Thrombomodulin as a regulator of the anticoagulant pathway: implication in the development of thrombosis

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Thrombomodulin is a cell surface-expressed glycoprotein that serves as a cofactor for thrombin-mediated activation of protein C (PC), an event further amplified by the endothelial cell PC receptor. The PC pathway is a major anticoagulant mechanism that downregulates thrombin formation and hedges thrombus formation. The objectives of this review were to review recent findings regarding thrombomodulin structure, its involvement in the regulation of hemostasis and further discuss the implication, if any, of the genetic polymorphisms in the *thrombomodulin* gene in the risk of development of thrombosis. We performed a literature search by using electronic bibliographic databases. Although the direct evaluation of risk situations associated with thrombomodulin mutations/ polymorphisms could be of clinical significance, it appears that mutations that affect the function of thrombomodulin are rarely associated with venous thromboembolism. However, several polymorphisms are reported to be associated with increased risk for arterial thrombosis. Additionally studies on knock out mice as well studies on humans bearing rare mutations suggest that thrombomodulin dysfunction may be implicated in the pathogenesis of myocardial infraction. *Blood Coagul Fibrinolysis* 23:1–10 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Venous thromboembolism (VTE) constitutes a major medical threat, affecting one to three in 1000 individuals annually in the western world. It is a multifactorial disease, which is triggered by the interaction between genetic and acquired risk factors that affect a delicate balance between procoagulant and anticoagulant forces. In the last 50 years, the molecular base of blood coagulation and the anticoagulant mechanisms that counteract it have been largely elucidated. The protein C (PC) pathway is a major anticoagulant mechanism that downregulates thrombin formation limits inflammatory responses and potentially decreases endothelial cell apoptosis. As far as regulation of thrombin generation regarded, the PC system controls the activation of procoagulant proteins factor X (FX) and FII (prothrombin) that promote fibrin formation [1–5]. The essential components of the pathway involve thrombin (T), thrombomodulin, the endothelial PC receptor (EPCR), PC and protein S. PC is a vitamin K-dependent protein that serves as the key component of the system after its activation by thrombin. Activation of PC is achieved on the surface of vascular endothelial cells by thrombin bound to the transmembrane glycoprotein thrombomodulin [6–8]. Activated PC (APC) modulates blood coagulation by cleaving peptide bonds in activated procoagulant factors VIII (FVIIIa) and V (FVa) that serve as cofactors in the activation of FX and prothrombin (FII), respectively, promotes fibrinolysis through the inhibition of plasminogen activator inhibitor-1 and finally reduces inflammation by decreasing white blood cell and nuclear factor-κB (NF-κB) activation. The APC-mediated inactivation of FVIIIa and FVa occurs on the surface of negatively charged phospholipid membranes. Although FVIIIa and FVa are highly sensitive to APC, they are partially protected in the assembled tenase (FIXa, FVIIIa) and prothrombinase (FXa, FVa, phospholipids, Ca^{++}) complexes, as their respective enzymes, FIXa and FXa, sterically hinder APC (Fig. 1). There are several APC-sensitive sites in both FVIIIa (Arg336 and Arg562) and FVa (Arg306, Arg506 and Arg679), and cleavage by APC results in loss of binding sites for the enzymes FIXa and FXa, respectively, dissociation of fragments and disintegration of the FVIIIa and FVa molecules [9–14].

Thrombomodulin is present on the surface of endothelial cells and binds with high affinity to thrombin that is generated in the vicinity of intact endothelium. This binding is associated with loss of the procoagulant activities of thrombin and gain of the ability to activate PC. The highest concentration of thrombomodulin in the circulation is detected in the capillary bed, where the surface to volume ratio reaches its maximum. The high

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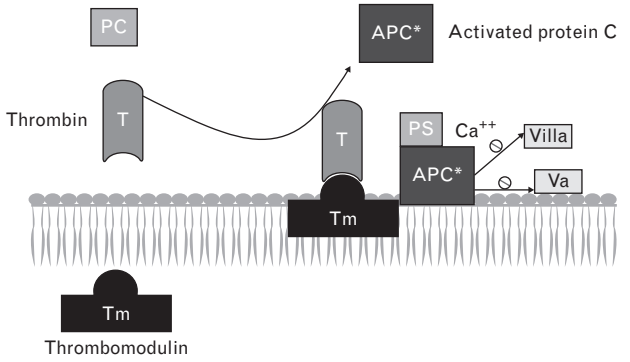
Fig. 1



