 1994; Varadi et al, 1995). Somewhat conflicting results were obtained whether or not FV is also a cofactor in the inactivation of FVa. Though extremely difficult to study in purified reaction systems, this issue was recently addressed (Cramer et al, 2010) and it was concluded that FV expresses activity in FVa inactivation.

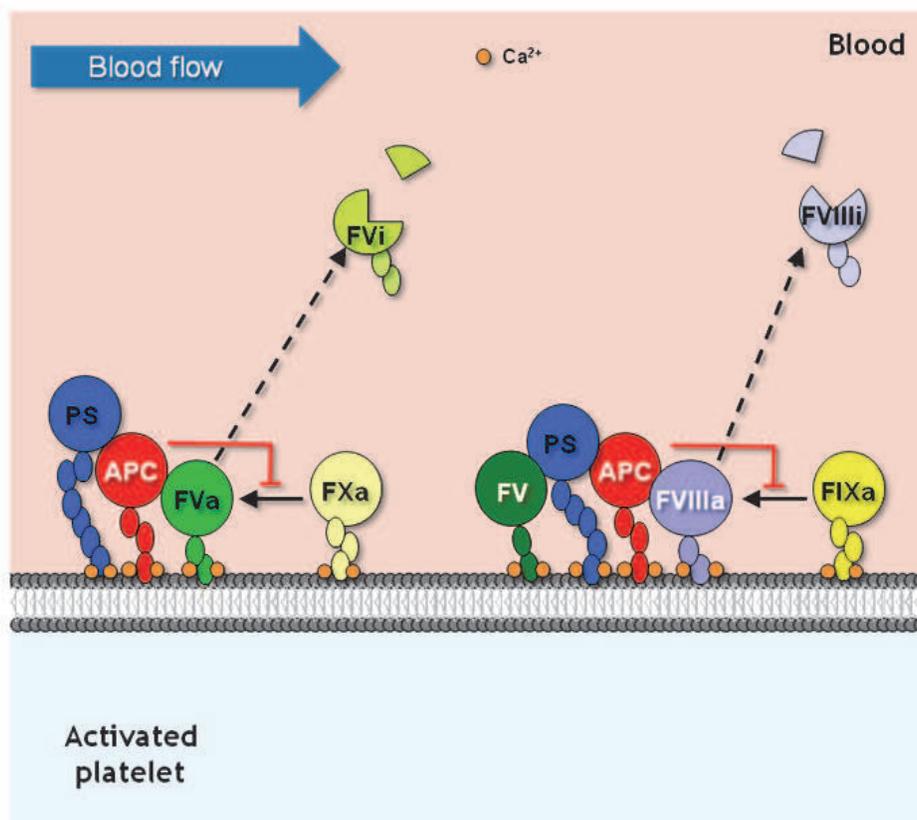


Fig. 2. APC catalyzed inactivation of FVa and FVIIIa. Left: APC, together with its cofactor protein S (PS) proteolytically inactivates factor Va (FVa) resulting in a loss of protein integrity and concomitant loss of cofactor activity. As a result, complex formation between FVa and factor Xa (FXa) is not possible, leading to lowered conversion of prothrombin by FXa. Right: analogous to regulation of FVa activity, factor VIIIa (FVIIIa) is cleaved by APC, resulting in dissociation of FVIIIa units and loss of activity. Factor V, FV. Factor IXa, FIXa.

In the absence of protein C, individuals are at high risk for the development of thrombosis, such as in the case of the classical homozygous protein C deficiencies that are seen in neonates where purpura fulminans develop (Dreyfus et al, 1991, 1995). This indicates the important function that the protein C pathway has, in particular in the microcirculation. Not only the deficiencies of the zymogen protein C, also those of the cofactor protein S are associated with a prothrombotic state. Deficiency of the other APC cofactor, FV, is by itself not associated with thrombosis, which indicates that, particularly at low FV levels, the procoagulant properties of this coagulation protein are dominant (Govers-Riemslog et al, 2002; Duckers et al, 2009). There are however reports in literature in which cases were described where individuals who developed autoantibodies against FV have suffered from

thrombosis rather than from bleeding problems (Ortel, 1999). It was hypothesized that in these cases antibodies may specifically target the anticoagulant properties of FV.

### 3. Structure and function of coagulations factors V and VIII

FV and FVIII are large plasma glycoproteins, primarily synthesized in hepatocytes, with relative molecular masses of ~330 kDa that share a common architecture. Both proteins have a mosaic domain structure of A1-A2-B-A3-C1-C2 (Fig. 3). FV and FVIII share a common ancestral gene and are consequently structurally related, with 40% amino acid sequence identity in their A and C domains. The B-domain that connects the A1-A2 heavy chains and the A3-C1-C2 light chains are much less (~15%) conserved. Both FV and FVIII are heavily glycosylated, and the presence of the glycans is required for a proper folding and functioning of the cofactor proteins (Nicolaes et al, 1999; Yamazaki et al, 2010).

