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Identification of the novel G250R variant indicates a role for Thrombomodulin in modulating the risk for venous thromboembolism

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ABSTRACT

Thrombomodulin (THBD) plays a key role in the regulation of thrombin activity, acting as an intrinsic anticoagulant and leading to protein C activation (Martin FA M. R., 2013). Results from animal models support the hypothesis that an impairment of THBD anticoagulation properties may predispose to thrombosis (Weiler-Guettler H, 1998) (Isermann B, 2001). In this study, we want investigate possible causative genetic defects in samples obtained from Italian families with venous thrombosis. The understanding of this would be of worth for understanding the genetic basis of the disease, and also how it is transmitted. In close relatives who had a history of recurrent VTE, a missense variant (G250R) in the EGF-1 domain was identified. Evaluation of cells transfected with the wild-type or the mutant construct by imaging technologies and flow cytometry provided evidences that the 250R variant significantly decreased its localization on the cell membrane. Functional analyses of the 250R variant increase our understanding of the

role of Thrombomodulin in coagulation and support the hypothesis that gene variants reducing protein functionality cause deficiency and behave as a thrombophilic risk factor.

INTRODUCTION

I. Venous thromboembolism (VTE)

Venous thromboembolism (VTE) is a common disease that has a high morbidity and mortality rate shortly after the event (Flinterman, 2012). Despite significant advances in diagnosis and treatment, VTE remains one of the major causes of morbidity in the world. It is a common preventable cause of death (Gould MK, 2012) (Laryea J, 2013) in hospitalized patients. The incidence of thromboembolic complications reported in literature reaches up to 80% (Clagett GP, 1998) (Ulrych J, 2016), with a mortality rate of 33.3% in the United States (Gould MK, 2012). The incidence is higher in patients with malignant disease (Laryea J, 2013).

Venous thrombosis is caused by the formation of a blood clot (thrombosis) inside a deep vein of the body, which causes partial or complete obstruction to the flow of blood. Blood is meant to flow. If it becomes stagnant, there is a potential for it to clot. The

blood in veins constantly forms microscopic clots that are routinely broken down. If the balance of clot formation and clot breakdown is altered, significant clotting may occur. A blood clot (thrombus) in the deep venous system of the leg or arm, in itself, is not dangerous. It becomes potentially life-threatening when a piece of the blood clot breaks off and embolizes, travels through the circulation system through the heart, and enters into one of the pulmonary arteries and becomes lodged. (Rosendaal FR, 2009) This can prevent blood from flowing properly through the lung and decreasing the amount of oxygen absorbed and distributed back to the body. Venous thrombosis is caused by both genetic and environmental factors. (Morange, 2010) (Blom, 2005). A thrombus can form if one or a combination of the following situations occurs: immobility, prolonged travel and sitting, hospitalization, surgery, pregnancy, obesity, smoking, hereditary predisposition to clot formation, cancer (Christiansen, 2006), (Souto, 2000). It is thought that it can affect the elderly population more, but it can actually strike at any age (Zhen Zhang, 2017). Cardiovascular diseases are the first cause of mortality in Europe (A Naess, 2007). Despite significant advances in diagnosis and treatment, heart disease remains one

of the major causes of mortality in the world. At an estimated incidence rate of 1-2 per 1000 persons every year, (White R, 2003) VTE is the third most common cardiovascular disease and is associated with substantial short-and long-term morbidity and mortality. Short-term consequences of venous thrombosis include the absolute need for anticoagulant therapy, which is inevitably associated with an increased bleeding risk; the estimated case-fatality rate is approximately 6% after 30 days. Long-term consequences concern the risk of disease recurrence, as approximately 20-25% of all patients have a recurrence within 5 years (Kyrle PA, 2010). Early epidemiological studies have suggested that the directly standardized incidence of all VT events is significantly higher among African Americans (138–141 cases per 100,000 individuals per year) than among Caucasians (80–117 cases per 100,000 individuals per year), significantly lower among Hispanic populations (55–61.5 cases per 100,000 individuals per year), and strikingly lower among Asians and Pacific Islanders (21–29 cases per 100,000 individuals per year) (Stein, 2004) (White R. Z., 2005). Ethnic differences are implicated in the inherited traits of VT (Seligsohn, 2001). A poor APC (plasma to activated protein C)

response caused by the factor-V-Leiden mutation in the coding sequence of F5 (MIM 612309), (Dahlba¨ck B. C., 1993) (Bertina, 1994) a common prothrombin-G20210A mutation in the 30 UTR of F2 (MIM 176930) (Poort, 1996) and the antithrombin-Cambridge-II mutation in SERPINC1 (MIM 107300) (Corral, Antithrombin Cambridge II (A384S): an underestimated genetic risk factor for venous thrombosis., 2007) are common genetic risk factors for VT in whites. However, despite the discoveries described above, the underlying molecular mechanisms of a considerable number of inherited thrombotic events remain Unsolved (Corral, Antithrombin Cambridge II (A384S): an underestimated genetic risk factor for venous thrombosis., 2007). Mounting evidence indicates that genetic variation within the procoagulant, anticoagulant, and fibrinolytic pathways might be a potential risk factor for VT (Dahlba¨ck B. , 2008) (Saposnik, 2004).

The first discoveries of the genetic causes of thrombosis date back to 1965 when Egeberg identified the first defect leading to thrombophilia, describing a family with hereditary antithrombin (AT) deficiency. In the late 70s the ABO-blood group was related to the risk of venous thrombosis (Paul A Kyrle, 2004) . In the

early 1980s deficiencies of protein C and protein S were identified as causes of heritable thrombophilia and in the 1990s, factor V Leiden and prothrombin 20210A were discovered. The deficiencies of the natural anticoagulants were viewed as strong risk factors, and FV Leiden and prothrombin 20210A as moderate risk factors. More recently reported variants are very common, but weak risk factors for thrombosis, increasing risk no more than 1.5-fold. Until now none of the genetic risk factors for a first episode of venous thrombosis have much effect on the chance of recurrence (Wei Huang, 2014) .

I. I. Factors responsible for thrombosis

To better understand the pathophysiology of deep vein thrombosis (DVT), reference should be made to the Virchow triad, which describes the three main categories of factors responsible for thrombosis:

1. Hemodynamic changes (stasis, turbulence);
2. Endothelial lesions;
3. State of hypercoagulability.

Stasis phenomena occur in the presence of venous stasis, varicose veins, immobility prolonged, mitral stenosis, pneumoperitoneum. Endothelial injuries and / or trauma result from vascular rupture, damage from cuts (surgery, trauma), hypertension, contact with procoagulant surfaces (bacteria, foreign materials, implants biomaterials or medical devices, activated platelet membranes and monocytes during chronic inflammation). States of hypercoagulability are present in the course of blood hyperviscosity, AT, PC, PS or factor V deficiency Leiden, nephrotic syndrome, severe trauma or burns, malignant tumor pathology, advanced pregnancy, cigarette smoking, oral contraceptives and obesity.

In the guidelines for the prevention of VTE in non-orthopedic surgical patients, the risk of VTE varies according to patient-specific factors and specific procedures (Gould MK, 2012). Patient-specific factors include: previous age> 60 years

VTE, cancer, duration of anesthesia ≥ 2 hours, bed rest ≥ 4 days, male, sepsis, pregnancy, puerperium, central venous catheterization, hospitalization > 2 days, post-operative complications (infections, acute renal failure, post-operative

transfusions, acute peri-operative myocardial infarction and pneumonia).

I. II. Hemocoagulative and immunological parameters

Below is a brief discussion of the function of each hemocoagulative and immunological parameter.

- The prothrombin time (PT) or "Quick time" measures the quality of the way extrinsic coagulation. It is used to determine the tendency to clot, by measuring coagulation factors I (Fibrinogen), II, V, VII and X, to adjust the Warfarin dosage, to determine the severity of liver diseases and vitamin k status. The normal range of PT is 10 - 13 seconds. It is the time taken for the plasma to coagulate after addition of the tissue factor.

- Partial thromboplastin time (PTT) evaluates the effectiveness of the intrinsic and common pathway of coagulation. The activated partial thromboplastin time (aPTT) is the most sensitive version of PTT and is used for checking the patient's response to heparin

in the treatment of thrombo-embolic disorders, to characterize clots and for research purposes in hemophilias (Kozek-Langenecker SA, 2013). The aPTT evaluates factors I (fibrinogen), II (prothrombin), V, VIII, IX, X, XI and XII, but any shortcoming will not be detected unless a decrease ranging from 30% to 40% of the normal value. A high aPTT value (> 30 - 40 seconds) indicates a defect of one or more coagulation factors, use of heparin, lupus anticoagulans, the presence of a non-specific inhibitor of clotting factors. A drop in aPTT indicates an increase in the factor VIII for acute or chronic pathology or inflammation or difficult sampling.

- Fibrinogen is a plasma glycoprotein synthesized by the liver and by endothelial tissue. It is transformed into fibrin by thrombin, which is needed to the formation of the hemostatic thrombus.

- D-dimer is a product of fibrin degradation by the plasmin. Theoretically it should not be present in plasma, except when the coagulation system is activated. In the real life, low concentrations of D-dimer are present in healthy subjects, indicating a balance dynamic between the formation of fibrin and

its degradation (balance hemostatic). D-dimer levels increase in physiological conditions such as age> 65 years (atherosclerosis), neonatal period, pregnancy (hypercoagulability), e pathological (hospitalization, infections, major trauma, stroke, decompensation heart disease, neoplasms, disseminated intravascular coagulation (DIC), surgery, liver and kidney disease, inflammatory diseases chronic, venous thromboembolism, thrombolytic therapy).

- Antithrombin (AT) is a 58,000 Dalton glycoprotein synthesized from the liver and with a half-life of about 3 days. It mainly inhibits the thrombin (factor IIa) and factors Xa, XIIa, XIa and IXa, and kallikrein. The action of AT is accelerated by heparin (heparin cofactor activity). The activity of the AT is directly linked to its structure which it characteristically has 2 active sites, one for binding with serine proteases, the other with heparin. The AT binding site to serum proteases allows the formation of stable (irreversible) and inactive equimolecular complexes, rapidly purified from the liver. The complex with heparin causes a change of AT, which makes the binding site to serine proteases accessible, accelerating its inhibition. After facilitating the

formation of the AT complex - serine protease, the heparin detaches, and binds to new AT molecules. Acquired deficiencies of AT are related to: heparin treatment (10 - 20% reduction for a faster elimination of AT linked to heparin); postoperative period for trauma with consumption coagulopathy (10-15% reduction), DIC, cirrhosis, nephrotic syndrome.