The protein C pathway. PS, protein S.

thrombomodulin concentration in the microcirculation is crucial for local PC activation and blood anticoagulation [15–17]. Additionally, EPCR, another endothelial cell protein, binds to the Gla domain of PC and enhances activation of PC by the thrombin–thrombomodulin complex. The generated APC has a relatively long half-life in the circulation (approximately 20 min) and is slowly inhibited by PC inhibitor, α1-antitrypsin or α2-macroglobulin [18–20].

The activity of APC is stimulated by protein S, a vitamin K-dependent protein cofactor. Protein S is sufficient for the inactivation of FVa, whereas regulation of FVIIIa in the tenase complex requires the synergistic contribution of both protein S and FV, suggesting that FV potentially expresses both procoagulant and anticoagulant properties [2,13]. Plasma concentration of FVIII is almost two orders of magnitude lower than that of FV. As a consequence, during activation of coagulation, tenase complexes are scarce in comparison to the abundant prothrombinase complexes. This may explain the need for the two APC cofactors – protein S and FV – for the regulation of tenase, whereas one cofactor – protein S – suffices in the regulation of prothrombinase complexes [4,5,21–23].

Anticoagulant proteins are not only essential to maintain blood hemostasis but are also implicated in the supply of oxygen and nutrients to tissue cells and the removal of toxic byproducts from metabolism [24]. Normal APC generation depends on a precise complex formation composed of at least four proteins on the surface of endothelial cells: thrombin, thrombomodulin, PC and EPCR [5,13]. Genetic and acquired alterations of the anticoagulant properties of the PC system constitute major risk factors for venous thrombosis [25–27]. Congenital or/and acquired deficiencies of PC, protein S and antithrombin can lead to development of deep vein thrombosis with the possibility of producing lung emboli as well as fetal growth restriction and miscarriage [28,29]. Point mutations in the APC cleavage sites of FVIIIa and FVa lead to resistance to APC and, thus, modify the risk for development of thrombosis. FV Leiden (FV 1691G/A) is the most common mutation that is associated with increased risk for development of thrombosis, especially when associated with environmental stimuli such as trauma, labor, surgery and prolonged immobilization [30–37]. Several reports support the association between mutations in *thrombomodulin* and *EPCR* genes and venous and/or arterial thrombosis [38–40]. In this review, we summarize the current available literature on thrombomodulin structure and function as well as the involvement in the development of thrombosis. Finally we discuss the recent advances in the contribution of *thrombomodulin* gene mutations and/or polymorphisms in the risk of venous and/or arterial thrombosis. We performed a comprehensive literature search by using electronic bibliographic databases (*PubMed*), using the following as keywords: thrombomodulin, protein C pathway, venous thrombosis, arterial thrombosis and our search spanned years 1980–2011.

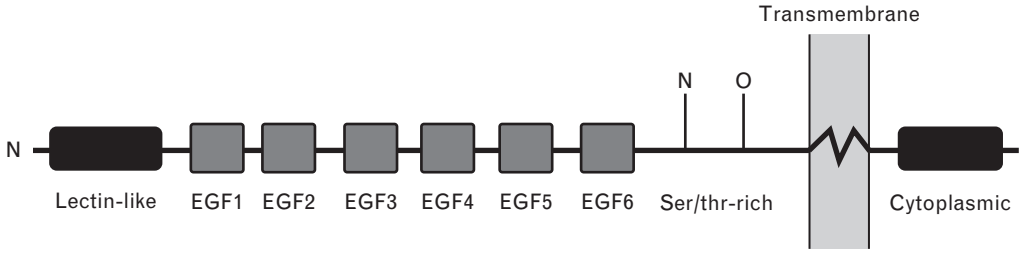
Thrombomodulin: structure and function

Thrombomodulin is a transmembrane endothelial cell surface glycoprotein of blood vessels, which is involved in coagulation, inflammation and cancer development and plays a role during embryogenesis. The *human thrombomodulin intronless* gene is located on chromosome 20p12-cem and its expression is controlled by areas within the promoter. These include a cap site at −164, a TATA box at −194, a GCAAT 'CAT' homology sequence at −278 as well as three GGGCGG hexanucleotide motifs and a complementary sequence at positions −132, −144, −168 and −210 upstream of the cap site, which are regions for interaction with the Sp1 transcription factor [41].

