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REVIEW

Blood coagulation and its regulation by anticoagulant pathways: genetic pathogenesis of bleeding and thrombotic diseases

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Platelet-mediated primary haemostasis and blood coagulation have evolved as important defence mechanisms against bleeding. The formation of the platelet plug provides the initial occlusion of the vascular lesion. This is temporally co-ordinated with the activation of the coagulation system, which occurs in response to the rupture of endothelium and the exposure of blood to the extravascular tissue. The reactions of blood coagulation are carefully controlled by several anticoagulant mechanisms and under normal conditions they prevail over the procoagulant forces. Genetic or acquired disturbances of the natural balance between the pro- and anticoagulant systems may result in bleeding or thrombotic diseases.

Keywords: APC resistance, factor V Leiden, haemophilia, protein C, protein S, von Willebrand's disease.

Primary haemostasis mediated by platelet-protein interactions

Primary haemostasis events are triggered in response to damage of the vascular wall by the exposure of blood to subendothelial tissue. A multitude of coordinated interactions amongst tissue components, plasma proteins, and receptors on platelets result in the initial sealing of the damaged area. Platelets undergo a series of reactions such as adhesion, aggregation, release of granule content, and morphological changes that lead to the formation of the platelet plug [1, 2]. The primary platelet adhesion is dependent on the interaction between platelets and the von Willebrand factor (VWF), a large plasma protein composed of multiple disulphide-linked subunits [3, 4]. When secreted into the blood, the VWF multimers can be >20 million Da in mass and 4 μm in length. The VWF undergoes proteolytic processing in plasma mediated by a metalloprotease called ADAMTS 13, which generates VWF multimers of all sizes and with different functional efficiency [5]. The larger VWF multimers are more efficient in platelet adhesion than the smaller ones and dysregulation of multimer processing is associated with disease (see below). The VWF mediates platelet adhesion by serving as a bridge between the tissue and the platelets, binding both to collagen exposed at sites of vascular injury and to the platelet membrane glycoprotein Ib-V-IX

(GPIb-V-IX) [6]. The adhesion of platelets functions better under conditions of high shear stress, i.e. it is more efficient in small arterioles than in veins. The mechanism is that the high shear unfolds the VWF thus exposing the binding sites for GPIb-V-IX. The platelet surface also contains receptors for collagen (glycoprotein Ia-IIa, GPIa-IIa) that contribute to the anchoring of the platelets to the damaged tissue [7]. Platelet adhesion is accompanied by major morphological changes of the platelets with rearrangement of the membrane and exposure of negatively charged phospholipids (see below) and formation of extensive pseudopodia that help anchor the platelets [2]. Concomitant formation and secretion of thromboxane A2 and release of ADP, calcium, and serotonin from the platelet granules result in the activation of additional platelets and contraction of smooth muscle cells of the vessel wall. An inside-out signal over the platelet membrane generates a conformational change of the platelet integrin glycoprotein IIb-IIIa (GPIIb-IIIa) and exposure of binding sites for the adhesive proteins fibrinogen, VWF, fibronectin, and thrombospondin [8]. These proteins form bridges between the platelets during the formation of the platelet aggregate. The formation of the primary platelet plug is temporally and spatially coordinated with the activation of the blood coagulation system leading to the generation of thrombin and the formation of the fibrin net [9].

Activation and propagation of the blood coagulation system

The precise and balanced generation of thrombin at sites of vascular injury is the result of an ordered series of reactions collectively referred to as blood coagulation [10–15]. Thrombin is the key effector enzyme of coagulation fulfilling many biologically important functions. It feedback amplifies coagulation by activating factor V (FV), factor VIII (FVIII), factor XI (FXI), and factor XIII (FXIII), it cleaves off fibrinopeptides A and B from fibrinogen, which results in the polymerization of fibrin monomers to a fibrin network, and it activates platelets by cleaving protease activated receptor-1 (PAR-1) [14, 16].

The exposure of tissue factor (TF) to blood and the subsequent binding of factor VII (FVII) to TF trigger the initiation of the coagulation system (Figs 1 and 4). TF is abundantly present in the membranes of cells surrounding the vascular bed but is normally not in contact with blood [17–19]. A fraction of FVII in blood is activated (FVIIa) and binding of FVIIa to TF initiates coagulation. Circulating factor IX (FIX) and factor X (FX) are converted by the FVIIa-TF-complex to active enzymes FIXa and FXa, which feedback amplify the system by activating FVII that is bound to TF. FIXa and FXa may remain associated with the TF-bearing cell or diffuse into fluid phase and bind to the membrane of activated platelets [9, 20, 21]. Negatively charged phospholipids that are

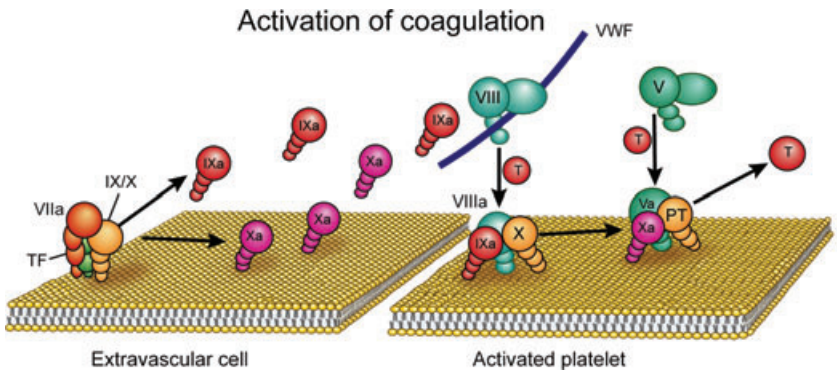

Fig. 1 Phospholipid-bound reactions that are involved in the activation of coagulation. TF (green) is exposed on extravascular cells after vascular injury and binds circulating FVIIa. The TF-FVIIa complex activates the vitamin K-dependent FIX and FX with high efficiency and the activated factors either remain on the surface of the activating cell or diffuse into solution and binds to the platelets. On the surface of activated platelets, the enzymes FIXa and FXa together with their respective cofactor FVIIIa and FVa form the tenase and prothrombinase complexes that activate FX and prothrombin respectively. Thrombin-mediated activation of FV and FVIII give positive feedback amplification of the system. FVIII circulates in plasma bound to the high molecular weight von Willebrand factor (VWF) and thrombin activation results in the liberation of the FVIIIa from VWF. The FVIIIa binds to the platelet surface and form the tenase complex with FIXa. Activated FV (FVa) forms the prothrombinase complex with FXa, which efficiently generates thrombin from prothrombin.

exposed upon activation of platelets have the potential to bind several of the coagulation factors and enzyme-cofactor complexes are assembled on the platelet membrane (Fig. 1). These complexes that are extremely efficient are crucially important for efficient propagation of the coagulation system [22–24].