- Protein C (PC) is a circulating zymogen which is activated by thrombin when complexed with the endothelial receptor of thrombomodulin. The resulting activated PC, inactivates factors Va and VIIIa, amplified by the PS cofactor, and suppresses the generation of thrombin and further thrombus (Van de Wouwer M, 2004). The anticoagulant function of the PC is expressed mostly on the endothelium rather than on the platelet surface (CT. E. , 2004).

- Protein S (PS) is a vitamin k - dependent protein and is mainly synthesized by the liver and endothelial cells. PS functions as a cofactor of protein C and enhances its degradation activity of factor V and VIII. It is also very important in phagocytosis of apoptotic cells, mediates the binding between phagocytes and the

dying cell from eliminate, making a bridge. PS is also a physiological anticoagulant and a decrease of it or a disturbed functionality leads to factors V and VIII reduced degradation, and therefore to an increased risk of venous thrombotic. The acquired PS deficiency occurs as a consequence of warfarin treatment, hormone replacement therapy, pregnancy, liver diseases, and chronic infections (HIV). A deficit of PS can cause DIC, DVT and pulmonary embolism.

- Antiphospholipid antibodies are a heterogeneous group of autoantibodies produced by the body according to mechanisms not yet fully known. Antibodies are directed against phospholipids and plasma proteins that have affinity for negatively charged surfaces, such as β2microglobulin, PT, PC, PS, thrombomodulin, annexin V, tissue plasminogen activator, kininogens, coagulation factor XII, low density lipoproteins oxidized (ox-LDL). Antiphospholipid antibodies are divided into three classes (lupus anticoagulans, anti-β2glycoprotein and anti-cardiolipin antibodies) and - inhibit the inactivation of factor V activated by the PC on a surface phospholipid, - inhibit tissue factor, - expose anionic phospholipids following the dislocation

of annexin V, - reduce fibrinolysis because of the reduction of the self-activation of factor XII of the phospholipid-dependent coagulation, - inhibit complexes heparin - antithrombin, stimulate the synthesis and exposure of the tissue factor on monocytes and endothelial cells, damage the endothelium causing it apoptosis, promote cell adhesion to vascular surfaces, induce platelet activation, cross-react with oxidized LDL, increase endothelin 1, alter the synthesis of eicosanoids and increase PAI-1. The most commonly altered parameters are aPTT and specific factors of the coagulation based on aPTT (Hoffman M, 2000).

- Lupus-like anticoagulant (LAC) is an anticoagulant factor that causes an increase in activated partial thromboplastin time (aPTT), carrying out its action mainly at the level of phospholipids with the addition of platelet or plasma phospholipids. In patients with diseases of the connective tissue, anti-phospholipid antibodies are associated with arterial and venous thromboembolic manifestations, thrombocytopenia and miscarriages.

- β2glycoprotein (old name of apolipoprotein H) alters ADP-mediated platelet aggregation, as it inhibits the release of serotonin by the platelets and thus prevents platelet aggregation ADP-induced. It also inhibits the production of factor Xa and the activation of factor XIIa. β2glycoprotein is therefore an anticoagulant, but it can have pro-coagulant activity under certain conditions. It inhibits activity of PC, which is involved in the degradation of the Va factor. With the addiction of phospholipids, the inhibitory activity is reduced. Anti β2glycoprotein antibodies (anti-lipoprotein H antibodies) inhibit the anticoagulant activity in the presence of β2glycoprotein, interfere with the inhibition of factor X stimulated by β2glycoprotein, inhibit self-activation of factor XII, increase thrombin formation by amplification of pro-coagulant reactions.

- Cardiolipin is a phospholipid of the mitochondrial inner membrane, and is responsible for impermeability to protons. Its name is due to the fact which was discovered in heart cells. In diseases where there is a strong cell damaging (syphilis, antiphospholipid antibody syndrome, vasculitis, Behcet's syndrome, SLE, idiopathic spontaneous abortion), it can be

released from the cells and stimulate the formation of specific antibodies against it, called anti-cardiolipin antibodies (ACA) or “reagine”. They they can appear as Ig-M, Ig-G or Ig-A, or be β2glycoprotei- dependent or independent. Only a subset of ACA binds the β2glycoprotein, with a consequent increase in thrombotic risk.

- The thromboelastogram measures the viscoelastic properties of the blood coagulated and not (citrated) after induction of coagulation under conditions similar to venous rheological properties in vivo. The type of change in viscoelasticity reflects the kinetics of all phases leading to thrombus formation (reaction (r), coagulation time (k), time of clot (CT) and clot formation time (CFT)), the stability of the clot, which is a function of the interaction between platelets and fibrin and of the fibrin polymerization (maximum amplitude (MA), maximum stability blood clot (MCF)), as well as its dissolution (fibrinolysis) (Kozek-Langenecker SA, 2013).

I. III. Coagulation patway

The blood coagulation system is characterized by a rapid activation of a series of serum proteases, mainly on cellular plasma membranes, culminating in the production of thrombin resulting in the conversion of fibrinogen into a fibrin clot.

Hemostasis is the result of the activation of two different systems, the intrinsic pathway and the via extrinsic coagulation, which are activated in contact with surfaces of platelets, foreign particles, biomaterials, or activated endothelium (Adams RL, 2009).

Tissue factor (TF) is the key promoter of the blood coagulation cascade and is mostly expressed by subendothelial vascular parietal cells and fibroblasts of adventitia. According to some scholars, TF is also produced by monocytes and by neutrophils in microparticles or in soluble form. Following the damage of the vascular endothelium, the TF is exposed to blood circulation and forms a complex with activated factor VII, activating factors IX and X. Both pathways haemocoagulatives culminate in the release of factor Xa.

Factor Xa converts prothrombin to thrombin, activating factors V and VIII. The activated factors VIII and V are respectively

cofactors for the activated factor IX, which in turn activates the X factor, and for the activated X factor, which converts prothrombin into thrombin. Thrombin induces the transformation of fibrinogen in a fibrin clot, which is solidified by activated factor XI, while the activated factor XIII acts in the cross-linking of fibrin, forming a clot that serves as a barrier to prevent the spread of any infection.

Activated platelets aggregate to TF, promoting thrombus formation e coagulation.

Thrombin formation is tightly regulated to ensure that the clot formation is not excessive and remains localized. They were main anti-coagulant mechanisms identified: the TF pathway inhibitor (TFPI), antithrombin and activated PC (Fig.1).

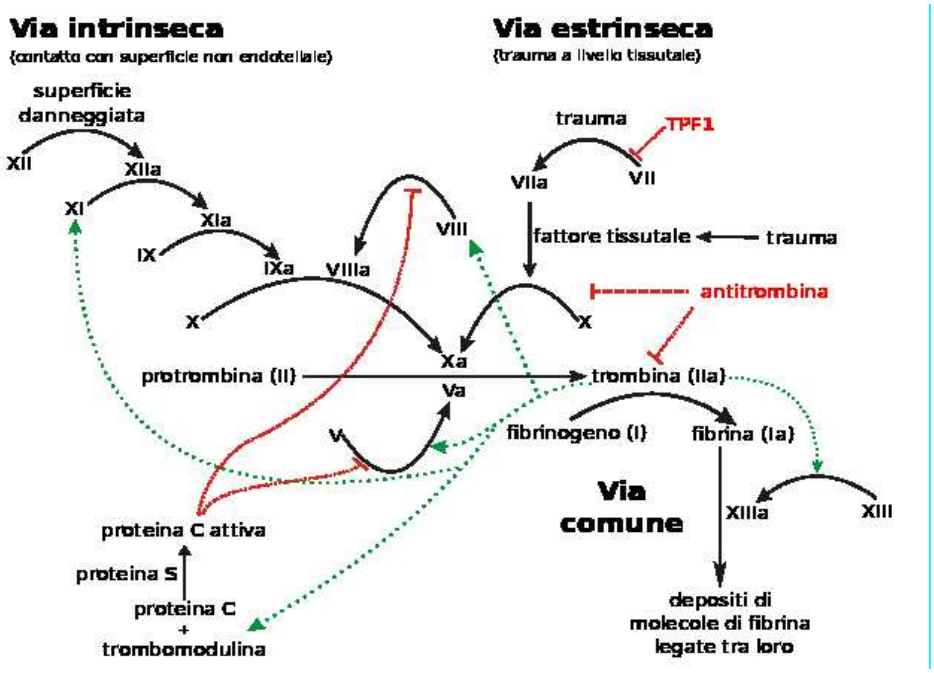


Fig.1 (The coagulation cascade, Wikipedia))

The modulation of the clot extension and the restoration of vascular integrity with healing, is mediated by the fibrinolytic system, which involves a distinct enzymatic cascade that leads to the removal of fibrin deposits. The key component of the fibrinolytic system is plasminogen, which is activated in plasmin from two serine proteases: the tissue plasminogen activator and the plasminogen activator urokinase. The first is involved in fibrinolysis; the second one in the production of plasmin. Plasmin is a powerful proteolytic enzyme which breaks down fibrin clots

into breakdown products, which are chemotactic for neutrophils. Plasmin also contributes to the inflammatory response by activating the classical way of the complement (Fig.2).