The human mature single-chain glycoprotein is 557 amino acids long including the first methionine and the signal peptide and does not possess intrinsic enzymatic activity. The main regions of the signal peptide, spanning 16 amino acids, are a hydrophobic core and a termination region enriched in prolines. Thrombomodulin is also expressed in the placental syncytiotrophoblast in which its expression and functional activity increase with gestational age. The mature protein is composed of five structural domains: an N-terminal lectin-like module, six endothelial growth factor (EGF)-like repeats, a Ser/Thr-rich region, a transmembrane region and a short cytoplasmic tail (Fig. 2) [42–48].

The N-terminal domain is a 154-amino acid residue module with homology to other C-type lectins and comprises about half of the extracellular part of the molecule [48,49]. Electron microscopy and computer models indicate that the lectin-like domain of thrombomodulin is globular and situated furthest away from the plasma membrane, so that it might effectively and easily interact with other molecules [50,51]. This domain is essential for receptor endocytosis and participates in the regulation of

Fig. 2



Domain structure of thrombomodulin. EGF, endothelial growth factor.

tumor growth. It also downregulates NF-κB and mitogen-activated protein kinase (MAPK) pathways, which are involved in endothelial cell activation and in setting the scenery for endothelial cell dysfunction [52]. Early studies confirmed, however, that although this domain lacks anticoagulant function, it is involved in inflammation and cell adhesion [43,44,49–51,53–67].

The best characterized domain is composed of six EGF-like repeats, which form an extended stalk in the extracellular part of thrombomodulin, providing a structure that is optimally suited for complex protein–protein interactions. This domain has mitogenic effects on cultured fibroblasts and vascular smooth muscle cells mediated via activation of protein kinase C and MAPKs. The clinical significance of these findings has not been elucidated, but a possible role in cellular proliferation and atherogenesis is suggested [68,69]. Although the function of the first two EGF-like repeats (furthest from the Ser/Thr-rich region) remains unknown, the others are crucial for activation of PC and thrombin activatable fibrinolysis inhibitor (TAFI) by thrombin [70–73]. EGF5–6 is crucial for thrombin binding (via its anion-binding exosite I), whereas EGF4–6 is required for activation of PC [74]. In contrast, activation of TAFI by thrombin–thrombomodulin requires EGF3–6 [75]. Additional antifibrinolytic activity is supported by the EGF-like repeats of thrombomodulin, as they also accelerate thrombin-mediated conversion of single-chain urokinase-type plasminogen activator to thrombin-cleaved 2-chain urokinase-type plasminogen activator [76], thereby interfering with the generation of plasmin [77,78]. In addition to their antifibrinolytic function via TAFI activation, the EGF-like repeats of thrombomodulin also accelerate thrombin-mediated inactivation of single-chain urokinase-type plasminogen activator. For this reaction to proceed, EGF5-6 and chondroitin sulfate, a stretch of approximately 20 repeating disaccharide units with a trisaccharide terminus, are required. The chondroitin sulfate, which may be variably expressed in different vascular beds, has additional functions (see below) [76].

A Ser/Thr-rich region extends between the sixth EGF module and the transmembrane domain and contains seven N-glycosylation and O-glycosylation sites, which support the attachment of chondroitin sulfate. Once modified with the chondroitin sulfate, thrombomodulin enhances the PC activity, accelerates the neutralization of thrombin by heparin–antithrombin and by the PC inhibitor and facilitates binding of platelet factor 4 to PC to accelerate its activation [79]. Thrombomodulin has a short cytoplasmic tail, deletion of which has no effect on development, survival, coagulation or inflammation in mice [47].