FXa and its cofactor-activated FV (FVa) assemble on the activated platelets to form the 'prothrombinase' complex that efficiently activates prothrombin to thrombin [14, 25]. Circulating FV can be activated directly by FXa on the platelets but the majority of FV is activated by the first thrombin that is generated during the coagulation process. Thrombin also activates FVIII and the generated FVIIIa serves as an important cofactor to FIXa in the 'tenase' complex that converts FX to FXa (Figs 1 and 4). FVIII in blood circulates is bound to the VWF but is liberated during the thrombin-mediated activation process enabling free FVIIIa to bind to the platelet membrane and participate in the tenase complex [26, 27]. The prothrombinase and tenase complexes are extremely efficient activators of their respective substrates prothrombin and FX converting several thousand substrate molecules per minute, whereas the free enzymes FXa and FIXa are inefficient. Thus, the assembly of enzymes and cofactors on the phospholipid surface is a prerequisite for the propagation of the coagulation system, and in addition it counteracts regulation by anticoagulant mechanisms.

The negative charge of the activated platelet phospholipid membrane upon which the tenase and prothrombinase complexes are assembled is due to the presence of phosphatidyl serine [14, 15, 23, 28]. Under normal conditions phosphatidyl serine is located in the inner layer leaflet of the plasma cell membrane of platelets but it is translocated to the outer layer during platelet activation [23]. All the participating proteins of the tenase and prothrombinase complexes have affinity for the negatively charged phospholipid surface, i.e. the enzymes (FIXa and FXa), the cofactors (FVIIIa and FVa), and the substrates (FX and prothrombin). The enzymes and the substrates bind to the phospholipid membrane via their amino-terminal domains, which contain γ-carboxy glutamic acid (Gla) residues. Gla is generated through a vitamin K-dependent posttranslational modification of glutamic acid residues in the Gla domains, and Gla residues are uniquely present in the vitamin K-dependent proteins [24, 29]. The Gla residues bind calcium, which is important for the correct folding of the Gla domain. Vitamin K antagonists, e.g. warfarin that is commonly used to treat thrombosis, inhibit posttranslational modification, resulting in misfolded Gla domains that are unable to bind negatively charged phospholipid membranes.

Prothrombin activation continues after the formation of the fibrin network and most thrombin is in fact formed after the generation of the blood clot [14, 30]. This thrombin is important for activation of FXIII and the thrombin activatable fibrinolysis inhibitor (TAFI). The fibrin clot is stabilized by activated FXIII (FXIIIa), a transglutaminase that catalyses covalent cross-linkage of fibrinogen [31]. TAFI is a carboxypeptidase that cleaves off the carboxy terminal lysines from fibrin. These lysines are important for the binding of fibrinolytic enzymes to fibrin and TAFI activation results in inhibition of fibrinolytic attack [32].

The TF-dependent activation of coagulation has traditionally been referred to as the 'extrinsic' pathway [17–19]. This is the mechanism by which coagulation is initiated *in vivo* in response to trauma. An alternative pathway by which the coagulation system can be initiated involves factor XII, high-molecular weight kininogen, prekallikrein and FXI and results in the generation of FXIa, which in turn can activate FIX [33]. These reactions are collectively called the 'intrinsic' pathway and their physiological importance is not fully understood. However, it is evident that the intrinsic pathway is not important in trauma-initiated coagulation because inherited deficiency of the intrinsic pathway protein FXII is not associated with bleeding problems. On the contrary, FXI deficiency yields a moderately severe bleeding disorder [34].

Various coagulation proteins circulate in blood at very different concentrations related to their specific roles in the blood coagulation system [10–12, 24]. Thus, early components of the pathway circulate at lower concentrations than those factors that participate at later stages, which is consistent with the principal organization of the system with multiple reactions and amplification potential. Fibrinogen is the predominant clotting factor (10 μmol L^{-1}), having a concentration that is approximately 50 000-fold higher than that of FVIII (0.2 nmol L^{-1}). The high level of fibrinogen is required for the formation of the fibrin clot, whereas

the low concentration of FVIII is more than sufficient to support FIXa in the activation of FX. Amongst the vitamin K-dependent proteins, FVII (10 nmol L^{-1}) is the least abundant, FIX and FX being at intermediate levels (100 nmol L^{-1}) and prothrombin circulating at the highest concentration (2 μmol L^{-1}).

Knockout mice technology has been instrumental in the elucidation of the relative importance of various coagulation factors *in vivo* [35]. The embryonic lethal phenotype associated with TF deficiency demonstrates the crucial importance of the TF pathway [36–38]. In contrast, mice deficient in FVII develop normally *in utero* but die shortly after birth from severe bleeding [39]. The difference in severity between TF and FVII knockout animals suggests a role for TF during embryogenesis beyond fibrin formation. Prothrombin and FV deficiency are associated with partial embryonic lethality and fatal haemorrhage [40–42]. In contrast, mice deficient in FIX and FVIII develop normally *in utero* but attain haemophilia-like disease after birth [43, 44]. Fibrinogen-deficient mice have normal fetal development and suffer a moderate-to-severe bleeding phenotype similar to that of human fibrinogen deficiency [45]. This shows that thrombin generation is more important than fibrin deposition. Mice deficient in TAFI have no phenotype demonstrating that TAFI deficiency is fully compatible with life [46].

Anticoagulant pathways regulating blood coagulation

Blood coagulation is potentially dangerous and regulation is exerted at each level of the pathway. Different anticoagulant principles are utilized such as enzyme inhibition and proteolytic degradation of the enzyme cofactors FVa and FVIIIa. The tissue factor pathway inhibitor (TFPI) regulates the initial steps of blood coagulation involving FVIIa and TF [47, 48]. There are no deficiency states of TFPI described in humans, which may indicate that lack of TFPI is incompatible with life. TFPI knockout mice have a lethal phenotype, the mice suffering from uncontrolled activation of coagulation with consumption of coagulation factors [49].