The activated forms of FV and FVIII, called FVa and FVIIIa respectively, are required for full expression of activity by the prothrombinase and intrinsic tenase complexes respectively. In fact, FVa and FVIIIa are essential non-enzymatic cofactors: in the absence of the cofactors the prothrombinase and tenase complex are virtually inactive.

To protect FVIII and to prevent premature expression of its cofactor activity, FVIII circulates in plasma in complex with von Willebrand factor (VWF), whereas FV in plasma is in free form (Weiss, 1977). In thrombocytes however, FV is bound to multimerin 1 (MMRN1), a large protein much like VWF, that protects FV from expression of its activity and presumably from intracellular degradation (Hayward, 1995). Upon activation of FV, the binding affinity of multimerin for FVa is decreased slightly, which will allow dissociation of the multimerin-FVa complex (Jeimy, 2008). A similar mechanism, modification of affinity by activation, has also been described for the binding between FVIII and VWF (Lollar, 1988). The circulating procofactors FV and FVIII possess negligible activity and for them to gain activity, they need to be proteolyzed. During activation, the large B-domain will be excised from the molecule. In fact it has been shown for FV that the presence of the B-domain itself is the reason that the cofactor is not active and that proteolysis is needed to eliminate steric and/or conformational restraints that are imposed on the cofactor by the B-domain. A lifting of these restraints by removal of the B-domain allows the availability of discrete binding interactions between FVa and its binding partners FXa and prothrombin (Kane, 1990; Keller 1995; Toso, 2004). Activation of FV and FVIII is essentially a positive feedback reaction since the potential activators are alpha-thrombin (Kane et al, 1981; Suzuki et al, 1982), meizothrombin (Tans et al, 1994), FXa (Monkovic & Tracy, 1990), FXIa (Whelihan et al, 2010) and tissue factor-FVIIa (Safa et al, 1999). Of these, activation by alpha-thrombin, the very enzyme that is produced by the prothrombinase complex, is regarded as most important.

The cleavages by thrombin and FXa are indicated in Fig 3. For both cofactors these result in the release of the B-domain, a process that is accompanied by expression of cofactor activity. The molecular weight of the FVa light chain is not unique due to incomplete N-glycosylation at Asn2181 in the C2 domain (Nicolaes et al, 1999; Kim et al, 1999). As a result of this variable glycosylation, two different forms of FV are present in human blood. These two glycoisoforms express different activities in both pro- and anticoagulation pathways (Váradi et al 1996, Hoekema et al, 1997) and glycosylation of the FVa light chain, more precisely the N-linked glycosylation at Asn2181, has been implicated in the pathogenesis of venous thrombosis (Yamazaki et al, 2002, 2010).