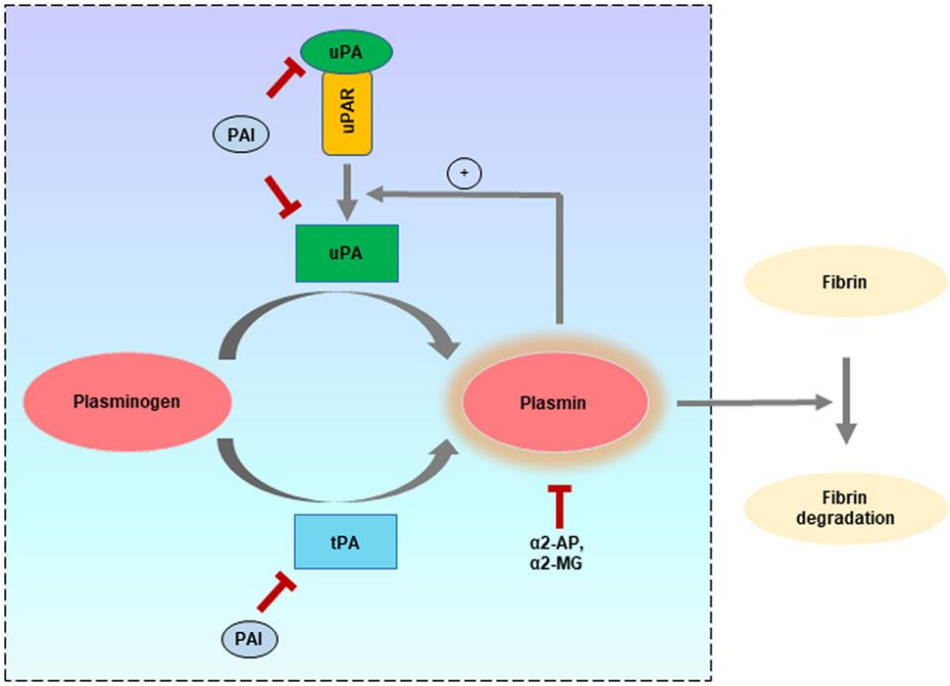


Fig.2 (Niaz Mahmood, 2018)

(Different components of the plasminogen activator (PA) system and role in fibrinolysis.)

The fibrinolytic system modulates the immune system, is regulated by mediators inflammatory and is also recruited by pathogens, often to the detriment of the organism.

The endothelial cell promotes a balance between antithrombotic and prothrombotic activities, acting on all sides of the hemostatic

process (Fig.3). The integrity of the endothelial surface itself prevents deposition, as well as adhesion platelet; endothelial cells also secrete potent PGI2 and NO vasodilators and antiplatelet agents. The endothelium also expresses thrombomodulin, a thrombin receptor that makes it similar to protein C. Enhanced by binding with protein S, mediated by heparan sulfate chains, it catalyses the interaction between AT and thrombin. Finally, the blood flow stimulates the endothelial secretion of tPA and uPA.

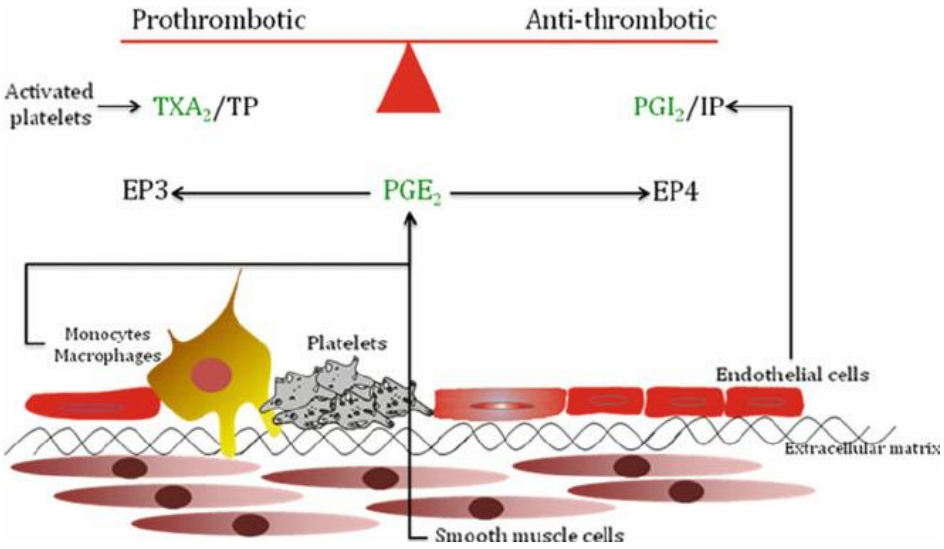


Fig.3 (Alexandre Kauskot, 2012)

(The balance between thrombotic and antithrombotic effects of prostanoids. In response to vascular injury, PGI 2 produced by endothelial cells opposes the enhanced prothrombotic effect of TXA 2 produced by platelets.)

On the other hand, the luminal surface of the vessels constitutes the main source of production of PAF (platelet activating factor) and vWF, powerful platelet aggregating activators. Endotoxins or cytokines, such as TNF and IL1, induce the production of TF, triggering the extrinsic pathway of coagulation (Fig.4). Finally, fibrinolysis can be inactivated by the release of PAI, a plasminogen activator inhibitor. Under physiological conditions, the endothelial hemostatic balance however puts more towards the production of antithrombotic factors. These biochemical processes are physiologically relevant, as theirs perturbations often predispose to thrombotic disorders.

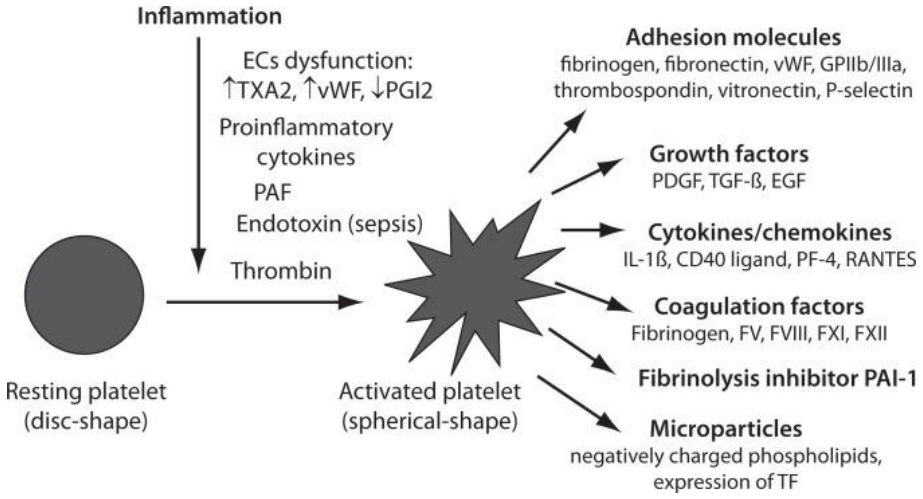


Fig.4 (Margetic, 2012)
(Platelet activation induced by inflammation).

II. Protein C

Protein C is a vitamin K-dependent coagulation protein that serves a critical role in the regulation of thrombin (B., 1995). PC is a double-chain plasma protein of p. m. 62,000 and mean plasma concentration of 4 mg / L (Kisiel, 1979). In the full-term newborn, CP is about 30% of the norm. Functionally active PC synthesis requires the supply of vitamin K and therefore behaves like all other coagulation proteins with procoagulant activity (factors IX, X, VII and II) whose synthesis is reduced by drugs that antagonize vitamin K (dicumarolics) (Lei Shen, 2013). PC circulates in plasma in an inactive form (zymogen), its activation occurs by thrombin through a proteolysis limited to the heavy chain; the detachment of a peptide of 12 amino acids exposes the active site of the activated PC, which thus acquires serinprotease properties. Protein C is synthesized in hepatocytes and circulates in plasma in a very low concentration of approximately 70 nm (Winter, 2020). Plasma protein C is activated after complex formation with thrombin on the endothelial cell receptor thrombomodulin; this activation is facilitated by protein C binding to the endothelial protein C receptor (EPCR). Activated

protein C (APC), in keeping with protein cofactors (protein S and factor V) and lipid cofactors (high-density lipoprotein and anionic phospholipids), cleaves critical sites in the activated procoagulant factors V and VIII, thus inactivating these enzymes (Fig.5). Patients with protein C deficiency have a decreased capacity to down-regulate the propagation of thrombin generation by factor Va and VIIIa once they have been activated by the small amounts of thrombin generated in the initiation phase of coagulation activation. Activated protein C also participates in the regulation of inflammation. During sepsis, signalling by inflammatory cytokines interleukin-1 and tumour necrosis factor mediates altered protein transcription in the systemic inflammatory response (SIR). SIR results in decreased synthesis of the regulatory proteins antithrombin, protein C and protein S, with increased synthesis of prothrombotic proteins factor VIII, von Willebrand factor, and fibrinogen. APC bound to EPCR cleaves the endothelial cell protease activated receptor-1 and, in addition to altered coagulation profiles, causes down-regulation of proinflammatory and proapoptotic mediators, up-regulation of antiinflammatory and antiapoptotic pathways, and stabilization of endothelial cell barrier functions (Mosnier LO,

2007). The clinical influence of SIR in the pathophysiology of sepsis and the importance of APC in dampening this pathway was demonstrated in the PROWESS trial, in which infusions of recombinant APC resulted in a significant decrease in the mortality of adults with sepsis (Cobas MM, 2006). Of note, patients with genetic protein C deficiency are not known to have increased susceptibility to sepsis or an altered inflammatory response. Levels of protein C raise later during the life than many other coagulation proteins. The mean plasma concentration of protein C in a healthy term infant is 40 IU dL^{-1} , with a lower limit of normal of 25 IU dL^{-1} . Protein C concentration increases from birth until 6 months of age when the 50th percentile of paediatric level is equivalent to the 10th percentile of healthy adults approximately 60 IU dL^{-1}. Protein C concentration remains slightly low through childhood and achieves the adult range after puberty (Peta¨ ja¨ J, Protein C pathway in infants and children., 2003). Healthy adults show a wide observed range of plasma protein C activity of approximately 65–135 IU dL^{-1} (RM., 2000). Authors of this review employ a nomenclature of mild protein C deficiency to indicate activity levels greater than 20 IU dL^{-1} but below the age-appropriate lower limit of normal

values, moderately severe protein C deficiency as activity levels in the range of 1–20 IU dL^{-1}, and severe deficiency for activity levels less than 1 IU dL^{-1} . Most neonatal presentations occur in infants with severe protein C deficiency in whom protein C activity is undetectable. However, rarely, patients with moderate protein C activity have also presented with neonatal PF (Tcheng WY, 2008). Protein C deficiency may be acquired and caused by increased consumption (e.g. overt DIC, severe infection without overt DIC, acute VTE) or by decreased synthesis of the active carboxylated protein (e.g. administration of vitamin K antagonists, severe hepatic synthetic dysfunction, complications of prematurity). Ill preterm infants may have very low levels of protein C activity (e.g. <10 IU dL)1) as an acquired deficiency superimposed on physiologically decreased levels at this age; these low levels may contribute to thrombotic complications in intensively supported preterm infants (Peta¨ ja¨ J, Protein C pathway in infants and children., 2003) (Manco-Johnson MJ, 1991). Rarely, antiphospholipid antibodies (APA) may also cause acquired protein C deficiency via antibody-mediated clearance.