The serine protease inhibitor (serpin) antithrombin (AT) inhibits enzymes of the coagulation system [50]. The physiological role of AT is to protect the circulation from liberated enzymes and to limit the coagulation process to sites of vascular injury. This is consistent with the observation that the free enzymes are more readily inhibited by AT than those taking part in the assembled tenase or prothrombinase complexes. Circulating AT is a relatively inefficient serpin, but its activity is stimulated by heparin and presumably by heparin-like molecules such as heparan sulphates or chondroitine sulphates that are present on the surface of endothelial cells [51]. The potentiation of AT efficiency by heparin is the molecular basis for the use of heparin as a therapeutic anticoagulant. Homozygous AT knockout mice have a lethal phenotype, demonstrating the importance of the protein for control of coagulation [52].

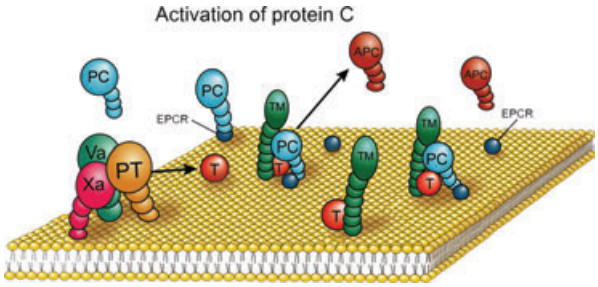

Fig. 2 Activation of protein C on the surface of endothelial cells. Thrombin (T) generated in the vicinity of intact endothelial cells does not work as a procoagulant but rather activates the protein C anticoagulant system after binding to thrombomodulin (TM). TM is present on all endothelial cells and binds thrombin with high affinity. The endothelial protein C receptor (EPCR, which is in dark blue) is another endothelial-bound protein that stimulates the activation of protein C by binding to the protein C Gla-domain, which helps orient protein C to the T-TM activation complex. Activated protein C (APC) is carried with the blood flow and fulfils an anticoagulant function throughout the vascular system.

The protein C anticoagulant system inhibits the procoagulant functions of FVIIIa and FVa, i.e. the cofactors in the tenase and prothrombinase complexes respectively [53–56]. The key component in the system is protein C, a vitamin K-dependent zymogen (proenzyme) to an anticoagulant protease. Protein C is activated by thrombin that is bound to the membrane protein thrombomodulin (TM) on the surface of intact endothelial cells. A recently described endothelial protein C receptor (EPCR) stimulates protein C activation (Figs 2 and 4). The thrombin-mediated activation of protein C demonstrates that thrombin has the capacity to express both pro- and anticoagulant functions. The procoagulant effects of thrombin are fully expressed at

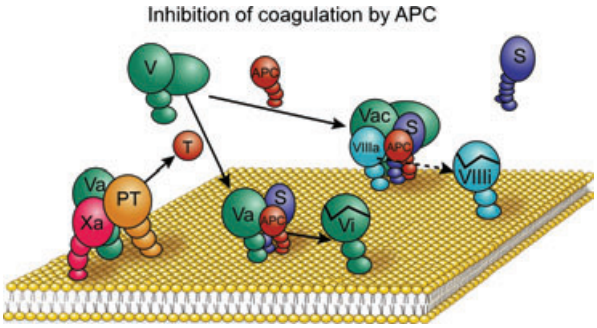

Fig. 3 Regulation of coagulation by activated protein C. APC and protein S form a complex on negatively charged phospholipid membranes that inactivate FVa and FVIIIa, which result in downregulation of the coagulation system. Not only protein S but also FV stimulate the degradation of FVIIIa. Thus, in this context, FV functions as an anticoagulant factor (Vac). Proteolytic modification of circulating FV determines whether FV will be converted to procoagulant FVa or to anticoagulant FVac. Thrombin or FXa that are generated as sites of vascular injury are the enzyme that convert FV to FVa, whereas cleavage by APC that is generated in intact vessels converts FV to anticoagulant FVac.

sites of vascular disrupture, whereas thrombin has anticoagulant functions in intact vessels where it binds to TM and activates protein C. Activated protein C (APC) inhibits coagulation by cleaving a few peptide bonds in each of the phospholipid membrane-bound cofactors FVa and FVIIIa [54, 57]. Protein S, a vitamin K-dependent plasma protein, supports the anticoagulant activity of APC in the degradation of FVa and FVIIIa (Figs 3 and 4). In human plasma, approximately 30% of protein S circulates as free protein, the remainder being bound to the complement regulatory protein C4b-binding protein (C4BP) [58, 59]. Only the free form of protein S functions as cofactor to APC. Protein S that is complexed with C4BP is instrumental for the localization of the C4BP to negatively charged phospholipid membranes. This is a unique way of providing local complement regulatory activity, which can be important for control of complement on the surface of cells undergoing apoptosis, a process associated with exposure of negatively charged phospholipids [59–61].

In the circulation, FVIII is bound to VWF, which not only stabilizes the labile FVIII molecule but also prevents it from interacting with the phospholipid membranes [26, 27, 62, 63]. The binding to VWF also protects the FVIII molecule from cleavage by APC. The situation is different for FV, which binds phospholipids equally well as FVa. As a consequence, APC can cleave the intact form of FV, a reaction that converts FV to an anticoagulant cofactor to APC, working in synergy with protein S in the degradation of FVIIIa in the tenase complex (Figs 3 and 4). Thus, FV, like thrombin, can function both as a procoagulant and as an anticoagulant factor, the procoagulant FVa being generated after activation by thrombin or FXa, whereas anticoagulant FV function is generated when FV is cleaved by APC [64, 65].

The protein C system is physiologically very important, which is most clearly demonstrated by the severe thromboembolic disease that is associated with homozygous deficiency of protein C in both man and mice [66]. Mice lacking the protein C or TM genes are affected by a lethal phenotype, the TM deficiency being particularly severe affecting embryogenesis even before the development of a functional cardiovascular system [67, 68].

In recent years, it has been proposed that APC also has anti-inflammatory and anti-apoptotic properties [56, 69–71]. These affects appear to be dependent on the EPCR that binds APC and changes the substrate specificity of APC. When interacting with EPCR, APC can cleave and activate PAR-1 whereas it is less efficient in cleaving the coagulation cofactors FVa and FVIIIa. The physiological importance of these novel activities of APC is not yet fully understood.

