Congenital Prothrombin Deficiency: Diagnosis and Management

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7.1 Introduction

Coagulation factor (F) II (prothrombin) is a vitamin K-dependent coagulation factor, which plays a pivotal role in blood coagulation cascade. Prothrombin is a 72-kDa glycoprotein that synthesized as an inactive zymogen by hepatocytes [1]. Prothrombin is activated to thrombin by prothrombinase complex, which consists of the activated FX (FXa), FVa, and calcium [2]. Thrombin is a multifunctional enzyme, which converts fibrinogen to fibrin in the blood coagulation cascade. In addition, this protein has different roles including platelet, FXIII, FV, FVIII, and protein C activation [3, 4]. Congenital FII deficiency, which was first reported by Quick in 1947, is among the rarest autosomal recessive coagulation disorder (similar to FXIII deficiency, please refer to Chap. 13) with incidence of 1:two million in the general population. This disorder mostly presents in regions where parental consanguinity is commonly practiced [5]. Based on the FII activity level, prothrombin deficiency is classified into three groups including mild, moderate, and severe forms with >10%, <10%, and undetectable levels of FII activity, respectively [6]. Moreover, this disorder can be classified into 4 main phenotypes including hypoprothrombinemia (type I deficiency) with concomitant decrease of both FII activity and antigen levels, dysprothrombinemia (type II deficiency) with normal or slightly decreased level of FII antigen, but low level of activity, hypo–dys or dys–dys forms, and combined deficiency of prothrombin and other γ-carboxylated coagulation factors [6, 7]. The most common clinical manifestations of this disorder are mucosal bleeding,

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_7

hematoma, and post-surgical bleeding [3]. Life-threatening bleedings including central nervous system (CNS) bleeding, gastrointestinal (GI) bleeding, and umbilical cord (UC) bleeding are rarely presentations in affected patients [3, 8]. About 60 causing mutations in *F2* gene are identified, and 80% of them are missense. Other mutations are insertion/deletion (10%), nonsense (6%), and splice site mutations (4%) [9]. FII deficiency is diagnosed based on family history, clinical manifestations, and routine and specific coagulation laboratory tests. FII deficiency is suspected through prolonged prothrombin time (PT) and activates thromboplastin time (aPTT) and is confirmed by FII assays. The one-stage PT-based assay is the most commonly used FII activity assay technique [3]. Since there are no specific available prothrombin concentrates, prothrombin complex concentrate (PCC) and fresh frozen plasma (FFP) are the treatments of choice [10].

7.2 Coagulation Factor II (Prothrombin) Structure

Coagulation FII (prothrombin) is a vitamin K-dependent glycoprotein, which plays a pivotal role in blood coagulation system. Prothrombin is a 72-KDa glycoprotein and synthesized in liver and needs post-translational carboxylation to become active. This protein circulates in blood stream at the concentration of 0.1 mg/mL with half-life of 60 h [6].

Prothrombin mapped to centromeric region of 11p11-q12 with length about 21 Kb and composed of 14 exons and 13 introns (Fig. 7.1). This protein consists of 4 fragments and 579 amino acid residues including gamma carboxyglutamic (Gla)