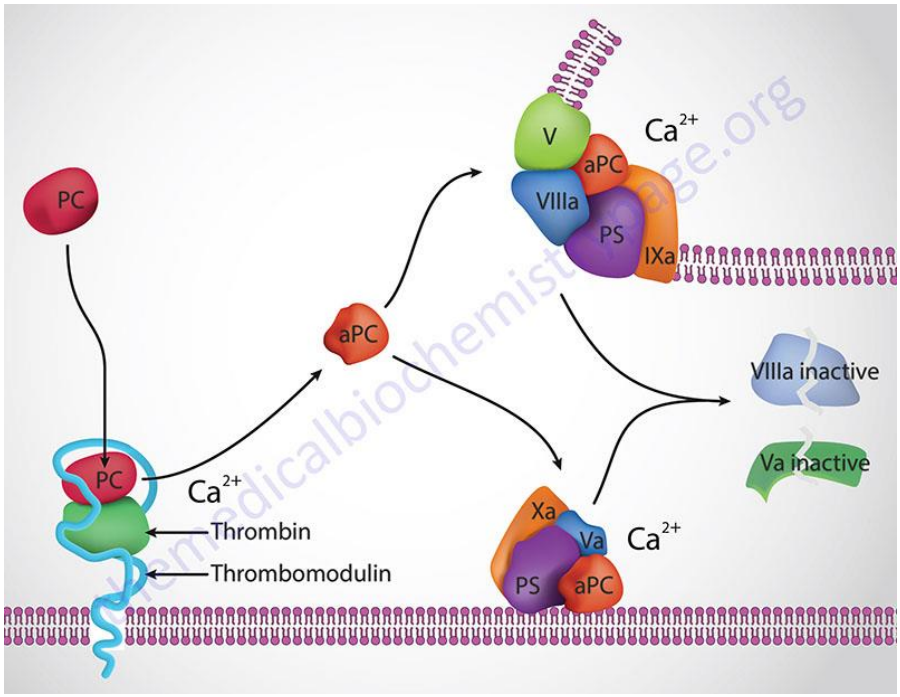


Fig.5 (themedicalbiochemistrypage)

(**Thrombin-mediated activation of Protein C (PC):** Thrombomodulin serves as a receptor for thrombin and protein C allowing an interaction between the two proteins. Thrombin cleaves protein C resulting in its activation (aPC). Following thrombin cleavage aPC hydrolyzes both factor Va and VIIIa rendering them inactive. This results in loss of active "tenase" complexes and active prothrombinase complexes.)

II. I. Protein C activation pathways and clinical events associated with defects of these pathways

The protein C pathway starts with the interaction between thrombin and thrombomodulin that transforms thrombin in an enzyme that starts an anti-coagulant response. Due to the common polymorphism of protein C, it is possible to examine the

relationship between the resistance to its activated form and cardiovascular diseases. The altered function of protein C contributes to the increased risk of myocardial infarction. The activation of protein C is almost certainly due to the formation of the thrombin-TM complex. It can be assumed that low TM levels weaken the anti-coagulant response when systemic thrombin levels rise, thus promoting the formation of the clot on sites of vascular damage. It has been demonstrated that the presence of TM diminishes the ability of thrombin to activate cells through thrombin receptors. These studies have been conducted on cells that express thrombin receptors, but not TM receptors. These cells were transfected with TM, and the response to thrombin could be attenuated according to the rise in TM concentrations. The inhibory effect can be at least in part explained by the fact that the thrombin and the thrombomodulin receptors share some common binding sites. This demonstrates that thrombomodulin inhibits thrombin mediated cellular activation by acting on thrombin's receptors. Keeping in mind that the same regulation also takes place on the endothelium, the reduced expression of TM, conditioned by the mutation on the 5' region of the gene, probably can favour the activation of endothelial cells and the

consequent expression of the adhesion molecules. Consequently, the loss of functions of TM can contribute locally to the development of atherosclerosis and to the breaking up of the plaque. The low TM levels in patients with mutations of the 5' region of the TM gene can generate a protein C variant that behaves less actively during transitory ischemic attacks and can consequently increase the probability of cardiovascular injury. Thrombomodulin also shows a growth factor like function. It should be remembered that the repeated EGF like sequences of the soluble TM form, corresponding to the proteolytically degraded TM, have a promoting action on mitosis of fibroblasts and endothelial cell lines (LIP GY, 1997).

II. II. Physiopathological characteristics

In endothelial injury determinism, a great importance must be attributed to the components that modulate TM expression and consequently influence protein C, S and the processes associated to these proteins (inflammation, immune responses). Protein C plays the fundamental role in endothelial injury physiopathology

by blocking the inflammatory reactions and the injury secondary to liberation of inflammatory peptides during coagulation, fibrinolysis and complement activation. Naturally, in this process trombomodulin plays a fundamental role due to its ability to activate protein C. Above protein C, we can find the regulation of cell surface TM expression that is influenced by other substances. Different peptides, such as bradychinine, complement factor C3a, fibrinogen degradation products function as stimuli for inflammatory cell (neutrophils, monocytes, macrophages) production of cytokines (LIP GY, 1997). Cytokines such as IL-1 and TNF down regulate the expression of TM, in particular by acting on the membrane form. Also TNF, endotoxins such as lipopolysaccharide (LPS), neutrophil elastase, oxygen free radicals, hydrogen peroxide and anaphilotoxin are capable of reducing TM activity by influencing the “cleavage” of the extracytoplasmatic part (MACGREGOR IR, 1997). The cytokines that down regulate TM concentration on endothelial cells also influence protein C. In this situation an increase of tPA and PAI-1 takes place (WADA H, 1998).

III. Thrombomodulin

Thrombomodulin (TM, encoded by THBD [MIM 188040]) is a membrane glycoprotein expressed on endothelial cells of blood vessels. (LOUGHEED JC B. C., 1995). Thrombomodulin plays a key role in regulating thrombin activity, acting as an intrinsic anticoagulant, the anticoagulant property of TM is mediated from its interaction with thrombin (the activated form of prothrombin) leading to the activation of protein C (PC, encoded by PROC [MIM 612283]). The high-affinity binding of thrombin to TM results in a greater than 1,000-fold amplification of the rate of thrombin-dependent PC activation. Thus, APC (the activated form of PC) inhibits the coagulation pathway by proteolysis of coagulation factor Va (the activated form of coagulation factor V) and coagulation factor VIIIa (the activated form of coagulation factor VIII, encoded by F8 [MIM 300841]) (Dahlba¨ck B. a., 2005). Akin to the cofactor function in anticoagulation, TM is also reported to be associated with fibrinolysis, embryonic development, arteriosclerosis, inflammation regulation, and tumor growth and metastasis

(Weiler, 2003), (Wu, 2012). Results from animal models support the hypothesis that an impairment of THBD anticoagulation properties may predispose to thrombosis. Thrombomodulin is present in the body in two forms (A K Ohlin, 1996); the first one has a higher molecular weight, and it is bound to the cytoplasmatic membrane of endothelial cells; the second form has a lower molecular weight and represents the soluble or plasmatic form (TOMURA S, 1994). The concentration of TM bound to the cell surface is regulated by genetic factors. The presence of gene mutations of the coding region or mutations of the regulatory sequences of TM transcription give place to the production of a molecule with impaired function or its lower expression (IRELAND H K. G., 1997). The concentration of serum TM levels varies accordingly. The proteinaceous component of the TM molecule has multiple moieties, each formed by about forty amino acid residues, called domains, that influence the tertiary structure and are responsible for the specificity of action of the glycoprotein. Different domains have different functions. These amino acid sequences can act as cofactors in the activation of protein C and have an anticoagulant effect by binding to thrombin. Thrombomodulin can stimulate

endothelial cell growth. This characteristic of TM depends from the molecular substrate of the sequences, known as EGF-like (epidermal growth factor-like). The binding of thrombin to thrombomodulin activates protein C and the trombin-thrombomodulin complex functions as an anticoagulant and anti-inflammatory stimulus (CT. E. , 1995). These reactions are regulated by the concentration of TM expressed on the cellular surface and also by the function of protein C, and is modulated by genetic mutations that can alter the anticoagulant efficacy. Many factors such as cytokines (IL-1, TNF) and neutrophils can have a regulatory role on TM activity. Cytokines reduce surface TM expression and cleave the molecule when activation of inflammatory processes takes place (MACGREGOR IR, 1997). Neutrophils, on the other hand, influence TM activity by the enzyme elastase.

III. I. Molecular structure

Thrombomodulin is a surface glycoprotein of endothelial cells, whose gene, localized on chromosome 207 does not contain

introns (IRELAND H K. K., 1996). Thrombomodulin is found in the body in two forms with different molecular weight. The heavy form weighs 150 kDa and the light form 69 kDa. THBD is an intrinsic membrane protein of 575 amino acid residues located on the luminal side of the endothelium (Martin FA M. R., 2013). The domains with epidermal growth factor-like structure play an important role as cofactors. These domains are formed by sequences of about forty amino acids, with six cystein residues that form three disulphuric bonds. The molecular sequence of thrombomodulin contains an N-terminal lectin-like element (1-154 residues), an hydrophobic region (155-222 residues), six EGF-like modules (223-462 residues), one dominium rich in Ser/Thr (463-497 residues), a trans-membrane moiety composed by 23 amino acids (498-521 residues) and a tail of 35 cytoplasmic amino acids (522-557 residues) (Fig.6) (Yi-Heng Li, 2012).

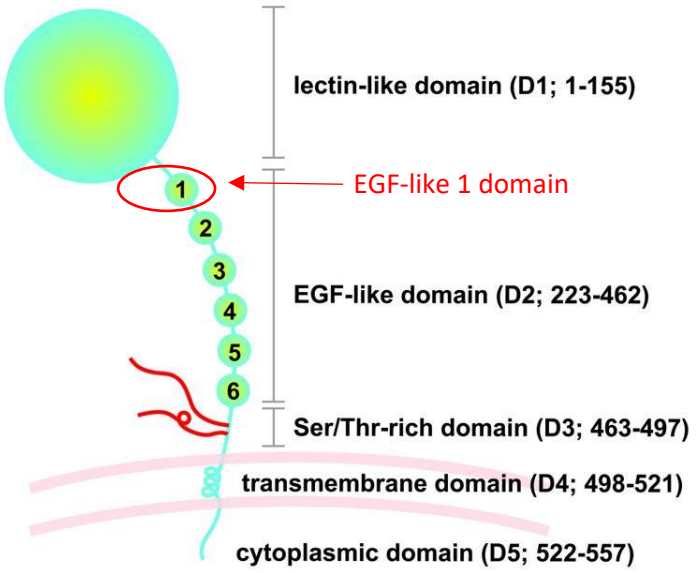


Fig.6 (Yi-Heng Li, 2012)

(Schematic presentation of structural domains of TM with corresponding sequence of amino acid. EGF, epidermal growth factor. Ser, serine; Thr, threonine; D1, domain 1; D2, domain 2; D3, domain 3; D4, domain 4; D5, domain 5.)