Inherited and acquired bleeding disorders

Defective platelet function or thrombocytopenia can cause bleeding problems but clinically important bleeding only occurs when the abnormalities are severe [1, 2]. The bleeding symptoms associated with the inherited platelet disorders are mucocutaneous, whereas haemarthrosis and intracerebral haemorrhage are rare. Deficiency of membrane receptor GPIIb-IIIa (Glanzmann's thrombastenia) is inherited as an autosomal recessive bleeding disease, affected individuals having intermittent and minor bleeding problems. Inherited deficiency of GPIb-V-IX (Bernard–Soulier syndrome) is characterized by thrombocytopenia, giant platelets and lack of VWF binding and affected individuals have bleeding tendency. Lack of the collagen-receptor GPIa-IIa is associated with mildly increased bleeding. Acquired platelet function defects can be caused by different medication (e.g. aspirin) and by different chronic

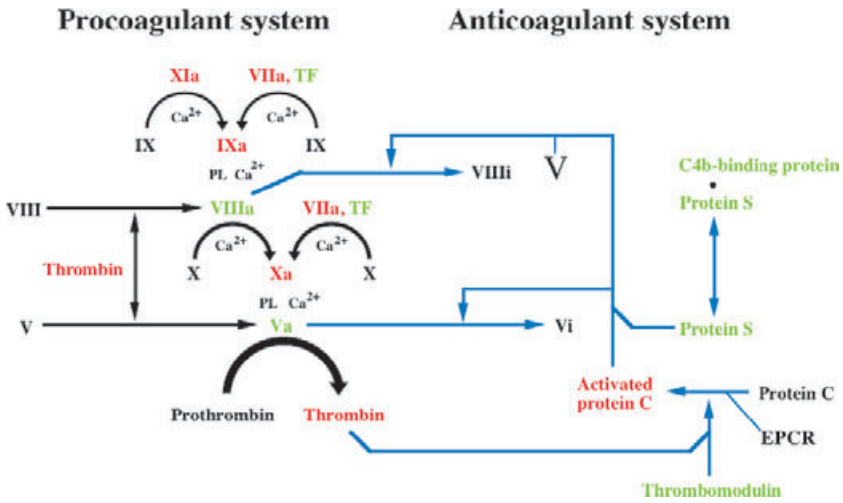

Fig. 4 Schematic representation of the reactions of blood coagulation and the protein C anticoagulant system. The reactions that are illustrated in Figs 1–3 are schematically presented here. The exposure of tissue factor (TF) to blood and the subsequent binding of FVIIa to TF result in the initiation of blood coagulation. The FVIIa-TF complex activates FIX and FX as illustrated in Fig. 1. FIX is alternatively activated by FXIa, which can be generated as a result of activation of the contact system (not shown in figure but discussed in the text) or by feedback activation of FXI by thrombin. FIXa and FVIIIa form the tenase complex in the presence of calcium and negatively charged phospholipid membranes (see Fig. 1) and FXa and FVa form the prothrombinase complex. Thrombin feedback activates coagulation by activation of FVIII and FV. Thrombin has multiple procoagulant functions such as activation of platelets, conversion of fibrinogen to a fibrin gel, and activation of FXIII (see text). In the presence of intact endothelium, thrombin binds to thrombomodulin and activates protein C (see Fig. 2). Endothelial protein C receptor (EPCR) stimulates the activation of protein C. Activated protein C counteracts coagulation by cleaving and inhibiting the cofactors FVa and FVIIIa. The free form of protein S in blood serves as cofactor to activated protein C. In the regulation of the tenase complex, FV plays an anticoagulant role as cofactor to activated protein C (see Fig. 3).

diseases, e.g. renal failure, autoimmune diseases, and myeloproliferative disorders.

The most common forms of haemophilia are due to different types of genetic defects (Table 1) resulting in inherited deficiency of FVIII (haemophilia A) or FIX (haemophilia B). Other coagulation factor deficiencies are very rare [34, 72]. The genes for FVIII and FIX are located on the X-chromosome, which stands in contrast to the rest of the coagulation factor genes that are located on other chromosomes (Fig. 5). This is why with few exceptions only males are affected by haemophilia A and B, whereas females are carriers of the disease. Haemophilias due to deficiency of one of the other coagulation factors are predominantly inherited as autosomal recessive traits in both sexes, although occasionally, individuals with heterozygous deficiencies have bleeding symptoms. The prevalence of haemophilia A in the male population is approximately 1/5000 and that of haemophilia B 1/30 000. In approximately 30% of haemophiliacs, the disease arises from novel mutations and haemophilia boys are therefore often born in families with no previous history of the disease. In a large number of cases, the genetic defects causing haemophilia A and B have been determined. In around 40% of the cases with haemophilia A the disease is caused by a gross FVIII gene rearrangement (Fig. 6). A large inversion due to an intra-chromosomal homologous recombination event results in the translocation of exons 1–22 (together with introns) of the FVIII gene away from exons 23–26 [72].

Haemophilias occur in severe, moderate and mild forms, corresponding plasma levels being <1% of the

Table 1 Different types of genetic defects affecting coagulation factor genes

Deletions – large or small (loss of exons/introns – frameshift)
Insertions – large or small (frameshift with premature stop codon)
Inversions [involving F8A gene in the FVIII (F8) gene]
Point mutations
In exons
Missense – changing amino acid residue
Nonsense – stop codon
In introns
Abnormal splicing
In noncoding regulatory 5′ or 3′ elements