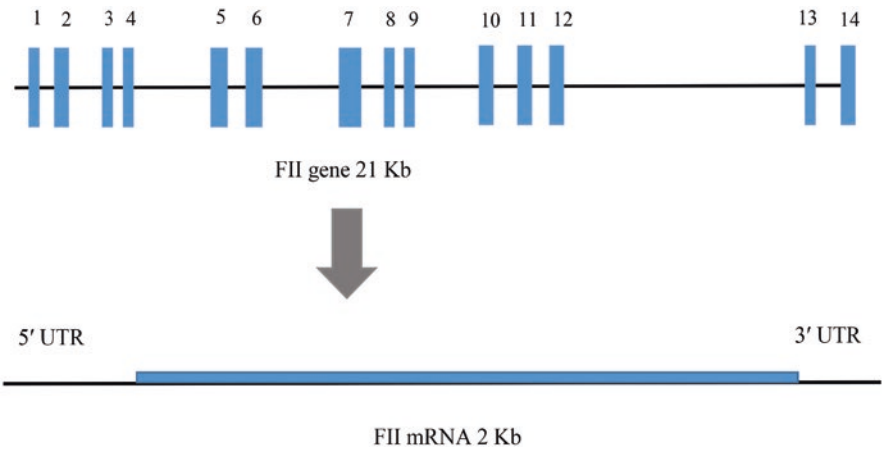

Fig. 7.1 factor (F) II structure. The schematic presentation of *F2* gene. The *F2* gene is composed of 14 exons and 13 introns covering a 21 kb region. This protein is mapped on short arm of chromosome 11 (11p11-q12)

domain (residues 1–46), kringle-1 (residues 65–143), kringle-2 (residues 170–248), and protease domain (residues 285–579). Protease domain includes chain A (residues 285–320) and chain B (residues 321–579) [1].

Thrombin is highly homologous with serine proteinases including chymotrypsin. Crystal structure of thrombin shows that this protein consists of the active site, exosites I and II, and different loops including γ-loop, 60-loop, and loop which contains Na^+ binding site [11]. Thrombin like chymotrypsin has serine residue (Ser195) which in conjugation with other residues including His57 and Asp189 forms the active site that is necessary for target peptide bond's nucleophilic attack [12]. In addition, surface of this thrombin is exposed to loops and is charged patched which is known as exosites around various residues in the active site. Thrombin contains two exosites including I and II which centered different residues including Lys36, His71, Arg73, Arg75, Tyr76, Arg77a, Lys109/110 and Arg93, Lys236, Lys 240, Arg101, Arg233, respectively. The roles of exosites are interaction with thrombin's cofactor and substrates [13, 14].

Moreover, thrombin contains different loops such as γ-loop and 60-loop that surround the active site. The 60-loop, which causes structural rigidity, interacts with residues in substrate's amino-terminal side, while the γ-loop, which is more mobile, interacts with substrate's residues in the carboxyl terminal side. In addition, thrombin has another loop that contains Na^+ binding sites. This loop influences thrombin function allosterically by promoting the binding and hydrolysis of its substrates. These substrates include fibrinogen, FV, FVIII, and PAR1 [11, 15].

7.3 Synthesis of Prothrombin

Initially, prothrombin is produced in liver as a prepro-prothrombin which consists of signal peptide. Following the removal of signal peptide by signal peptidase, pro-prothrombin is generated. Pro-prothrombin has a Gla domain which contains 10 glutamic acid residues in the N-terminal region [16]. Vitamin K-dependent carboxylase catalyzes the conversion of all 10 glutamic acid residues of pro-prothrombin to Gla (γ-carboxylation) and produces the prothrombin (Fig. 7.2). Gla domain is involved in binding of prothrombin to the anionic phospholipid surfaces on the activated platelets and also on vascular injuries in the presence of Ca^{++} [1, 17, 18]. Following the γ-carboxylation, the propeptide is removed and the mature zymogen is generated. Then three N-linked carbohydrates were added which are located in kringle-1 and serine protease domain [16, 19].