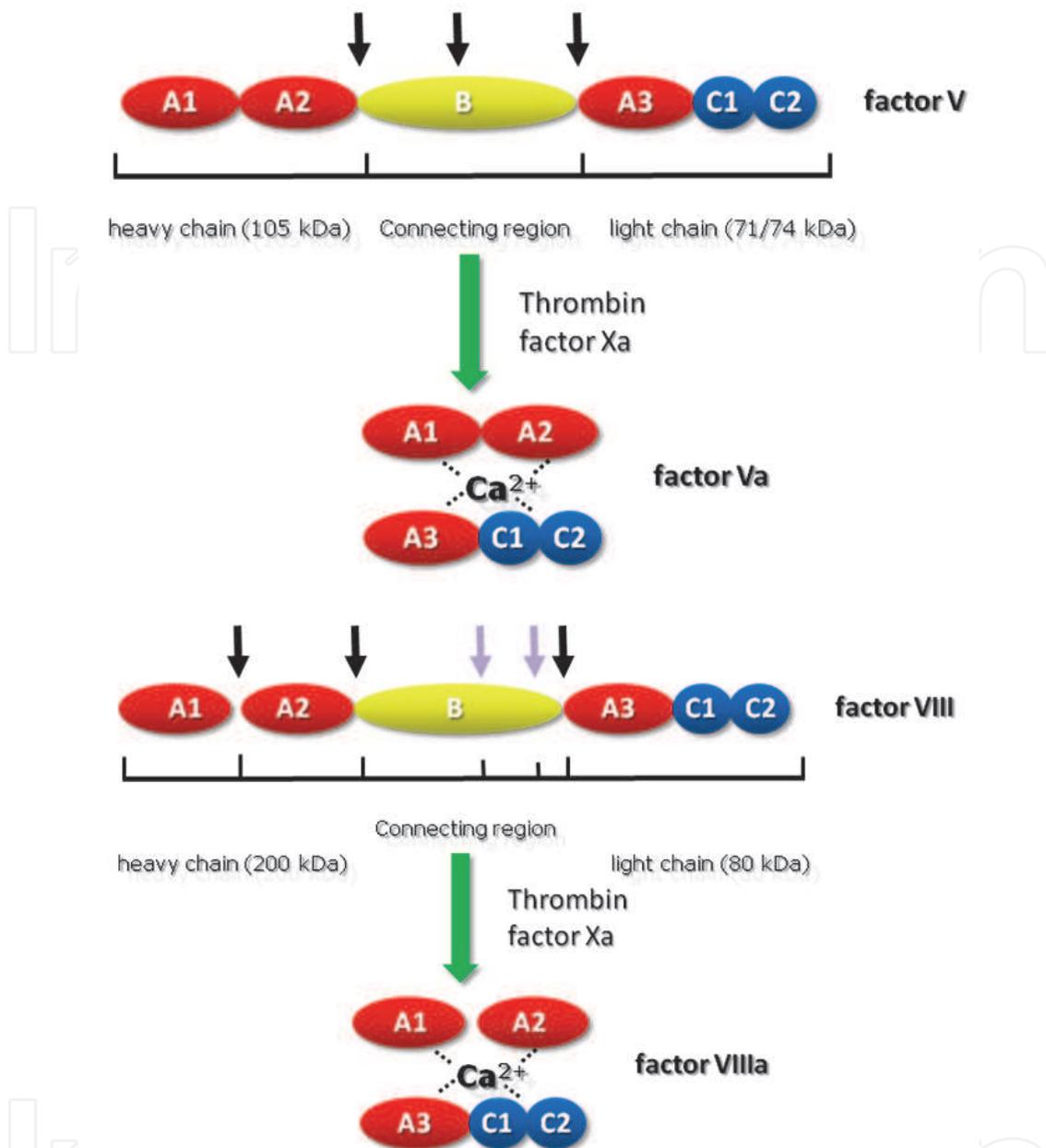


Fig. 3. Activation of FV and FVIII. The domain structures of FV (top) and FVIII (bottom) are indicated. FV is activated by thrombin or FXa at Arg709, Arg1018, and Arg1545, as indicated by the black arrows. The active cofactor is formed by the association of the A1-A2 heavy chain with the A3-C1-C2 light chain via calcium-dependent noncovalent bonding. FVIII is cleaved at residues Arg1313 and Arg1648 (upper arrows in purple) upon secretion from the cell, yielding a 200-kDa fragment (also referred to as the heavy chain, consisting of the A1, A2 and part of the B-domain) and an 80-kDa light chain. FVIII thus circulates as a dimer. Activation of FVIII by thrombin or FXa occurs through proteolytic cleavage at Arg372, Arg740, and Arg1689 (black arrows), yielding the heterotrimeric FVIIIa, consisting of a 50-kDa A1 domain-derived polypeptide, a 43-kDa A2 domain-derived polypeptide, and the 73-kDa A3-C1-C2-derived light chain, which are noncovalently associated via divalent metal ions.

The function of the cofactors FVa and FVIIIa is, like their structure, very similar. Both proteins express their cofactor activities when assembled in a membrane-bound complex that furthermore comprises a serine protease (FXa and FIXa respectively) and a zymogen-substrate (prothrombin and FX respectively). A functional complex is only formed in the presence of calcium. Calcium is needed for the Gla-domains of the vitamin K dependent proteins involved to reach their calcium-induced active conformation (Huang et al, 2004) and furthermore for the occupation of the single calcium binding sites in FVa and FVIIIa, which are necessary for expression of cofactor activity.

Involvement of the cofactor protein Va and VIIIa increases the  $V_{max}$  of the prothrombinase and tenase complex respectively, by several orders of magnitude. This implies that the presence of FVa or FVIIIa is essential for the formation of thrombin or FXa under physiological conditions (Nesheim et al, 1979; Rosing et al, 1980, van Diejjen, 1981).

#### 4. Regulation of FVa and FVIIIa activities

Given their potency and essential character, it is of prime importance for homeostasis that the activities of FVa and FVIIIa are tightly regulated. As mentioned above, the main proteolytic process responsible for FVa/FVIIIa regulation is limited proteolysis by the serine protease activated protein C (APC).