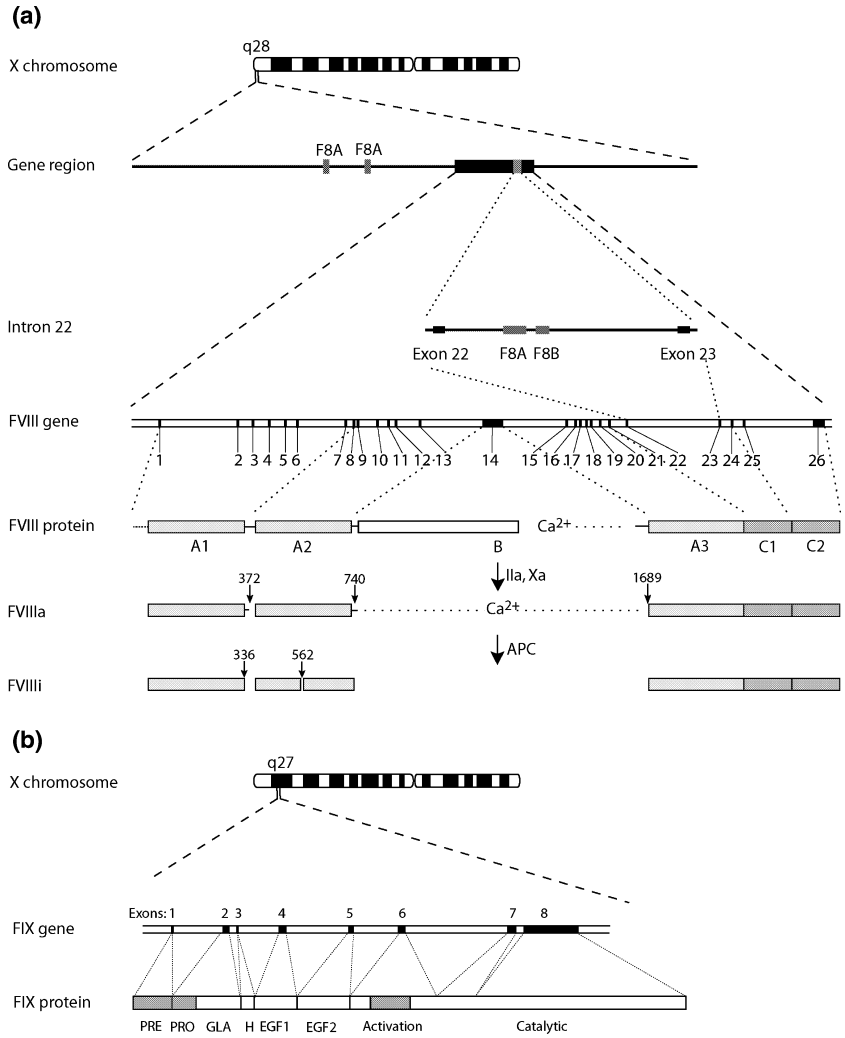

Fig. 5 Genomic organization of factor VIII and FIX genes on the X chromosome. The FVIII (F8) and FIX (F9) genes are located on q28 and q27, respectively, on the X chromosome, which explains the X-linked inheritance of haemophilia A and B. (a) The FVIII gene is composed of 26 exons. Of particular importance for the pathogenesis of haemophilia A is the presence of two genes, F8A and F8 B, in intron 22 of the FVIII gene. Two additional copies of the F8A gene are located closer to the q-terminal region of the chromosome. The F8A genes may cause an inversion of the FVIII gene as detailed in Fig. 6. The 26 exons encode the FVIII protein, which has a protein domain organization that it shares with the homologous FV protein (Fig. 7). The FVIII protein comprises a heavy chain composed of the A1-A2-B-domains, whereas the light chain is composed of the A3-C1-C2-domains. During coagulation, thrombin (IIa) or FXa activates FVIII by proteolytic cleavages at three sites (372, 740, and 1689), which results in the liberation of the B domain and the dissociation of FVIIIa from the VWF. The FVIIIa subunits are noncovalently linked by calcium ion-dependent interactions. FVIIIa is degraded by APC-mediated cleavages at positions 336 and 562, which results in loss of FVIIIa activity. (b) The eight exons of the FIX gene encode the different domains of the FIX protein. The boxes denoted PRE and PRO correspond to domains that are important for the intracellular processing of the protein and are cleaved off before secretion. GLA stands for the vitamin K-dependent domain that contains γ-carboxy glutamic acid residues and binds calcium and negatively charged phospholipid membranes. H is the helical region, EGF1 and EGF2 are the epidermal growth factor-like domains. The activation peptide is cleaved off during activation of FIX to a catalytically active serine protease that activates FX. Catalytic stands for the active serine protease domain. Several other vitamin K-dependent coagulation factors, including FVII, FX, and protein C, have similar genomic organization and domain composition.

normal plasma concentration, 1–5% and 5–30% respectively [72]. Thus, the normal plasma levels of FIX and FVIII are far higher than what is required for a normal physiological response. In mild haemophilia bleeding is usually only after trauma or surgery, whereas severe haemophilia is associated with apparently spontaneous bleeding episodes affecting joints, muscles, internal organs, and the

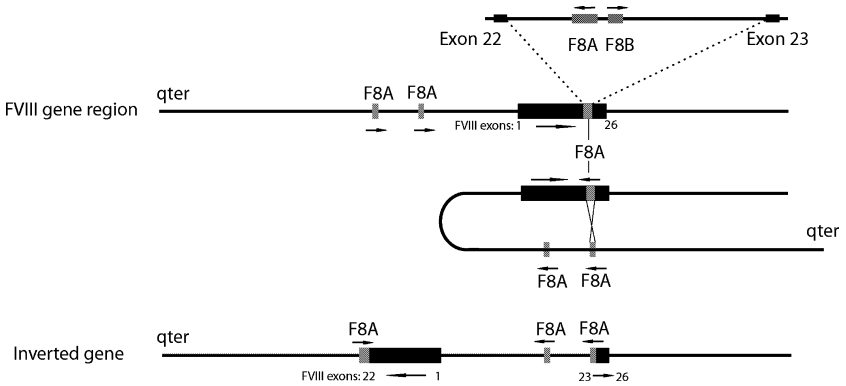

Fig. 6 Inversion of the FVIII gene. In around 40% of haemophilia A patients, the genetic defect is an inversion involving small genes (denoted F8A) that are located both outside the FVIII gene and in intron 22. The F8A genes lack introns and the genes that are located between the qter and the FVIII gene have the same orientation as the FVIII gene. In contrast, the F8A gene in intron 22 has the opposite directions. The inversion is due to a recombination event between the F8A gene in intron 22 and one of the two F8A genes outside the FVIII gene, as illustrated in the figure. After inversion, the FVIII gene is disrupted with the first 22 exons having a different orientation than exons 23–26. As a result, no intact FVIII protein can be synthesized.

brain. Joint bleeding (haemarthrosis) is the most characteristic feature of severe haemophilia, chronic arthropathy with loss of joint movement, fixed flexion contracture and severe muscle wasting being the result of repeated bleeds. The first bleeding manifestations do not appear in the neonatal period but rather in early childhood. A common early manifestation of haemophilia is bleeding from the mouth caused by the eruption of the teeth. Crawling and walking efforts are associated with haemorrhages in muscles and joints following minor twists or knocks. As primary haemostasis is unaffected, the patients do not usually experience major problems with bleeding from mucous membranes and minor skin lesions.