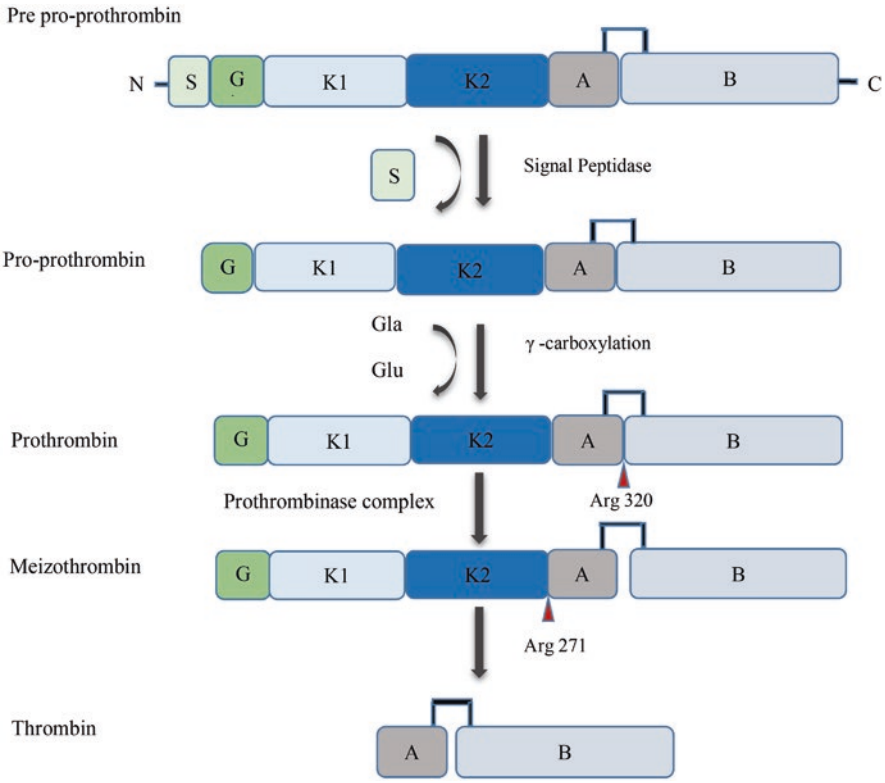

Fig. 7.2 Prothrombin synthesis and thrombin generation. Pre-pro-prothrombin is a precursor of prothrombin, which is synthesized in the liver. Following the removal of signal peptide by signal peptidase the pro-prothrombin which consists of Gla domain is generated. The vitamin K-dependent carboxylase catalyzes the conversion of glutamic acid residues of pro-prothrombin to Gla (γ-carboxylation) and prothrombin is produced. Prothrombinase complex which consists of factor (F) Xa and its cofactor (FVa) cleaves prothrombin and thrombin is produced. In an alternative pathway, prothrombinase complex cleaves prothrombin in two sites, the first cleavage occurs at Arg 320 residue and active intermediate meizothrombin is generated. The second cleavage occurs at Arg271 residue that results in conversion of meizothrombin to α-thrombin. *S* signal peptide, *G* Gamma-carboxyglutamic acid-rich domain, *K1* Kringle-1 domain, *K2* Kringle-2 domain

7.4 Thrombin Generation

Prothrombin activation which leads to α-thrombin generation is a critical step in coagulation cascade. α-thrombin generation is mediated by prothrombinase complex which is composed of FXa and its cofactor FVa that are assembled in negatively charge surface provided by activated platelet in presence of Ca^{++} ions [2]. Recent cryo-EM studies have provided the structural details with a resolution of 4.1 Å of the interaction between the prothrombinase complex and the zymogen prothrombin molecule [20]. This study showed that in the prothrombin-fVa–fXa

complex, the Gla domains of fXa and prothrombin align on a plane with the C1 and C2 domains of fVa for interaction with membranes. Prothrombin and fXa emerge from this membrane plane in curved conformations able to bring their protease domains in contact with each other against the A2 domain of fVa. The ^{672}ESTVMATRKMHDRLEPEDEE^{691} region of the A2 domain closes on the protease domain of fXa, resembling a lid to fix orientation of the active site. Consequently, the ^{696}YDYQNRL^{702} region of FXa binds to prothrombin and, by sequestering R271 against D697 and directing R320 toward the active site of fXa, makes possible the proteolytic activation of prothrombin [20].

Although FXa is capable of catalyzing this process, but the rate of prothrombin activation is markedly low. When prothrombinase complex is formed the rate of prothrombin activation is raised about 10^5-fold [21]. Prothrombin activation occurs by cleavages in two sites. The first cleavage occurs in Arg271 residue (between kringle-2 and A chain) and generates inactive intermediate fragment 1.2 and prothrombin-2. The second cleavage occurs at Arg320 residue and leads to conversion of prothrombin-2 to α-thrombin. In an alternative pathway, the first cleavage occurs in Arg 320 residue which leads to generation of active intermediate meizothrombin (mIIa) followed by cleavage at Arg 271 and generation of α-thrombin [1, 22]. The rate of prothrombin activation is controlled through cofactor Va and phospholipids. In the absence of FVa, prothrombin activates via prothrombin-2 pathway, while in the presence of FVa, the preferred pathway is meizothrombin intermediate [23].