Summarizing, thrombomodulin has several distinct functions that are located in different structural domains. Thrombomodoulin possesses at least three independent anticoagulant activities: it catalyzes thrombin activation of PC to APC, it binds to thrombin and alters thrombin substrate specificity by inhibiting thrombin-mediated clotting of fibrinogen, activation of platelets and procoagulant factors V, VIII, XI, XIII and finally it catalyzes the inhibition of thrombin by antithrombin. When thrombin is complexed with thrombomodulin *in vivo*, PC activation is enhanced over 1000-fold. PC activation by the thrombin–thrombomodulin complex is further enhanced 20-fold *in vivo* when PC is bound to EPCR [80,81].

Soluble thrombomodulin in health and disease

Thrombomodulin expression is not restricted to the cell membrane and also exists in a soluble form in plasma generated by enzymatic cleavage of the intact protein [82,83]. Under normal conditions, levels of soluble thrombomodulin in the plasma range from 3 to 50 ng/ml [52]. Higher levels of soluble thrombomodulin, possibly cleaved from endothelial cells by neutrophil-derived enzymes, indicate disorders associated with vascular damage, including a variety of infections, sepsis and inflammation [43,53,54]. Several studies suggest that plasma thrombomodulin levels may be inversely correlated with the development of coronary heart disease (CHD), implying that soluble forms of thrombomodulin may be vasculoprotective [55–57]. It is not yet clarified which proteolytic fragments of thrombomodulin provide

protection, although likely candidates include EGF1–6 and/or the lectin-like domain.

Thrombomodulin in inflammation

Thrombomodulin also possesses well defined anti-inflammatory properties, as coagulation proteases and their cofactors can modify the outcome of severe inflammation by engaging signaling-competent cell surface receptors. Thus, thrombomodulin provides a definite molecular bridge that links inflammation and coagulation. [15,16]. There are additional indirect mechanisms by which thrombomodulin may provide anti-inflammatory protection. It controls the complement arm of the innate immune system in a thrombin-dependent manner through activation of the thrombin activatable inhibitor of fibrinolysis (TAFI), and in a thrombin-independent, constitutive manner via its lectin-like extracellular domain; and inhibits the inflammatory effects of high-mobility box group 1 protein [84]. An intact thrombin–thrombomodulin complex is required for TAFIα to suppress complement activation (C5a and C3a). Furthermore, when associated with thrombomodulin, the pro-inflammatory properties of thrombin are abrogated and indeed reversed. As thrombomodulin redirects thrombin activity, thrombomodulin is effectively, however, indirectly an anti-inflammatory molecule by its trait of being a sink of thrombin [69,85,86].

The amino terminal C-type lectin-like domain of thrombomodulin has direct anti-inflammatory properties by mediating signals that interfere with MAPK and NF-κB pathways. The C-type lectin-like domain maintains the integrity of cell–cell interactions and, thus, might also prevent leukocyte transmigration. It extenuates the response of the vascular endothelium to pro-inflammatory stimuli by suppressing activation of well conserved intracellular pathways. Finally, it suppresses expression of intercellular adhesion molecule-1 [15,43].

Animal models

Animal models have added significant knowledge to our understanding of thrombomodulin function. The most convincing evidence that reduced thrombomodulin function enhances thrombus formation is derived from animal studies. Administration of thrombomodulin in mice and rats attenuates the consequences of thrombin-induced thromboembolism, whereas injection of antibodies that inhibit thrombomodulin-dependent PC activation aggravates the consequences of thrombin injection [87,88]. Weiler-Guettler *et al.* [89] demonstrated that knockin mice with a thrombomodulin mutant bearing a Glu387Pro single nucleotide polymorphism (SNP) corresponding to human thrombomodulin exhibit a prothrombotic disorder. This amino acid change is located between the interdomain loop of the fourth and fifth EGF-like domains and abolishes the ability of soluble thrombomodulin (sTM) to catalyze in-vitro thrombin activation of PC to APC. Transgenic mice with single nucleotide modification leading to Glu387Pro substitution (TMPro) develop normally and are viable, although PC activation is reduced to less than 5%, and thrombomodulin surface expression is only one-third of normal. The mice show a mild hypercoagulable state associated with vascular bed-specific fibrin deposition, moderately accelerated platelet thrombus formation and a strong predilection for stasis-induced thrombosis. TMPro animals do not develop spontaneous thrombosis. Superimposed fibrinolytic defects (i.e. tissue plasminogen activator deficiency) elicit spontaneous myocardial microvascular thrombosis in TMPro mice [90]. Substantial loss of thrombomodulin cofactor function in TMPro mice produces only a mild hypercoagulable state, and overt thrombosis occurs only in the presence of additional genetic defects or pathological challenges.