Combined FVIII and FV deficiency (multiple coagulation factor deficiency, MCFD) is a rare cause of bleeding [29]. Recently, defects in the genes of one of two intracellular proteins were found to cause the disease; either mannose-binding lectin-1 gene (LMAN1, also called ERGIC-53) or the protein encoded by the MCFD2 gene. These proteins form a complex in the ER–Golgi intermediate compartment functioning as a cargo receptor for the ER to Golgi transport of FVIII and FV. Both FV and FVIII have unique carbohydrate-rich regions that interact with LMAN1, an interaction important for the secretion of these proteins from the synthesizing cells. Combined deficiency of the vitamin K-dependent coagulation proteins is another very rare inherited cause of bleeding. This may be caused by genetic defects in one of the two known enzymes that are involved in the γ-carboxylation of the vitamin K-dependent coagulation proteins, i.e. the γ-carboxylase and the vitamin K epoxide reductase [29].

von Willebrand's disease (VWD) is a relatively common bleeding disorder affecting both males and females [3–5, 73–75]. It is caused by quantitative or qualitative defects of the VWF and the prevalence is around one symptomatic individual in 10 000 people. VWF is important for platelet adhesion and for maintaining the normal level of FVIII [3, 4]. Patients with VWD have a primary haemostasis defect caused by deficient adhesion of platelets to exposed subendothelial collagen. Bleeding from skin and mucous membranes is a common clinical manifestation and the bleeding symptoms often begin soon after birth. The disease is clinically heterogeneous and the severity of the symptoms depends not only on the nature of the disease-causing mutation, but also on whether both alleles are affected or not. Three major types of VWD are distinguished. Type 1 refers to partial deficiency (heterozygous) and is inherited in an autosomal dominant fashion. Type 2 (several subtypes distinguished) is associated with a qualitative defect in the VWF, which often affects the multimeric structure of the protein. Type 3 refers to total deficiency (homozygous or compound heterozygous) and is inherited as an autosomal recessive disease. Severe forms of VWD have a secondary deficiency of FVIII, as the

VWF is a carrier of FVIII in blood. Hyperactive VWF is also associated with disease and constitutes some of the type 2 variants. As discussed above, larger VWF multimers are more efficient in inducing platelet adhesion than smaller multimers and under normal conditions the nascent huge VWF multimers are processed to smaller ones by proteolytic modification mediated by ADAMTS 13. In rare cases with inherited deficiency of ADAMTS 13, VWF is not properly processed and the hyperactive platelet responsiveness results in excessive intravascular platelet adhesion and platelet depletion, a condition known as thrombotic thrombocytopen purpura [5, 76–79].

Bleeding disease can be due to the development of autoantibodies against a coagulation factor, the most common antibodies being directed against FVIII [80–83]. These conditions, which may be very severe and occasionally life threatening, mainly affect elderly people. The molecular mechanisms responsible for the development of the autoantibodies are poorly understood. Acquired bleeding problems can also be related to deficiency of vitamin K, which is required in the biosynthesis of many of the coagulation proteins. Malabsorption of the lipid-soluble vitamin K results in deficient γ-carboxylation of the vitamin K-dependent coagulation proteins, which in severe cases may result in increased bleeding. Even more common is vitamin K deficiency due to excessive intake of vitamin K antagonists, e.g. warfarin that is used as anticoagulant therapy. Poor liver function due to severe liver disease may also cause bleeding due to decreased synthesis of coagulation factors. In disseminated intravascular coagulation (DIC), acquired bleeding is due to the consumption of platelets and coagulation factors due to widespread pathological proteolysis [84, 85]. In this condition, multiple proteolytic enzyme systems including the coagulation and fibrinolytic systems are activated causing microvascular thrombosis and major disturbances of the capillary circulation. DIC may complicate malignancy, traumatic injury, surgery or pregnancies, and is often caused by severe infections with septicaemia.

Laboratory evaluation of patients with bleeding problems

The initial evaluation of patients with bleeding symptoms includes a careful anamnesis, measurements of platelet counts, global clotting tests such as the activated partial thromboplastin time (APTT) (intrinsic pathway) and the prothrombin time (PT) (extrinsic or TF pathway), and functional assays for VWF. Bleeding-time tests have been used frequently in the past but as they have low predictive value they are no longer recommended in primary screening [86]. Platelet functions can be tested by platelet aggregometry induced by different agonists such as thrombin, ADP, collagen, or ristocetin. The platelet surface receptors are investigated by fluorescense-activated cell sorting using specific antibodies to the various receptors. When a coagulation disorder is suspected, specific functional and immunological testing of coagulation factors can be performed at specialized laboratories. Severe haemophilia demonstrates prolonged clotting times in laboratory tests that are sensitive to the tenase complex, e.g. APTT but not PT, which is insensitive to the tenase complex due to the high TF concentrations used in the assay. Specific functional assays for FVIII and FIX are used to confirm the diagnosis of haemophilia. Identification of causative mutations has so far only been carried out in research laboratories and large databases with many different disease-generating mutations are available on the Internet. Mild and moderate forms of haemophilia may have either normal or only slightly prolonged APTT and the diagnosis is made after specific testing for the respective factor. The classification of VWD relies on the use of several types of tests including determinations of VWF concentration, the VWF function, and the multimeric pattern of VWF [4, 73–75]. Diagnosis of acquired bleeding disorders caused by autoantibodies is based on inhibition in clotting tests, e.g. APTT or PT, by antibodies. Identification of the coagulation factor that is recognized by the antibody involves specific coagulation or immunological tests, e.g. Western blotting that analyses the reactivity of the antibodies with various coagulation proteins. The laboratory diagnosis of vitamin K deficiency is based on the use of clotting tests examining the TF pathway, e.g. PT. APTT is usually normal in this condition. DIC is characterized by consumption of both platelets and coagulation factors [84, 85, 87]. Thus, platelets are low, the APTT prolonged and the levels of fibrinogen and FV decreased. Increased levels of fibrin degradation products, including D-dimers, result from activation of the fibrinolytic system.