Thrombin binds to the EGF-like modules and this bond is strengthened by the presence of the sulphuric glycosaminoglycans of the Ser/Thr rich region (IRELAND H K. K., 1996), via the chondroitin sulfate (shown as black lines from S-T regions), bind to CD14 and adhesion_molecules LFA-1 and MAC-1, modulating responses to endotoxin and regulating leukocyte trafficking. Connected to the ectodomain via the transmembrane region (Trans), the cytoplasmic region (Cyto)

binds, in some cells, to the adaptor protein ezrin, which alters the actin cytoskeleton and modulates cell migration. (Fig.7) (HouraLoghmani, 2018). The structure of the EGF shows two main rings of 9-15 amino acids. The N-terminal ring is formed by the amino acids located between the third and fourth cystein that make up two disulphuric bonds between the first and the third cystein and the second and fourth cystein, respectively. The C-terminal ring is formed by the amino acids between the fifth and the sixth cystein and is a simple ring with one disulphuric bond. The model considered shows that the third and the fourth cystein, in the EGF sequences, are close enough to form a disulphuric bond. The thrombomodulin region including the fifth and the sixth domain inhibits the breakdown of fibrinogen, but also plays a role as a competitive inhibitor of protein C activation, without however showing a cofactor-like activity. The fourth EGF-like domain is necessary for the cofactor activity. A fragment containing the fourth, fifth and sixth EGF-like moieties can solicit a structure variation at a distance of 15 A (Angstrom) from the active site of the thrombin molecule (LOUGHEED JC B. C., 1995). Thrombomodulin binds to thrombin by two of its structural distinctive features: the disulphuric bond (that forms a

cyclic struc ture compressing the C-terminal ring) and the conjunction of the tail amino acids to the C-terminal ring region. The cofactor activity of the fourth and sixth EGF-like domain depends on the presence of the fourth EGF-like domain. The fact that the fourth domain does not bind well to the thrombin molecule suggests that, although necessary for protein C activation, it is however unable to exert its action if not in conjunction with the fifth EGF-like domain. This leads to the conclusion that the main region were the thrombomodulin-thrombin bond is possible can be localized in the fifth EGF-like domain and in the region connecting the fifth to the sixth EGF-like domain. The structural characteristics of this region formed by the amino acids C409-E•426 is the cyclic structure formed by the disulphuric bond between C409 and C421 and by the bond between the amino acid tail and this ring. The ring can interact with the tail so as to determine specificity of the bond. Thrombomodulin cofactorial activity, evaluated by the quantity of protein C that can be activated by thrombin, is measured by incubation of thrombin and TM together with protein C, and than by dosage of the activated protein C. Interactions of TM with thrombin that generate APC and CPB2 that dampen coagulation,

inflammation, fibrinolysis, and complement are highlighted in the box on the left. A directly proportional relationship between activated protein C and concentration of TM has been thus demonstrated. The sequence of the TM structure that depends from the O region of the sugar, where the condroitin-sulphate bond takes place, favours the inhibition of thrombin by anti-thrombin III and raises the affinity of TM for thrombin (CT. E. , 1995). Functional studies indicate that thrombin binding is mediated by THBD EGF5-6 domains while the EGF-4 domain is required for protein C binding to the thrombin–thrombomodulin complex. EGF3-6 domains are essential for CPB2 (Plasma carboxypeptidase B2) activation. The lectin-like domain is essential for constitutive endocytosis. Thrombomodulin cofactorial activity, evaluated by the quantity of protein C that can be activated by thrombin, is measured by incubation of thrombin and TM together with protein C, and than by dosage of the activated protein C. The functions of the EGF1-2 domains remain unknown.

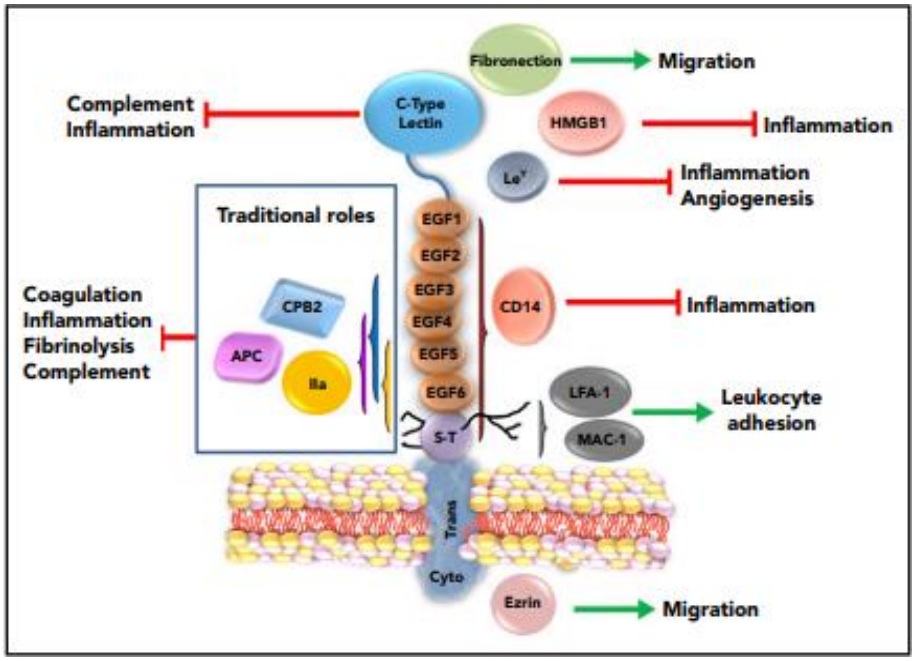


Fig.7 (HouraLoghmani, 2018)

(Schematic of TM structure with putative functional correlates.)

III. II. Physiology of TM

Thrombomodulin production is regulated by the sequences close to its gene (IRELAND H K. G., 1997) and depends from the vascular micro-environment. Many factors such as the presence of tPA (tissue plasminogen activator) and PAI-1 (Plaminogen activator inhibitor) (WADA H, 1998), inflammatory cytokines, immune reactions and coagulation cascade activation influence TM levels. An interesting observation is the sharing of the

receptor site of the thrombin molecule with that of the TM15 molecule, through a relationship based on the concentration of TM. When TM reaches high levels it binds to thrombin with a 1:1 ratio (LOUGHEED JC B. C., 1995), blocking the activation of the coagulation cascade and activating protein C. Both fibrinogen and factor VII participate in the formation of the clot. High factor VII levels favour the formation of thrombin (key enzyme for the development of the arterial thrombus) that is also a mitosis promoting factor for smooth muscle cells and fibroblasts. Thrombin is also a potent platelet activator. The platelet activating factor activates neutrophils and promotes the appearance of leucocyte adhesion molecules on the endothelial cells. Through interaction with thrombomodulin, thrombin can start an anticoagulant cascade, by the activation of protein C. Thrombin thus seems to be able to mediate vascular responses to events that favour coagulation, anti-coagulation, inflammation and proliferation (CT. E. , 1995). Two main mechanisms contribute to thrombotic disease in humans: the anticoagulant pathway of protein C and the heparinantithrombin mechanism. These two physiological systems work together to regulate the coagulation processes. The protein C pathway inhibits the

function of the regulatory proteins factor Va and factor VIIIa. The heparin-antithrombin system inhibits the coagulation proteases.

III. III. Metabolism

Thrombomodulin serum levels also depend from the degradation of cellular TM present on endothelial cells detached by natural turnover. The elimination of TM is guaranteed by renal and hepatic metabolism10. Thrombomodulin levels are elevated in renal failure and this increase shows a significant correlation with creatinine serum levels. Half life of TM is ten minutes (KUWASAKO K, 1997). Serum TM may be expressed as the ratio between soluble TM/serum creatinine, to distinguish the habitual elevation of TM occurring during renal failure from that due to endothelial cell damage. Endothelial cell production of TM is up regulated mainly by cAMP, retinoic acid and IL-4 and IL-1310.

III. IV. Genetic features

The importance of the primary, secondary and tertiary structure of TM is evident in patients with gene mutation. These subjects present an elevated risk of cardiovascular accidents. This risk naturally increases also in association with other metabolic factors. In a study conducted in patients surviving from a myocardial infarction, appeared to be an important predictive index of early myocardial infarction (DOGGEN JMC, 1998).

In the last years, the availability of powerful technologies allowed for a series of studies aimed at investigating novel VTE loci. Several loci have been suggested to affect the risk for VTE, most of them through gene expression in blood and liver tissue (Cushman M., 2016).

Loci acted in concert each other in an additive fashion to produce the final phenotype. A meta-analysis of genome-wide association studies found suggestive evidence for an association of a large series of common gene variants and VTE (Lindström S., 2019).

Clinical characteristics of patients carrying a strong risk factor are significantly different from people with a moderate risk factor. Usually, in subjects carrying a strong genetic risk factor,

VTE occurs at a younger age as an idiopathic or provoked episode, a family history of VTE is often present, and there is a high probability of recurrence (Bucciarelli P., 1999). Animal model data suggest that TM dysfunction or deficiency may be associated with a prothrombotic disorder (Kumada T., 1988) (Vicente C.P., 2012). Recently, transgenic mice with a missense mutation in the TM gene (THBD) corresponding to human E387P, developed a prothrombotic disorder showing an improvement in fibrin deposition (Weiler-Guettler H., 1998), probably due to the thrombomodulin inability to catalyze in vitro thrombin activation of protein C to APC. Although some studies suggested a role for THBD variants, results are conflicting. In addition, genome-wide studies also gave inconsistent findings (Burke J.P., 2005). The association between a disease and a gene variant arises because the latter is directly causative or is in strong association with a causative variant. The occurrence of several factors may explain inconsistencies among studies that addressed the identification of THBD disease-causing variants, including insufficient statistical power, population stratification, various forms of between-study heterogeneity, including differences in genetic ancestry, ascertainment schema, environmental

influences, and time-varying associations (Sugiyama S., 2007). Although it is difficult to known to what extent mutations in the thrombomodulin gene can actually modify its function as modulator of the coagulation and fibrinolysis pathways, it is conceivable that any impairment of TM exposure on the plasma membrane can produce a lower thrombin binding. The following increased amounts of unbounded thrombin are expected to induce an imbalance between its procoagulant (increased fibrin formation) and anticoagulant (impaired APC generation) properties. Indeed, the bleeding phenotype in patients carrying a THBD mutation associated with increased soluble protein levels and decreased thrombin generation further stresses the pivotal role of THBD as key regulator (Dargaud Y., 2015).