Thrombomodulin is also a substantial component of the anticoagulant mechanism for the prevention of thrombosis [91]. Generation of thrombomodulin-deficient ($TM^{-/-}$) chimeric mice by inactivating both alleles of the *thrombomodulin* gene in murine embryonic stem cells revealed that the actual physical size of the $TM^{-/-}$ area or procoagulant loci limits fibrin formation and deposition. However, the fibrin deposits were largely restricted to pulmonary vessels with a lumenal area greater than 100 μm^2. These observations suggest that localized thrombomodulin deficiency, as it occurs in atherosclerotic lesions or iatrogenic endothelial cell damage [92,93], triggers localized coagulation and thrombosis.

Isermann *et al.* [94] established a mouse model by conditional *thrombomodulin* gene ablation only in endothelial cells. Only 60% of the mutant mice survive beyond birth, yet they succumb to a severe hypercoagulable state and massive thrombosis after 3 weeks, terminating in a lethal consumptive coagulopathy. Disease onset and progression could be prevented by warfarin anticoagulation. Unexpectedly, the remaining 40% of mutant mice succumb to a novel developmental defect not observed in completely thrombomodulin-deficient mice.

Regulation of expression of thrombomodulin

The complex regulation of thrombomodulin underlines its importance in a wide variety of pathophysiologic conditions and biological systems. Thrombomodulin is transcriptionally upregulated by vascular EGF, histamine, dibutyryl cAMP, retinoic acid, theophylline, heat shock proteins and statins [16,95,96]. Its transcription is downregulated by shear stress, hemodynamic forces, hypoxia, oxidized low-density lipoprotein and transforming growth factor-β [97]. Although tumor necrosis factor-α (TNFα) and interleukin-1β upregulate thrombomodulin expression in THP-1 cells [98], these cytokines effectively suppress thrombomodulin messenger RNA and cell surface functional protein levels in endothelial cells via inhibition of transcription and stimulation of endocytosis [99,100]. Thrombomodulin PC cofactor

activity can be abrogated by oxidation of a methionine in the EGF-like repeat, as it might occur during inflammation as a result of neutrophil activation [101].

The involvement of thrombin in the regulation of expression of thrombomodulin is controversial. Thrombin is either suggested to upregulate thrombomodulin expression [16] or to result in reduced thrombomodulin expression in primary cultures of human endothelial cells by approximately 40% at the level of mRNA, protein and activity [102]. The latter results suggest that activation of the coagulation cascade may result in a positive feedback loop consisting of thrombin-mediated repression of thrombomodulin-dependent PC activation.

Polymorphisms/mutations in the *thrombomodulin* gene and venous thrombosis

Normal APC generation depends on the precise coupling of thrombin and PC to their respective receptors, thrombomodulin and EPCR on the surface of endothelial cells. Any change in the efficiency of this coupling may cause altered APC generation and modification in the risk of thrombosis. In fact, several polymorphisms or mutations in the coding region and the promoter region of the *thrombomodulin* gene have been studied regarding the association with the risk of venous thrombosis. The influence of these polymorphisms over the level or the activity of thrombomodulin is unclear.