The inactivation process occurs much in analogy to the activation described before: APC targets its substrates at multiple but specific cleavage sites, provided that both the substrate and the enzyme are bound to a membrane surface (Kalafatis et al, 1994; Nicolaes et al, 1995, Egan et al, 1997, Barhoover & Kalafatis, 2011). In the absence of a lipid surface, reactions occur too slowly to be physiologically relevant (Bakker et al., 1992; Nicolaes et al, 1995). Cleavage at each of the cleavage sites is characterized by its own kinetic parameters and since there is no specific order of cleavages, the rates of the cleavage reactions are being determined mostly by the local concentrations of FVa, APC and any cofactors or modifiers present. Though cleavages are random, the reaction products formed after cleavage at each of the single cleavage sites express different residual cofactor activities. For FVa this means that where cleavage at Arg306 results in a complete loss of protein integrity, cleavage at Arg506 results in a reaction product that has considerable remaining cofactor activity, an activity which will depend on the concentrations of other reactants present (e.g. FXa, Nicolaes et al., 1995).

At a concentration around or lower than the plasma concentration of FV (21 nM) the cleavage at Arg506 is the preferred cleavage however, being ~20 fold faster than cleavage at Arg306. The inactivation of FVa is enhanced by the presence of protein S which selectively appears to stimulate the slower cleavage at Arg306 by a factor of 20 (Walker, 1981; Rosing et al, 1995), the dominant cleavage at Arg506 is only stimulated 2-fold by protein S (Rosing et al, 1995; Norstrom et al, 2006). Interesting to note in this respect is a recent finding that protein S, which circulates in both a free form and in complex with C4b binding protein (C4BP) has different effects on APC-catalyzed FVa inactivation, depending on whether it is free or not. Whereas it had prior been deemed that only free protein S is active as a cofactor to APC, it was shown that also the protein S-C4BP complex is able to stimulate the Arg306 cleavage in FVa more than 10-fold, while cleavage at Arg506 is inhibited 3- to 4-fold (Maurissen et al, 2008). In the absence of protein S, FVa when incorporated in the prothrombinase complex, is protected from inactivation by APC. FXa specifically protects FVa from cleavage at Arg506 (Rosing et al, 1995; Norstrom et al, 2006), whereas

prothrombin has no preferred protection and attenuates the cleavage at both Arg306 and Arg506 (Rosing et al, 1995; Smirnov et al, 1999; Tran et al, 2008).

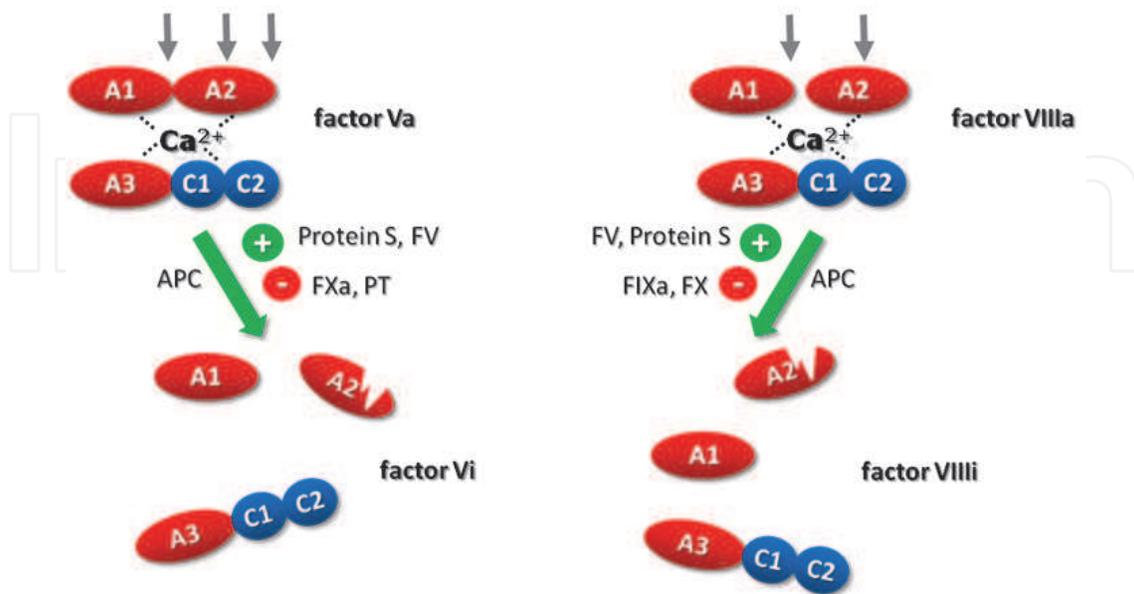


Fig. 4. APC-catalyzed inactivation of FVa and FVIIIa. FVa Inactivation proceeds primarily via cleavages after residues Arg306 and Arg506 and to a lesser extent at Arg679 in the heavy chain domain (Kalafatis et al, 1994; Nicolaes et al, 1995) (upper grey arrows). In FVIIIa, APC targets the peptide bonds after Arg336 and Arg562 (Fay et al, 1991), (upper grey arrows). For FVa, cleavage at Arg506 is preferred over that at Arg306. Full loss of activity requires cleavage at Arg306. Complete cleavage by APC then results in a loss of protein integrity, generating inactivated FVa, FVi, and inactivated FVIIIa, FVIIIi. The disintegration is accompanied by a loss of protein activity. Cofactors that influence the reactions are indicated: protein S and FV are able to enhance the cleavages in both FVa and FVIIIa, whereas FXa and prothrombin (PT) are able to protect FVa from inactivation by APC. Likewise, FIXa and FX are protective for FVIIIa.