AIM OF THE RESEARCH

The goal of the Project is first of all to identify in patients with a positive family history, without any known genetic risk factor, new genetic factors associated with an increased thrombotic risk.

Furthermore, it is important to investigate the unknown genetic causes of these forms of DVT that still do not have a clear diagnostic picture and to clarify the mechanisms of the disease.

Achievement of these results will provide the means to identify tools for correcting these mechanisms and to transform the results obtained from basic research into clinical applications.

Diagnosis and treatment of a DVT is meant to prevent pulmonary embolism which is one of the most dangerous causes for human life. We can further improve by developing, validating, and implementing risk assessment strategies, in order to improve and implement the methods of prevention, diagnosis and treatment of thromboembolic disease.

MATERIALS AND METHODS

I. Selection and Clinical evaluation of patients with DVT

An Italian family was investigated, consisting of close relatives who had a history of recurrent VTE without any identifiable cause despite extensive investigations. After the approval of the local ethics committee, the study was carried out according to the principles of the Declaration of Helsinki; informed consent was obtained from all subjects. For this purpose, a blood sample was collected. DNA samples were extracted from 0.2 mL of whole blood (EDTA-treated) according to standard protocols.

II. DNA Analysis

Patients II-4 and II-5 (Fig.8) were screened using Whole-Exome Sequencing (WES).

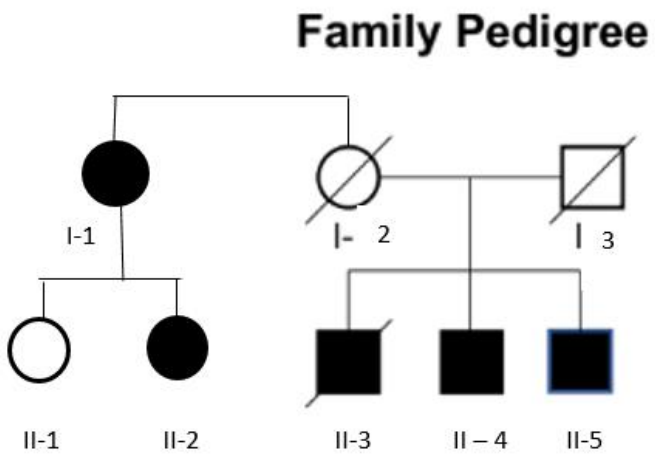


Fig.8

The presence of the variant was investigated by direct sequencing of genomic DNA in all available family members.

WES was outsourced to GATC Biotech (Konstanz, Germany). Sequencing (Next Generation Sequencing, NGS) performed with Illumina DNAPrep with Enrichment Kit on NextSeq 550 (Illumina) platform. Variant confirmation test performed by Sanger sequencing. Average coverage of sequenced regions: 355X. For the interpretation of the results, only the regions with a minimum reading depth of 20X were considered.

The test may not identify single exon duplications and deletions, multiexonic, whole gene, complex genomic rearrangements

expansion mutations of repeated sequences (dynamic mutations), which can be characterized by other techniques. The method has a limited resolution in the identification of mosaicisms. Due to any GC-rich regions and repeated sequences, alignment with the reference sequence may not be reliable. It is possible that a variant is not identifiable due to the failure to capture the genomic region in which it is located. The presence of any homologous chromosomal regions with high sequence similarity (pseudogenes) decreases the sensitivity and analytical specificity of the test. Furthermore, it is possible that a variant may not be recognized as a cause of the clinical phenotype due to incomplete scientific knowledge. The interpretation of the variants was performed on the basis of the indications and clinical data provided at the time of the test request.

III. Bioinformatic analysis

The bioinformatics analysis involves the use of the Variant Interpreter software (Illumina) and the sequences are aligned to the reference human genome GRCh37. For the allelic frequency

reference is made to the gnomAD v2.1.1 population database (Genome Aggregation Database).

Variant annotation and prioritization were performed with open-source software (Variant Studio, Illumina San Diego, Calif). Variants were classified according to American College of Medical Genetics and Genomics (ACMG) standards and guidelines as pathogenic, likely pathogenic, variants of uncertain significance (VUS), or benign (Richards S, 2015).

IV. Prediction of pathogenicity

The effect of the substitution was investigated by using in silico prediction of pathogenicity (Polyphen-2,http://genetics.bwh.harvard.edu/pph2/;SIFT,http://sift.jcvi.org/sift-bin/retrieve_enst.pl;). Thrombomodulin AlphaFold structure prediction model (AF-P07204-F1.pdb) (John J, 2021) was then used as a template to investigate the putative pathogenic effect of the substitution by using established available bioinformatics tools (MISSENSE3D and Swiss-PdbViewer).

V. Thrombin Generation asssay

Thrombin generation in platelet-poor plasma was measured by calibrated automated thrombogram (CAT) in triplicate. CAT is a system to measure Thrombin Generation based on fluorescence according to the Hemker & al. method. (Diagnostica Stago, Italy). PPP was prepared by two centrifugation steps at 3,000 g for 10 min and aliquots were stored at −80°C. TG was measured in all samples, simultaneously. To minimize variation, samples of normal pool plasma were measuredon the same plate.

VI. Analysis of the functional effects of the discovery variation c.748G>C

VI. I. Experimental design and mutational primers synthesis

The mutagenesis primers were designed according to specific guidelines below: the variation to be entered must be within the primer, between two regions of 15-18 mer; The length of the

primer should not exceed 45 mer (to avoid the formation of secondary structures), nor be less than 25 mer; The melting temperature (Tm) must not exceed 78 ° C. The sequence must have a GC content of around 40%; The sequence must end with one or more bases G or C.

PRIMERS THBD c. g748c
MUTATE NUCLEOTIDE SEQUENCE
3'-cgcacctcttgccgccgacgctc-5'
5'-gcgtggagaaccgcggctgcgag-3'

The Forward and Reverse primers have been tested in different annealing conditions (58 ° -60 °) in order to choose the optimal primer and temperature to obtain a greater yield of mutagenesis.

VI. II. Specific site mutagenesis experiment

To evaluate the functional consequences of the identified *THBD* variant, site-directed mutagenesis experiments were carried out to insert the identified mutation into a full-length human *THBD*

complementary DNA plasmid (pCMV6-Vector, Myc-DDK-tagged; OriGene, Rockville,Md) by using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technology, Santa Clara, CA). This is a method that allows mutations to be produced in a specific position in the DNA sequence in a gene. The method allows the insertion of point-like nucleotide variations or the insertion or deletion of short nucleotide sequences.

The PCR mix, by sample, was as follows:
Buffer 10X 5uL
dNTPs mix 1uL
Quicksolution reagent 1.5 uL
Primer F or R (125ng) 1uL
Template DNA 5 uL
ddH2O to a final volume of 50uL

Then add:

1 uL of QuickChange Lightning Enzyme

Cycling Parameters for the QuikChange Lightning Site-Directed Mutagenesis Method

Segment	Cycles	Temperature	Time
1	1	95°C	2 minutes
2	18	95°C	20 seconds
		60°C	10 seconds
		68°C	30 seconds/kb of plasmid length*
3	1	68°C	5 minutes

* For example, a 5-kb plasmid requires 2.5 minutes per cycle at 68°C

At the end of the amplification reaction, the PCR product, by definition without methylation, was digested with a restriction endonuclease (Dpn I: 1 mL) for 2 hours at 37 ° C. This procedure selectively eliminates parental DNA haemimethylated or methylated and therefore not mutagenized.

VI. III. Transformation of E. Coli with mutated DNA-maxiprep

The DNA obtained from digestion with Dpn I was used to transform a chemically competent E.coli strain. The transformed cells were expanded into 200 ml of liquid LB medium containing

ampicillin in order to obtain quantitative quantity of material (maxi prep).

VI. IV. Transfection of the cell line HEK293

Wild-type or mutated *THBD* cDNA plasmid were transiently transfected in human embryonic kidney (HEK) 293 cells (constitutively deficient in THBD). Transfections into HEK293 cells were performed with Turbofectin 8 (Origene, Rockville, MD, USA). The human embryonic kidney cell line "Human Embrionic Kidney" (HEK293) was easily used for transfection experiments. Cells are cultured in Dulbecco Modified medium (DMEM high glucose) supplemented with 10% fetal bovine serum (FCS), 2% Glutamine and 0.1% gentamicin (invitrogen, Milan, Italy) until use. The cells were kept in an incubator at 37 ° C with 5% CO2.

VI. V. Quantitative Real Time PCR

To assess amounts of expression of recombinant THBD, mRNA expression levels of wild type or mutant THBD were analyzed 48 hours after transfection by Quantitative Real Time PCR. Total RNA was extracted from transfected cells. The mRNA reverse transcription was performed by SuperScript IV First Strand Synthesis System (Life Technology, Brooklyn, NY, USA). All samples were assayed on a CFX96 Touch Real-Time PCR detection System using iQ SYBR green supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) (Ariano A, 2020). The relative amount of the transcripts of Wild-type or mutated THBD were evaluated. GAPDH was used as endogenous reference genes. CFX manager software were used to perform experiment setup and data analysis. (p value less than 0,05 was considered statistically significant). To analyzed recombinant THBD protein levels, Western blotting of cell lysates were performed using a monoclonal anti-THBD antibody [PBS-02] (ABCAM).

VI. VI. Western blotting

To analyzed recombinant THBD protein levels, Western blotting of cell lysates were performed using a monoclonal anti-THBD antibody [PBS-02] (ABCAM).