Le Flem *et al.* [103] analyzed the distal promoter region of the thrombomodulin in patients with VTE. Eight novel mutations were found, the more frequent being the −1748G/C and the del −1208/1209TT polymorphisms. The mutated alleles were not more frequent in patients than in controls: the odds ratios (OR) adjusted for age and sex were 0.90 and 0.92 for the −1748G/C and del −1208/1209TT mutations, respectively, suggesting that these mutations are not risk factors for thrombosis. Patients with the del −TT allele were more likely to have varicose veins of the lower limbs than patients with the wild-type allele (33 vs. 17%, $P = 0.007$). This deletion was also in tight linkage disequilibrium in cases and controls ($P < 0.01$) with the 1418C/T mutation. Furthermore, a point mutation in the promoter region (−33G/A) could not be clearly associated with increased risk for VTE [104].

The −33G/A mutation is located 7-nt upstream of the TATA box, within a promoter region important for basal *thrombomodulin* gene transcriptional activity and in the vicinity of the putative TNFα and heat shock responsive sequences. It is well established that efficient transcription initiation of a *human protein-encoding* gene requires assembly on the promoter DNA of several components such as a multiprotein complex containing RNA polymerase II, transcription factors and a consensus sequence (G/C-G/C-G/A-CGCC) located immediately upstream of the TATA box. These transcription factors can interact with transcription complexes and, thus, support transcription initiation. Being located in this consensus sequence, the −33G/A mutation might, therefore, induce a downregulation of the thrombomodulin promoter. Le Flem *et al.* [104] found that −33G/A mutation was more frequent in the patients (0.97%) than in the controls (0.25%) and might be a risk factor for venous thrombosis. To investigate the effect of this mutation on the thrombomodulin promoter activity, the proximal promoter region of the gene (either bearing or not bearing the −33G/A mutation) was inserted into a promotorless expression vector, upstream of the firefly *luciferase* gene and transiently transfected into EA.hy926 endothelial cells. The presence of −33G/A mutation was associated with mild decrease in the promoter activity. These results do not clearly support the hypothesis of the −33G/A mutation being a risk factor for VTE by lowering the expression of the *thrombomodulin* gene on vascular endothelial cells.

Heit *et al.* [105] reported that although mutations within the lectin, EGF6 and 3′-untranslated regions (127G/A, 1418T/C, del −1752C and 3645G/A, respectively) were more common than mutations within the thrombomodulin promoter, EGF1-5, Ser/Thr-rich region, transmembrane and cytoplasm regions, which were either absent or uncommon, none of them could be identified as a risk factor for incident VTE. The ORs for an association of these mutations with VTE were 1.07 [95% confidence interval (CI) 0.15–7.86], 1.09 (95% CI 0.73–1.62), 0.56 (95% CI 0.10–3.11) and 0.83 (95% CI 0.56–1.22) for the 127G/A, 1418T/C, del −1752C and 3645G/A, respectively. Null genetic marker allele frequencies did not differ significantly among cases and controls. Norlund *et al.* [106] reported a case of a heterozygous 127G/A mutation, which supports the hypothesis of an association between mutations in the *thrombomodulin* gene and venous thrombosis.

Ohlin and Marlar [107] identified another point mutation in the *thrombomodulin* gene occurring in a patient with pulmonary embolism. DNA sequence analysis revealed a 1456G/T substitution, which results in an Asp468Tyr change. This mutant was recently expressed in Cos cells and no impairment of thrombomodulin expression and function could be demonstrated [108]. Another study suggested that mutations that affect the function of thrombomodulin (1418C/T) are rarely in association with VTE and low sTM levels, as these levels are affected by a number of variables apart from gene integrity [109]. It appears that mutations that affect the function of thrombomodulin are rarely associated with VTE (Table 1) [103–105,109]. The fact that thrombomodulin is a key protein of the PC pathway and this pathway has a central role in coagulation inhibition is difficult to reconcile. A possible explanation is that severe deficiencies of thrombomodulin are incompatible with life. Moreover, a recent study on the expression of thrombomodulin in placental tissue from spontaneous recurrent miscarriage and

Table 1 Venous thromboembolism

Polymorphisms	Number of patients	Odds ratio	Reference
−1748 G/C	327	0.90	[105]
del −1208/1209 TT	327	0.92	[105]
−33 G/A	205	0.97	[106]
127 G/A	223	1.07	[107]
1418 C/T	223	1.09	[107]
del −1752 C	223	0.56	[107]
3645 G/A	223	0.83	[107]
1418 C/T	192	1.00	[111]

Association of thrombomodulin polymorphisms with the risk of developing venous thrombosis.

voluntary abortions revealed reduced expression in tissues originating from women with miscarriages compared with controls [110]. As no severe thrombomodulin deficiencies have been identified in patients with thrombosis, it can be postulated that thrombomodulin mutations, in the absence of PC deficiency, might not be associated with large vessel thrombosis, as thrombomodulin has a major role particularly in the capillary beds.