A quantitative explanation for the protection by FXa has recently been given, since both APC and FXa bind with similar affinity to similar/overlapping binding regions on the surface of FVa and thus are in direct competition for complex formation with FVa (Nicolaes et al, 2010). Interesting in this respect is the observation that APC, when bound to FVa can completely but reversibly inhibit the activity of FVa, even in the absence of irreversible cleavage of FVa by APC (Nicolaes et al, 2010).

APC-mediated cleavages in FVIIIa occur at Arg336-Met337 and Arg562-Gly563 (Fay et al, 1991) and are, like is the case for FVa, not ordered but rather determined by kinetic parameters and local concentrations of FVIIIa, APC and other proteins that may modify the reaction kinetics (Fig. 4, upper grey arrows). This means that Arg336 is usually cleaved first and, very similar to FVa, a secondary cleavage at Arg562 is required for a complete loss of activity (Varfaj et al, 2006; Gale et al., 2008). The relevance of APC-catalyzed inactivation of FVIIIa is not undisputed since, in contrast to FVa, FVIIIa is not stable and its activity is subject to rapid decay caused by dissociation of the A2 domain from the rest of the molecule. Note that FVIIIa is a heterotrimer, with the A1 domain being separated from the

A2 domain. It has been estimated that the majority of FVIIIa activity (70-80%) is lost spontaneously (Lollar et al, 1990).

Moreover, the affinity of APC for FVIIIa was estimated to be ~100-fold lower than the affinity of APC for FVa (Nicolaes et al, 2010), which may indicate the lesser importance of APC-catalyzed FVIIIa inactivation, especially if the 100-fold lower plasma concentration of FVIII, as compared to FV, is taken into account. When FVIIIa is incorporated in the tenase complex however, FVIIIa is much more stable and a role for FVIIIa regulation by APC becomes more evident. Like is the case for FVa, when incorporated in the prothrombinase complex, FVIIIa is protected from APC-catalyzed inactivation not only by increased stability of the FVIIIa heavy chain, FIXa and FX have been reported to selectively protect FVIIIa from cleavage at Arg336 and Arg562 (O'Brien et al, 2000).

APC-catalyzed FVIIIa inactivation is specifically enhanced by the synergistic cofactors protein S and FV. In FVIIIa, cleavage at Arg562 is most pronouncedly enhanced in the presence of protein S, though FV and protein S stimulate both APC cleavage sites in FVIIIa (Shen & Dahlbäck, 1994; Varadi et al, 1996; Lu et al, 1996; Gale et al, 2008). Resultingly, when both protein S and FV are present, cleavage at Arg336 and Arg562 occurs at similar rates in FVIIIa (Gale et al., 2008).

## 5. APC resistance: First observations

In 1993, a first report (Dahlbäck et al, 1993) was published in which three different families were described that presented an abnormal anticoagulant response to APC when the plasma of family members was tested in a classical activated partial thromboplastin time (APTT). In the plasma of normal individuals a prolongation of the APTT will occur when APC is added. However, for certain family members of the families studied, this prolongation was observed to be much less than for other family members or normal controls. The plasmas showed a poor response to APC and the term "APC resistance" was coined. APC resistance was found to be an inheritable trait that was hypothesized to be caused by a defective function of a hitherto unknown cofactor to APC (Dahlbäck & Hildebrand, 1994). A surprisingly large proportion of thrombophilic patients, viz. 20-60%, proved to be resistant to APC and thus the new discovery attracted broad scientific interest (Griffin et al, 1993; Koster et al, 1993; Svensson & Dahlbäck, 1994; Halbmayer et al, 1994).

To discover a molecular mechanism for APC resistance, attempts were made to isolate the unknown cofactor from normal plasma and this revealed that this factor was FV (Dahlbäck & Hildebrand, 1994). Addition of FV to an APC resistant plasma sample could normalize the response to APC. This was the first evidence that FV is not only a procoagulant protein, but these experiments established FV as well as an anticoagulant protein. Soon after, the involvement of the FV gene was confirmed. In 1994 several research groups succeeded simultaneously in the identification of the molecular cause for APC resistance by a thorough study of the FV gene of APC resistant individuals. The cause was identified as a single nucleotide polymorphism (SNP) at position 1691 which codes for a missense mutation at Arg506, replacing the arginine by glutamine, exactly at one of the APC cleavage sites in FVa. The FV gene containing the Arg506 mutation was since then described as FVLeiden, FVR506Q or FV:Q506. (Bertina et al, 1994; Zöller et al, 1994; Voorberg et al, 1994; Greengard et al, 1994).