VI. VII. **Fluorescence-activated Cell Sorting**

Cellular localization of expressed wild type and mutant THBD constructs was studied using immunofluorescent labelling and confocal microscopy analysis (d'Apolito M, 2019). Cell surface expression of wild type and mutant THBD constructs was investigated in non-permeabilized HEK293 cells using flow cytometry. Transfected HEK cells were trypsinized, rinsed with phosphate-buffered saline, and blocked in phosphate-buffered saline and 1% normal goat serum for 15 min. Cells were then labeled with an anti-VEGFR-2 antibody coupled to phycoerythrin for 1 h (R&D Systems Inc. Minneapolis, MN), washed with phosphate-buffered saline, fixed in 2.5% paraformaldehyde, rinsed, and sorted using AMNIS flow cytometer. (*Luminex Corporation*, Austin, USA) set on channel

2. Data were analyzed using Amnis IDEAS software subtracting the values of the negative control. Scatter plot was obtained by plotting fluorescence intensity (Ch 2) on x-axis vs. side scatter (Ch 06) on y-axis. the single and focus cells events were gated, and finally 20,000 single cell events for sample were acquired. The percentage of green positive cells (channel 2, 488 nm excitation laser) and mean fluorescence were analyzed using Amnis IDEAS software subtracting the values of the negative control. Brightfield and fluorescent images for any single cell event were collected.

VI. VIII. Confocal microscopy

Specific fluorescence was identified by confocal microscopy using the Leica TCS SP5 (Leica) equipped with argon-krypton (488 nm), green-neon (543 nm), and helium-neon (633 nm) lasers. To stain the nuclei, samples were incubated with TO-PRO (Invitrogen, Molecular Probe). The slides were then mounted in Gel Mount (Biomed Corp.) and sealed. Images viewed through a 60X oil immersion objective were acquired recorded at the same photo multiplier tube, pinhole aperture, and laser voltage setting,

and analyzed with Leica Application Suite Advanced Fluorescence software (LAS AF, Leica). In detail, each image was represented with 1,028 × 1,028 pixels measuring 387.5 × 387.5 μm^2 each, and recorded with a line mode to reduce background noise (average on two scanning images). Image acquisition was performed selecting specific domains of the emission spectrum.

RESULTS

An Italian family, in which many relatives had a history of recurrent VTE without any identifiable cause despite extensive investigations, was examined. (Fig.9) The index case (II-5) was a 53-year-old man, with a previous documented thrombosis (42 yrs) in the right-sided popliteal vein and pulmonary embolism. After cessation of the anticoagulation, a new episode of right-sided popliteal vein thrombosis occurred (49 yrs). A brother (II-4) suffered from a thrombotic event in the left-sided popliteal vein (32 yrs) and from a recurrent event 10-yrs later. The remaining brother (II-3) had a thrombosis in the right-sided popliteal vein (43 yrs). Then, he died of an accident at work (46 yrs). In addition, a first cousin of the propositus (II-2) suffered from a thrombotic event in the right-sided popliteal vein (61 yrs) and a child of her (III-3) of recurrent venous thromboses. Whole exome sequencing of the two living affected siblings (II-4, II-5), performed assuming a dominant mode of inheritance, identified the exon 1 *THBD* variant c.748G>C (NM_000361.3), predicting

an amino acid change protein (p.G250R) located in the EGF-1 domain. Direct Sanger sequencing (Applied Biosystem, Foster City, CA, USA) (Ariano A, 2020) (Sansonno D, 2014) confirmed the presence of the heterozygous missense mutations in both II-3 and II-2 and in a first cousin of the propositus (II-2) and her asymptomatic daughter.(III-4) (Fig.9). The *THBD* variant was novel, being unreported in GnomAD and in BRAVO, and *in silico* predicted to be deleterious.

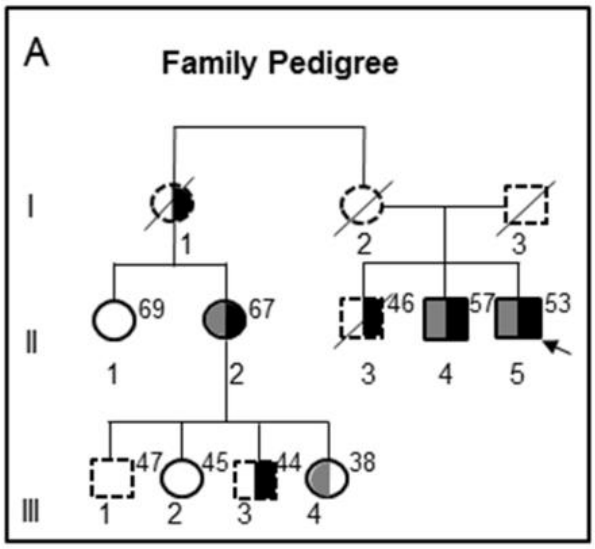


Fig.9

(Italian family investigated. Family tree showing cosegregation of the clinical phenotype (black half-filled symbol) and the THBD-250R variant (gray half-filled symbol). The dashed symbol indicates a family member who was not investigated. Current age years are indicated above the symbol.)

Multiple amino acid sequence alignment of THBD orthologs from different species showed that G250 was conserved across all the analysed species and suggested that this amino acid change might cause structural defect and modify THBD function. (Fig 10)

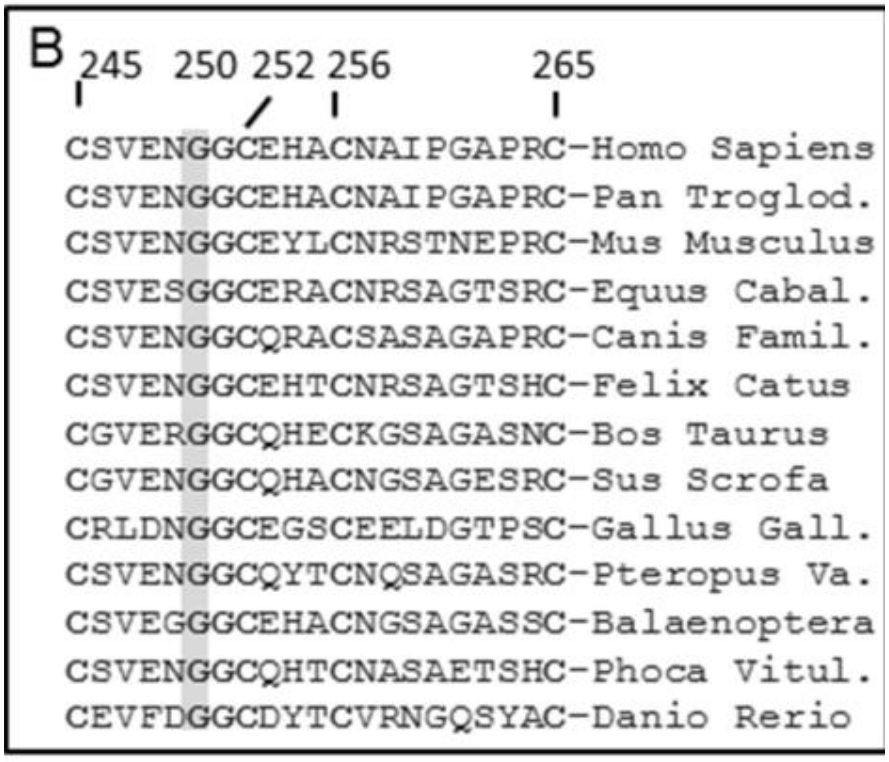


Fig.10

(Alignment of a portion of the THBD EGF-1 domain containing the amino acid position #250 (highlighted in gray) along different species. Conserved residues are indicated (bold).)

Thrombomodulin AlphaFold structure prediction model (AF-P07204-F1.pdb) was used as a template to investigate the putative pathogenic effect of the p.G250R.

The *in silico* analysis (MISSENSE3D; http://www.sbg.bio .ic.ac. uk/ missense3d/) predicted a detrimental effect on the domain structure (Fig.10). Indeed, the substitution replaces a buried uncharged residue (250G: Relative Solvent Accessibility, RSA, 0,0 %) with a charged residue (250R: RSA 4,4 %). This substitution triggers clash alert. The local clash score for wild type is 7,65 and local clash score mutant is 36,85. (Fig.11) shown comparison of the predicted structures of both wild type and mutant protein (Swiss-PdbViewer (https://spdbv.unil.ch/), Clashes is displayed in pink dotted lines in mutant protein.

GLY250 ARG250

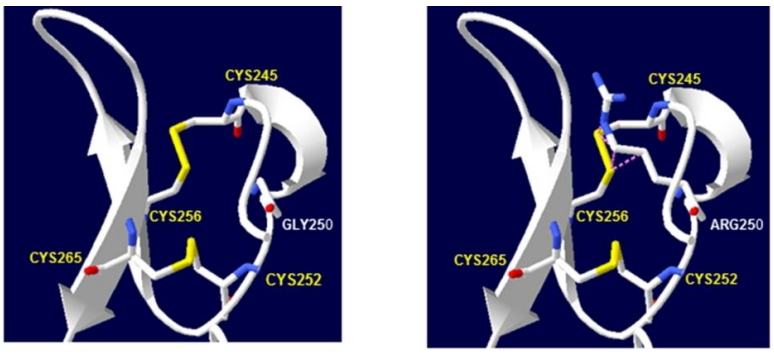
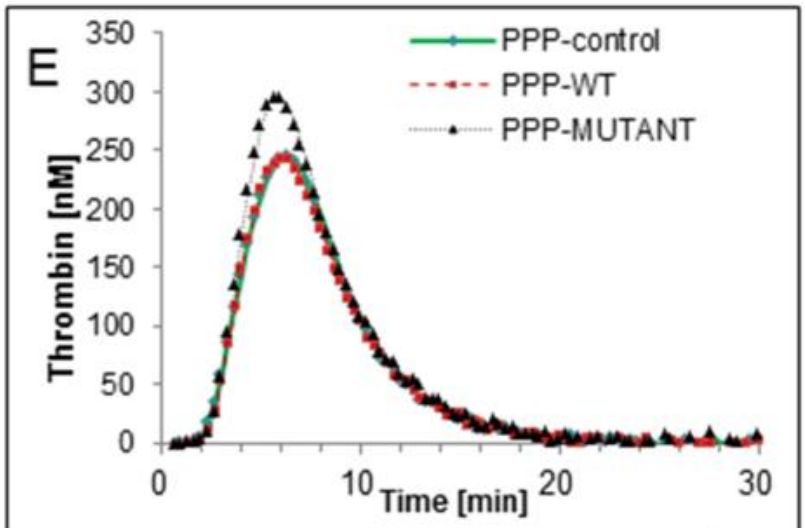


Fig.11

(Comparison of the predicted structures of both wild type and mutant protein using SwissPdbViewer (https://spdbv.unil.ch/), Clashes is displayed in pink dotted lines in mutant protein. THBD mRNA production and western blot analysis.)