Treatment of patients with bleeding problems

Treatment of haemophilia relies on the use of concentrates of FIX or FVIII that are derived either from plasma or produced by recombinant technologies [72, 88–91]. The concentrates are given on demand or as prophylactics. Intramuscular injections can trigger severe bleeding episodes and should therefore not be used. Moreover, drugs that inhibit platelet function, such as aspirin, should be avoided. The administration of FIX and FVIII concentrates is usually uneventful but virus transfer has, in the past, complicated the use of plasma-derived products. This has not been a problem associated with recombinant products. Both plasma-derived and recombinant factor concentrates occasionally induce antibody formation. The treatment of haemophiliacs having inhibitory antibodies is difficult and may include the use of so-called bypassing reagents such as recombinant FVIIa or activated prothrombin complex concentrate, or efforts to induce immune tolerance [90, 92, 93]. Gene therapy is not yet established as a therapeutic modality but research is intense [72, 94–97].

Severe forms of VWD are treated with concentrates of VWF in conjunction with bleeding episodes [73, 75]. The small peptide D-amino D-arginine vasopressin (DDAVP) increases the release of VWF and FVIII from endothelial cells and is therefore useful in the treatment of type 1 VWD [98, 99]. However, it is not effective in type 3 VWD because an efficient response to DDAVP requires that the VWF can be synthesized. The different type 2 variants demonstrate variation in the response to DDAVP and the substance should only be used after individual testing.

Recombinant APC concentrates are useful in the treatment of severe septic shock with associated DIC [55, 69, 70, 85, 100, 101]. It is not elucidated whether the beneficial effects are mainly dependent on the anticoagulant properties of APC or whether anti-inflammatory and anti-apoptotic effects are involved.

Inherited and acquired thrombotic disorders

Venous thrombosis annually affects one in 1000 individuals, with higher incidence amongst elderly people than in the young [102, 103]. In most cases the recovery is uneventful but pulmonary embolism or postthrombotic syndrome may complicate the disease. The pathogenesis of thrombosis involves inherited and acquired risk factors. Most acquired risk factors are of short duration, e.g. pregnancy, surgery, and immobilization, contrary to the lifelong inherited risk factors. Thus, a thrombotic episode may appear to be induced by an acquired risk factor when in fact the disease is due to a combination of genetic and acquired risk factors. The natural balance between pro- and anticoagulant forces is affected by most of the inherited risk factors for thrombosis. A majority of the genetic risk factors impair the function of the protein C anticoagulant system [103–105]. The most common, found in 20–40% of patients with thrombosis, is a single point mutation in the FV gene, which causes the APC resistance phenotype (Fig. 7). The FV mutation (G1691A) predicts the replacement of Arg506 in one of the APC cleavage sites in FV/FVa with a Gln. Mutant FV (denoted FVR506Q, FV:Q506 or FV Leiden) has full procoagulant capacity but affects the protein C anticoagulant system in two ways [64, 65]. The first is impaired degradation of mutant FVa by APC because the mutation eliminates one of three APC cleavage sites in FVa. The second is impaired degradation of FVIIIa because mutant FV is a poor cofactor to APC in the degradation of FVIIIa. FV Leiden is the result of a single mutation that took place around 30 000 years ago, i.e. it is the result of a founder effect and the mutation is predominantly found in whites but absent in Asians, Aboriginals in Australia and black Africans. The prevalence of FV Leiden is different in different European countries and with few exceptions there is a north to south gradient with highest prevalence (10–15%) in the north and lowest in the south (approximately 2%). In North America, where the population is of mixed ethnic background the prevalence is approximately 5% and in South America it is somewhat lower. Heterozygous individuals have a five- to 10-fold increased risk of thrombosis whereas homozygotes have 50–100-fold increased risk [103–105]. During evolution, carriership of the FV mutation may have provided a survival advantage, which would explain the high prevalence of the mutation in certain populations. Thus, women with FV Leiden have a reduced bleeding tendency after delivery, which in the history of mankind probably was a major survival benefit [106].

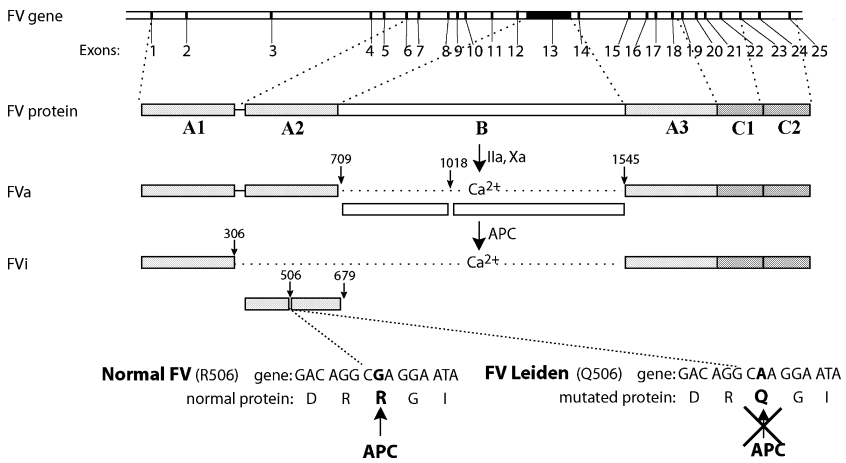

Fig. 7 Molecular basis of FV Leiden mutation. The FV gene (F5) is similar to the FVIII gene and contains 25 exons that encode the A1-A2-B-A3-C1-C2 domains of FV. Either thrombin (IIa) or FXa activate FV during the early phase of coagulation by cleavages at positions 709, 1018, and 1545, which results in the dissociation of two B-domain fragments. FVa is composed of the heavy (A1-A2) and light (A3-C1-C2) chains that are linked together by calcium-dependent noncovalent bonds. FVa serves as a cofactor to FXa in the activation of prothrombin and its activity is inhibited by APC, which cleaves at three positions (306, 506, and 679). The FV Leiden mutation [G–A nucleotide change in the codon for arginine (R) at position 506] results in the replacement of arginine 506(R506) with a glutamine (Q506). This affects the APC cleavage site and the consequence of the mutation is that APC is unable to cleave FVa at this position. In addition to the impaired FVa degradation, the mutation is also associated with loss of the anticoagulant activity of FV (see text and Fig. 3) that is important for regulation of the FVIIIa activity.