With a genetic cause unveiled and relatively easy DNA sequencing technologies becoming increasingly available, the allelic frequencies of the FVLeiden mutation was studied in

various patient and ethnic populations. The FVLeiden mutation was found in ~95% of families with APC resistance, which makes FVLeiden the major cause of hereditary APC resistance (Zöller et al, 1994). The FVLeiden mutation is very common in general populations though it is found exclusively in populations of Caucasian descent (~5% of Europeans are carrier of the mutation) and the high prevalence implied that, at the time, inherited APC resistance was 10 times more prevalent than the sum of all other hereditary causes of thrombophilia known (Rees et al, 1995, 1996). The FVLeiden mutation is known as the most common hereditary causal factor for thrombosis, by virtue of the APC resistance it causes in its carriers.

## 6. The molecular basis of FVLeiden related APC resistance

The role that the FVLeiden mutation, i.e. the replacement of arginine by a glutamine at position 506, has in the etiology of APC resistance been well studied. Several mechanisms contribute to the explanation of the prothrombotic tendency that is present in carriers of the mutation. First, given that the mutation abrogates the preferred mutation at Arg506, this means that one of the prime APC cleavage sites is lost in FVaLeiden. The absence of a cleavage site will impair efficient downregulation of procoagulant FVa activity (Kalafatis et al, 1994, Nicolaes et al, 1995). Second, it was discovered that for FV to act as a cofactor in the APC-catalyzed inactivation of FVIIIa, it must not be cleaved by thrombin (more precisely, the C-terminal region of the B domain must be intact) and furthermore, FV should be cleavable at Arg506 (Thorelli et al, 1998, 1999). This implies that FVLeiden, is not a cofactor in the inactivation of FVIIIa by APC, since it cannot be transformed into an anticoagulant molecule (Varadi et al, 1996). Third, it was found that APC also possesses a proteolysis-independent anticoagulant activity (Gale et al, 1997; Nicolaes et al, 2010). By virtue of its binding to FVa, thereby effectively competing with FXa for prothrombinase complex formation, APC is able to down-regulate thrombin formation in the absence of FVa cleavage. It was estimated that the non-enzymatic anticoagulant effect accounts for ~6% of the overall APC activity. In the case of FVaLeiden however, APC is not able to bind to the FVa region around the most favored cleavage site at Arg506 and consequently APC cannot regulate the activity of FVaLeiden via the proteolysis-independent mechanism.

Taken together, the FVLeiden mutation has an effect both on the inactivation of FVa and of FVIIIa, the two cofactors that are essential to thrombin formation, and the inactivation of which was shown to be both contributing to APC resistance in the plasma of FVLeiden carriers (Castoldi et al, 2004).

The *in vivo* effects of FVLeiden are perhaps most strikingly illustrated in the APC resistance phenotype that is observed in the plasma of so-called pseudo-homozygous APC resistant individuals. The individuals are genotyped as heterozygous FVLeiden carriers. However, their phenotype is that of a homozygous FVLeiden carrier. Due to a null-mutation in their normal FV allele, the normal FV is lacking in these individuals and only the FVLeiden allele is expressed (at ~50% of a normal FV level). The associated thrombosis risk in pseudo-homozygotes is in the same range as that of homozygous carriers of the FVLeiden mutation (50- to 80-fold increased). When purified FV is however added to pseudohomozygous plasma, the response to APC is corrected such that it will reach the same range as that of heterozygous carriers of the FVLeiden mutation. Heterozygous carriers however have an associated risk of thrombosis that is ~5-fold higher than normals. The increased risk of thrombosis in homozygous and pseudohomozygous carriers of the FVLeiden mutation

therefore appears not so much to be caused by a defect in the FVLeiden that is present, more likely it illustrates the absence of the anticoagulant normal FV (Simioni et al, 1996, 2005; Castoldi et al., 2004; Brugge et al, 2005).

## 7. Modifiers of the APC resistance phenotype

The discovery of the FVLeiden mutation boosted the research into APC resistance and APC resistance as such was established as an independent risk factor for thrombosis (de Visser et al, 1999) even when the FVLeiden mutation was not present. Research showed that in 10-15% of individuals who are APC resistant (as determined via an APTT-based assay), the FVLeiden mutation is not present (Toralunga et al, 2004; Tosetto et al, 2004). This implies that besides FVLeiden, other factors exist that may modify the outcome of an APC resistance assay. These modifying factors of the APC resistance phenotype can be roughly divided into genetic and acquired factors and of the genetic factors, those that originate from the FV gene have been best studied.