Thrombin generation (TG) assays are used widely to investigate the thrombin generating capacity in an asymptomatic THBD-250R carrier (III-4), not assuming oral anticoagulants, and her sister (III-2), who carries only the 250G allele. A samples of normal pool plasma were measured on the same plate. TG in PPP was measured by calibrated automated thrombogram (CAT) in triplicate (Fig.14). III-4 shown an higher thrombin generation with an increased ETP (nM*min) and peak (nM) values, indicating a hypercoagulable state as compared with her sister and controls.



	ETP_(nM•min)±SD	Peak_(nM)±SD
PPP-Control	1616,98±9,92	246,12±3,17
PPP-Wt	1607,42±11,56	244,15±6,33
PPP-Mutant	1818,81±11,4	300,46±2,59

Fig.14

THBD mRNA expression and western blot analysis (Fig.12F-G) showed that THBD expression was similar in transfected HEK293 cells with the THBD-250G or the THBD-250R allele (p > 0.05 NS non significance). Thus, the THBD-250R variant did not appear to modulate THBD mRNA synthesis and protein expression levels, suggesting that the new variant is unlikely to haveany significant effect on gene expression.

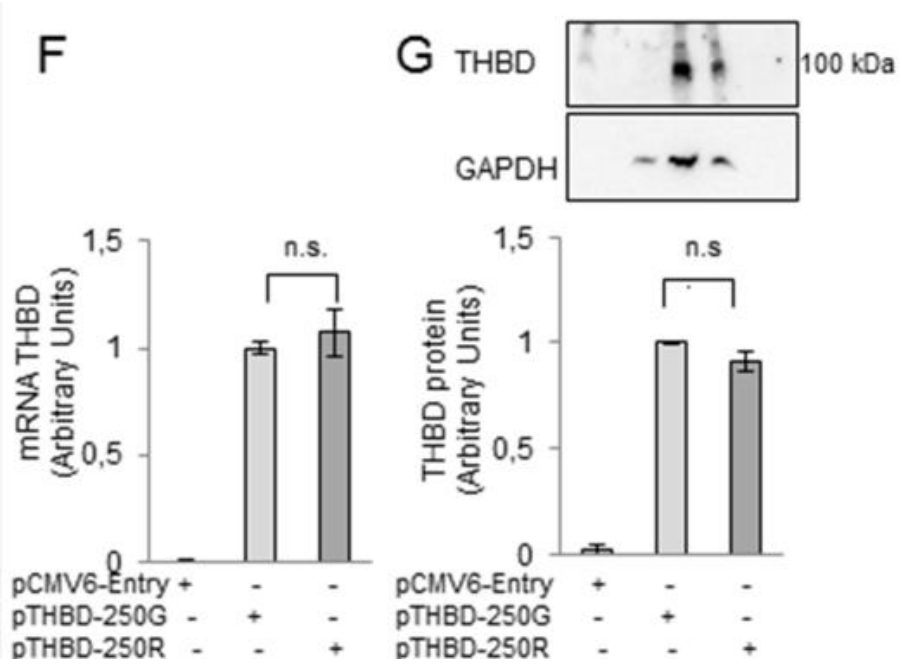


Fig.12

In permeabilized fixed THBD-250G overexpressing cells, the protein was distributed predominantly on the plasma membrane

surface, with little evidence of intracellular localization. (Fig. 13A) In contrast, in THBD-250R transfected cells, an intracellular punctate pattern was observed with a clear-cut decrease of the cell surface membrane fluorescence intensity. Indeed, the distribution of the signal of mutant protein throughout the cell suggests that the intracellular signal detected comes from the ER. The different pattern of cell distribution suggests that the THBD-250R variant decreases its localization on the cell membrane. The intracellular location was confirmed by parallel experiments in which permeabilization was not performed before fixation. In this case, fluorescence was markedly lower on the cell surface (Fig. 13B). Flow cytometry analysis detected lower amounts of cells expressing on surface THBD-250R (Fig. 13C), with an approximately 43% reduction of gate as compared to cells expressing THBD-250G, confirming that the THBD-250R subcellular distribution is significantly modified with a lower localization on the plasma membrane.

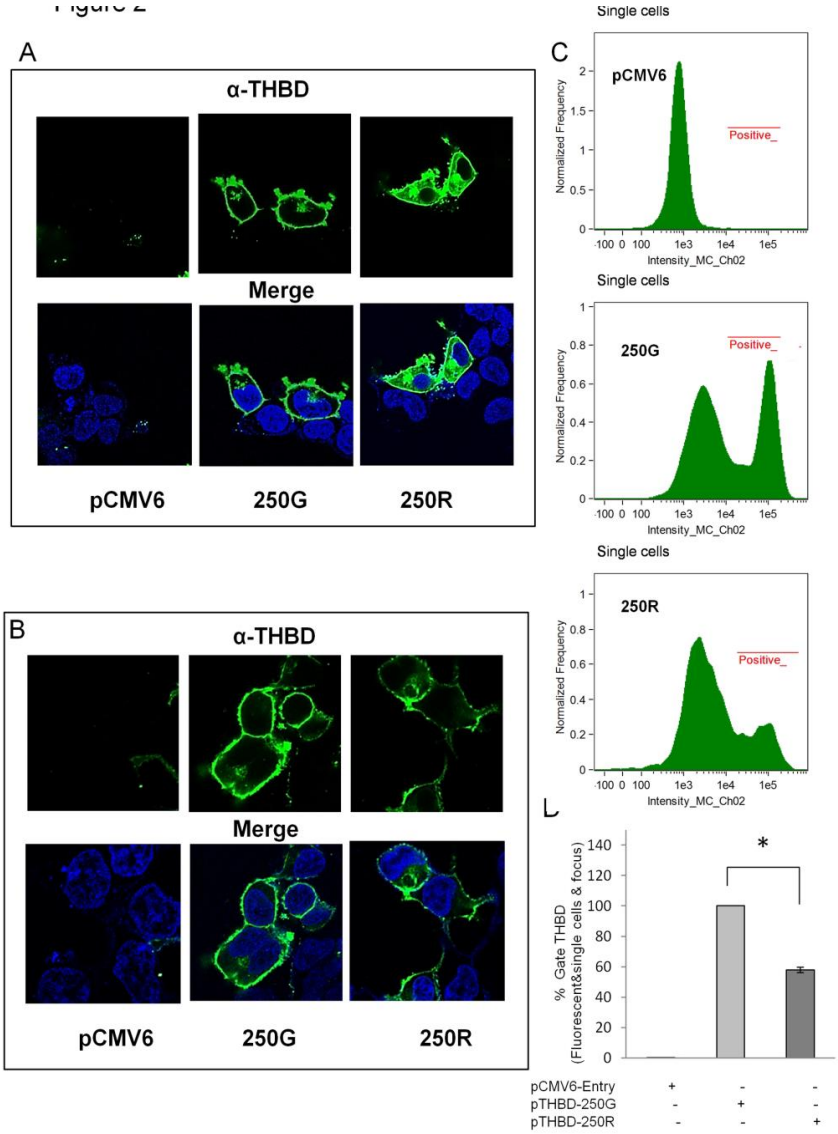


Fig.13

(Confocal microscopy in permeabilized (A) and not permeabilized (B) HEK293 cells transiently transfected with the wild-type and mutant construct. Green staining documents the presence of THBD. Blue staining identifies the nucleus. C. Graphical representation of THBD distribution as determined by flow cytometry analysis. p.CMV6: empty plasmid. 250G: wild-type allele; 250R: mutant p.250R allele. Similar data were found in two additional experiments. Data are the mean ± S.E. from three independent experiments. *p <0.01 Cells overexpressing the wild-type p.G250 allele (THBD-250G) compared to the mutant p.250R (THBD-250R). 190x253mm (300 x 300 DPI))

DISCUSSION

All data indicate that the THBD-250R variant is not able to sufficiently target the plasma membrane, likely preventing efficient post-translational processing or, alternatively, via an increased internalization. In the present study, was searched for new causative genetic defects in an Italian family with unexplained VTE using the whole exome sequence approach. Massive sequence analysis of two affected relatives improved diagnostic rate by facilitating sequence variant analysis and enabling detection of a novel disease-causing gene. In this family, was identified a novel rare THBD missense mutation, with an autosomal dominant inheritance, that resulted in a variant protein (250R). The G250R substitution greatly affected cellular localization of the protein, also in absence of thrombin binding. Because of the impairment of THBD exposure on the plasma membrane, the 250R variant can produce a lower thrombin binding. Indeed, the bleeding phenotype in patients carrying a

THBD mutation associated with increased soluble protein levels and decreased thrombin generation further stresses the pivotal role of THBD as key regulator (Langdown J, 2014). The following increased amounts of unbounded thrombin are expected to induce an imbalance between its procoagulant (increased fibrin formation) and anticoagulant (impaired APC generation) properties. The expression characterization of the previously unreported THBD 250R variant lends support to findings that carriership of THBD variants with an impairment of anticoagulation properties may represent a genetic risk factor for VTE. THBD is known to have additional functions and plays an important role in modulating inflammation through several indirect and direct pathways, which may also contribute to protection against venous thrombosis (Weiler H, 2003) . In mouse models, the lectin-like domain was demonstrated to play a pivotal role in the regulation of inflammation suggesting that reduced THBD exposure on the endothelial surface increase the risk for thrombosis (Conway EM, 2002). Downregulation of THBD may modulate the risk for thrombosis also via an impairment of its regulation of inflammation and complement system.

CONCLUSION

In conclusion, functional analyses of the THBD 250R variant show that defective THBD folding and trafficking to the plasma membrane could underlie the susceptibility to venous thrombosis in the family investigated, further suggesting that THBD loss of function mutants could be an underappreciated cause of thrombophilia. The result of this project helps to increase knowledge on the causes of thrombophilia, in order to stimulate new research to identify therapeutic alternatives, taking into account the genetic profile of the subject, giving patients the opportunity to have a significant improvement in therapy.

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