Polymorphisms/mutations in the *thrombomodulin* gene and arterial thrombosis

There are several data in the literature concerning the association between *thrombomodulin* gene polymorphisms and atherosclerosis or myocardial infraction (MI). This is surprising considering that other mutations disrupting the function of the PC pathway, such as the FV Leiden mutation, tend to increase the risk of venous, but affect marginally the risk for arterial thrombosis [111,112]. This discrepancy may reflect the fact that thrombomodulin can modulate inflammatory processes, complement activity and fibrinolysis in a manner that depends on PC activation.

As previously reported, the −33G/A SNP is not a well established risk factor for VTE. However, it was significantly associated with MI (OR 1.5, 95% CI 1.0–2.2) [113] and coronary artery disease (OR 1.7, $P = 0.031$) in a Chinese population [114]. Moreover, a dimorphism at codon 455, 1418C/T transition resulting in an Ala455Val substitution in the EGF6 domain of thrombomodulin, was also reported to be associated with MI. Wu *et al.* [115] suggested that this polymorphism has been associated with coronary events among African–Americans patients in the Arteriosclerosis Risk in Communities (ARIC) study. Presence of the T allele increased risk of CHD by 6.1-fold (risk ratio 6.1, 95% CI 1.7–22.9) in blacks, but did not significantly increase the risk in whites, when adjusted for age, sex and other CHD risk factors. In the Stroke Prevention in Young Women study [116], there was a significant relationship between 1418C/T polymorphism and risk of early-onset ischemic stroke. The CC genotype compared with the CT and TT genotypes combined was significantly associated with stroke (OR 1.9, 95% CI 1.1–3.3). The prevalence of the CC genotype was 81% (59 of 73) for blacks and 68% (93 of 137) for whites. Blacks were significantly more likely to have the CC genotype than the CT and TT genotypes (38.8 vs. 24.1%, $P < 0.05$). Ohlin *et al.* [117] demonstrated that that the C allele in the 1418C/T dimorphism is significantly more frequent among survivors of premature myocardial infarction.

Recently, the FINRISK national study analyzed eight common SNPs of the *thrombomodulin* gene (including 1418C/T, del −1208/1209TT and −33G/A) [118] and found no consistent association between *thrombomodulin* gene SNPs and incident coronary events, incident brain infarctions and total mortality. The SNP allelic frequencies did not differ significantly between the incident cases and members of the subcohort. The majority of the altogether 35 haplotypes observed in the datasets were rare: only seven haplotypes had a frequency of more than 5%. Olivot *et al.* [119] found that a polymorphism in coding sequence 1418C/T and two polymorphisms of *thrombomodulin* gene promoters −1748G/C and del −1208/1209TT were in linkage disequilibrium, whereas they were not associated with brain infarction risk by either single-locus analysis or haplotype analysis ($P = 0.94$). The thrombomodulin genotype distributions were compatible with Hardy–Weinberg equilibrium among cases and controls ($P > 0.24$). The −1748G/C was in negative linkage disequilibrium with del −1208/1209TT and 1418C/T in cases ($P < 0.10$) and controls ($P < 0.005$). A strong positive linkage disequilibrium between del −1208/1209TT and 1418C/T ($P < 0.0001$) was also reported. The three SNPs defined three major haplotypes accounting for 93.3% of all chromosomes.