Important to mention is the fact that, since APC resistance is diagnosed according to the function of APC in plasma, the very test that is used to determine the presence of APC resistance is of influence as to whether a certain individual is described as APC resistant or normal. This is illustrated by the observation that in the endogenous thrombin potential (ETP) -based APC resistance assay (Nicolaes et al, 1997), most of the non-FVLeiden APC resistant samples are caused by an abnormal female hormonal status (as in pregnancy, hormone replacement therapy or oral contraceptive (OC) use) (Rosing et al, 1997; Curvers et al, 2002). In the APTT-based APC resistance assays other factors besides OC use and pregnancy were found to be prevalent among cases on non-FVLeiden APC resistance. These include high FVIII levels, elevated prothrombin levels, malignancy and the presence of lupus anticoagulants (Henkens et al, 1995; Cumming et al, 1995; Laffan & Manning, 1996; Aznar et al, 1997; Tosetto et al, 1997; de Visser et al, 1999; Castaman et al, 2001; Tosetto et al, 2004; Toralunga et al, 2004; Sarig et al, 2005).

Several allelic variants of the FV gene have been described that contribute to the APC resistance phenotype and to venous thrombosis. FV Cambridge (Williamson et al, 1998) and FV Hong Kong (Chan et al, 1998) were both discovered in thrombosis patients. One of the predominant APC cleavage sites, at Arg306, is replaced in both these FV variants as a result of a missense mutation in the FV gene. In FV Cambridge Arg306 is replaced by Thr, and in FV Hong Kong Arg306 is replaced by a Gly residue. Interestingly, whereas FV Cambridge was discovered in the plasma of an individual with unexplained APC resistance, FV Hong Kong was not associated with APC resistance. This phenotypic difference has thus far not been explained. Investigations with recombinant FV variants that mimic FV Hong Kong and FV Cambridge have shown that both recombinant FV variants have only a slightly decreased APC response in a plasma system with an *in vitro* APC resistance phenotype being intermediate between those of normal FV and FVLeiden (Norstrom et al, 2002). Epidemiological studies have shown that FV Hong Kong is not a risk factor for venous thrombosis whereas more data on the rare FV Cambridge condition are needed to establish whether or not this FV genotype is associated with venous thrombosis.

The FV2 haplotype is characterized by several linked mutations (both missense and silent) in exons 4,8,13, 16, and 25 of the gene for FV associated with slightly reduced FV levels. It has a high incidence in the general population (10-15%) (Bernardi et al, 1997; de Visser et al, 2000). Especially when present in combination with FVLeiden, R2 FV may enhance the APC

resistance phenotype (the majority of circulating FV will be FVLeiden) and increase the risk of thrombosis (Faioni et al, 1999) Moreover, carriers of the R2 allele seem to have increased amounts of FV1 in their plasma (Castoldi et al, 2000; Hoekema et al, 2001). FV1 is a glycosylation isoform of FV that may be more thrombogenic than the other isoform, FV2 (Hoekema et al, 1997). Where the reduced FV levels in R2 FV carriers can be attributed to the Asp2194Gly mutation (Yamazaki et al, 2002, 2010) it remains questionable whether the R2 FV molecule itself is APC resistant.

Another FV-related cause for APC resistance is the so-called FVLiverpool. In this variant, which was found in two related individuals with severe thrombosis at a young age, Ile359 has been replaced by Thr (Mumford et al, 2003; Steen et al, 2004). This missense mutation introduces a novel site for N-linked glycosylation at Asn357. Due to the presence of an extra glycan structure, APC-catalyzed inactivation at Arg306 is hampered by steric hindrance. Like in the case of FVLeiden, FVLiverpool is not active as a cofactor to APC in the APC-catalyzed inactivation of FVIIIa such that the mutation affects both inactivation of FVa and FVIIIa.

Besides these mentioned mutated FV variants, also autoantibodies have been reported to be associated with APC resistance. In three cases, these antibodies were directed against FV (Ortel, 1999), however also antibodies against protein S (Nojima et al, 2009) and APC (Zivelin et al, 1999) have been described in this context. An exact causal mechanism for the thrombosis in these cases is not known, but may involve the broader context of an anti-phospholipid syndrome. In addition, the coverage of epitopes on the surface of FV, that are in particular important for the FV anticoagulant functions, have been suggested.

Given that APC resistance is diagnosed from plasma samples and knowing that human plasma contains many proteins that contribute to the functional assay outcome, it will be conceivable that APC resistance as such cannot be attributable to a single cause. Whether or not acquired factors such as pregnancy, malignancy, oral contraceptives or hormone replacement are involved, a final outcome of the interaction of genetic and acquired factors is the potential change of several important coagulation factors. A change in the level of these coagulation factors, and in particular, prothrombin, protein S, FVIII or tissue factor pathway inhibitor (TFPI) may influence assay outcome and render a plasma sample APC resistant (de Visser et al, 2005).