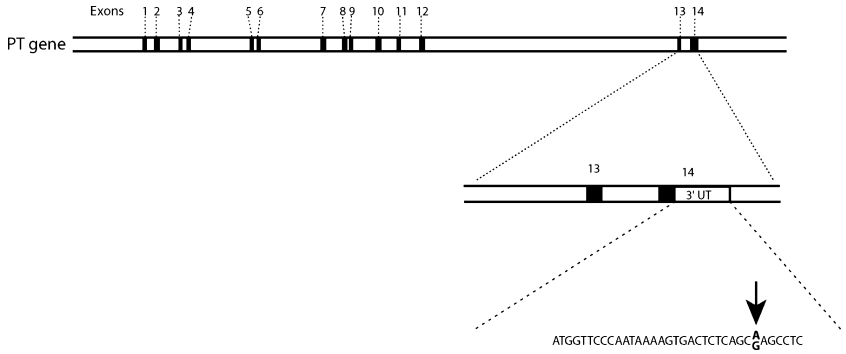

Fig. 8 Point mutation in prothrombin gene as a risk factor of thrombosis. A single G–A transition at position 20210 in the 3′ untranslated region of the prothrombin gene (F2) is identified as a common risk factor for venous thrombosis. The 20210 A allele is associated with slightly elevated levels of plasma prothrombin and an increased risk of thrombosis. The mutation does not affect the structure of the prothrombin molecule.

A single point mutation (G20210A) in the 3′ untranslated region of the prothrombin gene (Fig. 8) is the second most common genetic risk factor for thrombosis (three- to five-fold increased risk) found in 6–8% of patients with thrombosis and in around 2% of healthy controls [103, 105]. It is also the result of a single mutation event and a founder effect. The prothrombin function is unaffected by the mutation but the levels of prothrombin in plasma are slightly increased as a result of the mutation, which may be the basis for the increased risk. Other inherited risk factors of venous thrombosis include heterozygous deficiencies of protein C, protein S, or AT. Each of them is found in 1–3% of thrombosis patients and they are relatively rare in the general population, protein C or protein S deficiency in approximately 1/300 and AT deficiency in 1/2000 [107, 108]. The deficiencies are in contrast to the FV and prothrombin mutations caused by many different gene mutations. The thrombosis risk in deficiencies of protein C

or protein S appears to be similar to that of APC resistance, whereas AT deficiency is a somewhat stronger risk factor. Most individuals with a single genetic risk factor, although they have a lifelong increased risk, will not suffer from thrombosis during their lives because the associated risk is relatively low. People affected by more than one risk factor, either genetic or acquired are at higher risk and it is now considered that venous thromboembolism is a typical multigenetic/multifactorial disease.

The antiphospholipid syndrome (lupus anticoagulant) is an acquired risk factor for both arterial and venous thrombosis [109–111]. The antibodies are directed against a protein–lipid complex, the protein usually being β2-glycoprotein 1, but antibodies against other proteins such as protein S or prothrombin have occasionally been identified. Pregnant women with the antiphospholipid syndrome have an increased risk of spontaneous abortions.

Laboratory evaluation of thrombosis patients

FV Leiden can be diagnosed by direct DNA based assays or by functional APC resistance tests having close to 100% sensitivity and specificity for the FV mutation. DNA-based tests distinguish heterozygous from homozygous forms [105, 112]. Occasionally, the APC resistance test indicates a more severe phenotype than the DNA test, which may be due to pseudohomozygosity with one mutant FV allele and one null allele, i.e. heterozygous FV deficiency plus FV Leiden. Although DNA analysis points to heterozygosity, all FV molecules in plasma are APC resistant in this condition because the null allele is not expressed. The prothrombin mutation (G20210A) is diagnosed by DNA testing, whereas deficiencies of protein C, protein S, or AT are demonstrated by functional or immunological assays. Assays for the free form of protein S are preferred over those measuring the total protein S level as they have higher predictive value for protein S deficiency [58, 113, 114]. The antiphospholipid syndrome is often associated with prolonged clotting time in clotting tests because the antibodies disturb the interaction between the coagulation factors and the phospholipids [109–111]. The condition is therefore often discovered after observation of a prolonged APTT. Other clotting tests, e.g. the dilute Russell's viper venom (RVV) time, can be used to screen for the presence of a lupus anticoagulant. In the RVV assay, a prothrombinase complex is generated due to the activation of FX and FV by the RVV and the lupus antibodies can disturb the conversion of thrombin. The potency of the lupus anticoagulants is determined by testing the inhibitory activity in mixtures of normal and patient plasma. Neutralization assays using addition of excess phospholipids and different immunological tests designed to measure the binding of the patient antibodies to immobilized phospholipids (cardiolipin) or β2-glycoprotein 1 are also used in laboratory evaluation.

Treatment of thrombosis patients

In most cases, venous thrombosis is initially treated with a combination of heparin and vitamin K antagonists [102, 115, 116]. The heparin is either unfractionated (UFH) or low molecular weight (LMWH), which is prepared from UFH by either chemical or enzymatic cleavage methods. Laboratory monitoring of LMWH is not required and as LMWH has better pharmacokinetic properties than UFH, adequate haemostatic control is achieved with a single daily dose. Heparin is discontinued after a few days of the combined treatment when the levels of functional vitamin K-dependent coagulation proteins are in line with the therapeutic range. Vitamin K antagonist therapy is usually continued for 3–6 months. Its effect should be regularly monitored by prothrombin time-international normalized ratio. The benefits of the anticoagulation effect must always be weighed against the risk of bleeding complications, especially if an oral anticoagulant is used for periods exceeding 3–6 months, when the risk of thrombotic recurrence probably declines. Patients with combined genetic defects are at increased risk of recurrence, and long-term anticoagulation beyond 6 months can be considered. Prophylactic anticoagulant treatment is recommended for individuals with multiple genetic defects in situations known to be associated with a high risk of thromboembolic complications. In the years to come, alternative anticoagulant drugs, e.g. synthetic pentasaccharides as an alternative to LMWH or direct inhibitors of thrombin, FXa or FVIIa, will possibly replace the currently used therapeutic strategies [117–121].

Conflict of interest statement

I wish to declare that I am the patent holder of tests for APC resistance and free protein S and I receive royalties on sales of these tests. The diseases APC resistance and protein S deficiency are mentioned in the review and the laboratory procedures for detecting these diseases are described.

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