The 127G/A mutation predicting an Ala25Thr substitution within the lectin-like domain is associated with a two-fold increased risk for MI, and a positive interaction of this polymorphism with acquired risk factors has been demonstrated [120,121]. This point mutation does not alter the ability of thrombomodulin to activate PC [39], implying either linkage of this polymorphism with another mutation or impairment of PC-independent thrombomodulin functions of the lectin-like thrombomodulin domain [44]. Doggen *et al.* [121] studied the 127G/A mutation in two independent studies, a pilot study of 104 patients with MI and a larger 'Study of Myocardial Infarctions Leiden' (SMILE), and came to the conclusion that the 127G/A mutation in the *thrombomodulin* gene increases the risk of MI among men (OR 2.0, 95% CI 0.8–5.1). This risk increases even more in the presence of another cardiovascular risk factor such as smoking or a metabolic risk factor.

Five mutations (three distinct) were identified (−9/10 GG/AT, −33G /A, and −133C/A) in the promoter region of the *thrombomodulin* gene and were detected to be in close proximity to consensus sequences for transcription

Table 2 Arterial thrombosis

Polymorphisms	Event	Number of patients	Odds ratio	Reference
−33 G/A	MI	278	1.50	[115]
−33 G/A	CAD	320	1.70	[116]
1418 C/T	CHD	467	6.10	[117]
1418 C/T	IS	141	1.90	[118]
127 G/A	MI	560	2.00	[123]
−9/10 GG/AT, −33 G/A, −133 C/A	MI	104	5.20	[124]

Association of thrombomodulin polymorphisms with the risk of developing arterial thrombosis. CAD, coronary artery disease; CHD, coronary heart disease; IS, ischemic stroke; MI, myocardial infraction.

control elements within the *thrombomodulin* gene. Only one of these mutations was identified in the control group (−33G /A). The risk of MI was, therefore, approximately five times increased in patients with a mutation (OR 5.2, 95% CI 0.6–45.3) (Table 2) [113–116,121,122].

Conclusion

The importance of the PC pathway is endorsed by the observation that PC deficiency, protein S deficiency and FV Leiden mutation are associated with an increased risk of thromboembolic events that are either spontaneous or triggered by circumstantial risk factors [31,123–125]. Genetic abnormalities affecting thrombomodulin should theoretically result in a compromised PC pathway and be associated with thrombosis. Screening for *thrombomodulin* gene mutations in patients with thrombotic disease has revealed several mutations and/or polymorphisms, some of which are associated with venous and/or arterial thrombosis [30,36,103]. Mutations leading to severe deficiencies of thrombomodulin may be incompatible with life. Although still mostly circumstantial, current evidence supports that development of thrombosis might be associated with other *thrombomodulin* gene mutations. Although thrombomodulin is a potential mediator of thrombosis, development of the disease cannot be solely attributed to such mutations. Additional genetic or acquired risk factors seem to be involved in the pathogenesis of thrombosis. It is well established that the most prevalent risk factors for development of venous thrombosis [PC, protein S, antithrombin (AT), FV Leiden] are associated with gene products that exhibit reduced biological activity, as detected by functional testing. However, there is no extensive knowledge to which extent mutations within the *thrombomodulin* gene affect its biological activity. Combined defects such as FV Leiden and AT/PC/protein S deficiency are also frequent and associated with increased risk of thrombosis [126–128]. Other candidates might include genes coding for further anticoagulant components like prothrombin (FII), fibrinogen, factor XII, cystathionine-β-synthase, methylenetetrahydrofolate reductase and tissue factor pathway inhibitor. It is well documented that the risk of thrombosis is increased when several genes are affected. Nevertheless, it is still unclear whether *thrombomodulin* gene mutations are part of this complex multigenetic disorder.

The association between *thrombomodulin* gene polymorphisms and arterial thrombosis seems to be more established. This association may be attributed to the fact that thrombomodulin can modulate inflammatory processes, complement activity and fibrinolysis in a PC activation dependent way.

Further studies including a larger number of patients are needed in order to clarify the implication, of any, of genetic polymorphisms in the *thrombomodulin* gene in the risk of development of thrombosis. Finally, the interaction between well identified genetic and/or acquired risk factors for thrombosis and thrombomodulin mutations/polymorphisms should be investigated.

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Conflicts of interest

There are no conflicts of interest.

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