## 8. Genetic and acquired interactions determine thrombosis risks

Given that thrombosis is a multi-factorial disease, several factors can work in concert so as to disturb the haemostatic balance (Seligsohn & Zivelin, 1997; Rosendaal, 1999). Whether or not the presence of APC resistance, with its high prevalence in the general population, will result in a thrombosis, is dependent on the interplay between the various factors that influence the haemostatic balance in an individual (Martinelli, 2001). The contribution of inherited risk factors to the total risk for thrombosis development was estimated over 60%, and of these, the FVLeiden mutation is considered the most important by virtue of its very high prevalence.

Risk factors may show synergism in the events that cause a thromboembolic episode as was concluded from several studies where it was found that the prevalence of the FVLeiden mutation was much higher in thrombotic families with antithrombin, protein C or protein S deficiencies or with the HR2 haplotype or the prothrombin G20210A mutation, than in the general population. Not only is the prevalence of the FVLeiden mutation higher than

expected, also the combined risks for thrombosis in these families are much higher than what would be concluded from the sum of the risks associated with the single thrombophilic defects present (Koeleman et al, 1995; Gandrille et al, 1995; van Boven et al, 1999; Faioni et al, 1999; Salomon et al, 1999).

Not only gene-gene, also gene-environment (which is interpreted as interactions between a genetically determined risk factor and an acquired risk factor) contribute significantly to the overall risk for thrombosis. This type of interaction includes interactions between FVLeiden and the antiphospholipid syndrome (Simantov et al, 1996), or those between FVLeiden and long-distance travel or immobilization (Cannegieter et al, 2006; Schreijer et al, 2010).

group		Risk*	RR
FVLeiden	OC use		
no	no	0.8	1.0
no	yes	3.0	3.7
yes	no	5.7	6.9
yes	yes	28.5	34.7

\*Indicated are annual thrombosis risks (Risk) per 10,000 individuals and the related relative risk (RR) the data were obtained from Vandenbroucke et al, 1994

Table 1. Interaction between carriership of the FVLeiden mutation and OC use

The most studied interaction however, in this respect, is that between carriership of the FVLeiden mutation and the use of oral contraceptives (OC) (Vandenbroucke et al, 1994, 2001; Wu et al, 2005; van Hylckama Vlieg, 2009). Given the world-wide use of OC, this is an interaction of great importance. As is illustrated by Table 1, the relative risk for thrombosis is ~ 5 fold higher in FVLeiden carriers who use OC than in those who do not use OC. Interaction between the risk factors, both having effects on the protein C anticoagulant system, is the likely cause for the overall multiplicative risk which is higher than the sum of the individual risks. This is illustrated by the various changes in coagulation parameters that have been associated to the use of OC or pregnancy: lowering of protein S, rise in prothrombin levels, lowering of FV levels, rise in FVIII levels, a rise in the levels of FIX and FX and a decrease in the TFPI levels. Each of these changes can have an effect on the coagulability of the blood (Tchaikovsky & Rosing, 2010), which overall changes the APC resistance phenotype.

## 9. Conclusion

The protein C pathway is vital for a normal haemostatic balance in that it down-regulates thrombin formation by inactivation of the non-enzymatic cofactor molecules of the prothrombinase enzyme complex. Resistance to APC, or "APC resistance", is a functional defect of the protein C anticoagulant pathway, characterized by a reduced responsiveness of plasma to the addition of APC. Several factors, both genetic or acquired, can act in concert and result in an APC resistant phenotype. In a great majority of cases, the presence of the FVLeiden, a widespread hereditary variation of the gene product of FV, is involved. FVLeiden contributes to APC resistance in multiple ways, affecting both the inactivation of FVa and of FVIIIa. Given its high penetrance in the general population, the simultaneous occurrence of FVLeiden and other risk factors for thrombosis is common. Knowledge about

the interactions between various risk factors and the underlying mechanisms that result in the onset of thrombosis is vital to our understanding, diagnosis and treatment of thrombosis.

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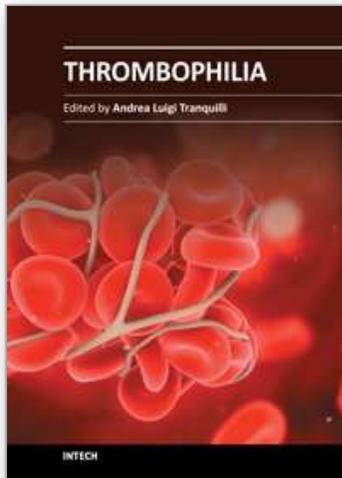
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## **Thrombophilia**

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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-gynecologists 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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Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821

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