The proteolytically active thrombin is comprised of two polypeptides, A-chain (36 residues) and the B-chain (259 residues) which are covalently linked by single disulfide bond through the Cys^1-Cys^{122} [24]. Autoproteolysis of thrombin in the A-chain at position of Arg 284-Thr 285, leads to removal of 13-residue N-terminal peptide of A-chain and generation of α-thrombin. In solution, α-thrombin automatically undergoes cleavage of the Arg62-Ile and Arg73-Asn bonds in B-chain and subsequently in the Arg123-Glu and Lys154-Gly bonds, giving rise to formation of β-thrombin and γ-thrombin, respectively. These forms of thrombin are much less active in comparison with α-thrombin [12, 16, 25].

7.5 Hemostatic Roles of Thrombin

Thrombin is a multifunctional serine protease which involved in regulation of numerous pathophysiological coagulation and inflammation processes [4].

7.5.1 Fibrin Formation

The primary role of thrombin is conversion of soluble fibrinogen into insoluble fibrin. During this reaction, thrombin binds to the central E nodule of fibrinogen and cleaves four specific Arg-Gly bonds at the N-terminal of both Aα and Bβ chains, leading to release of fibrinopeptide A (FPA) and B (FPB), respectively (Fig. 7.3). Following the cleavage of FPA, a fibrin monomer also termed as fibrin I is formed.

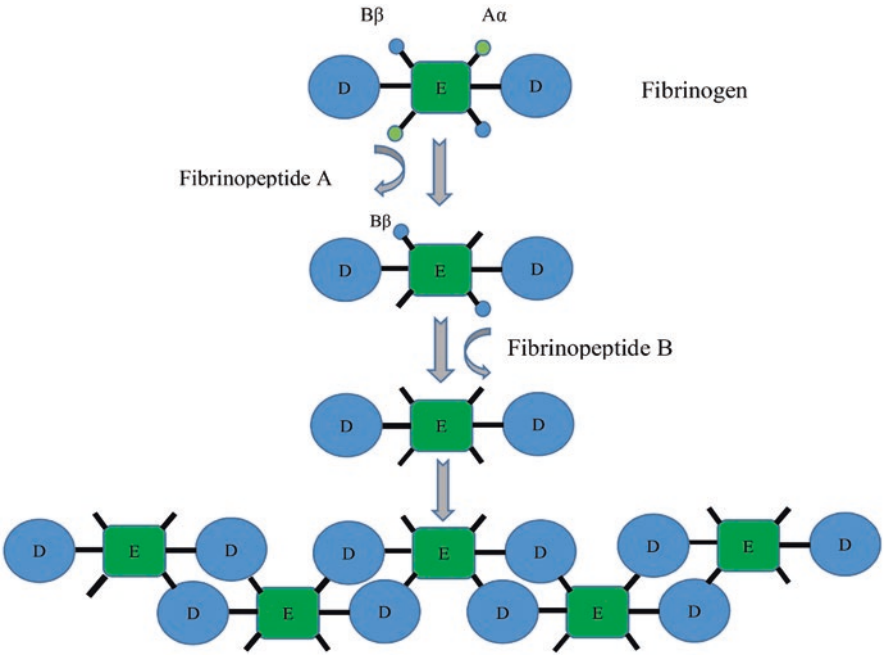

Fig. 7.3 Thrombin binds to the central E nodule of fibrinogen and cleaves this protein at four specific Arg-Gly bonds at the N-terminal of Aα and Bβ chains. This reaction leads to release of fibrinopeptide A (FPA) and B (FPB), respectively. Following the cleavage of FPA, a fibrin monomer which also termed as fibrin I is formed. Fibrin I polymerizes to protofibrils. The deposition of fibrin leads to extensive meshwork formation (please refer to Chap. 6, Congenital fibrinogen disorders, diagnosis, and management)

Then fibrin I spontaneously polymerizes to protofibrils. Cleavage of FPB results in generation of fibrin II protofibrills. The deposition of fibrin leads to formation of extensive meshwork which by surrounding the platelets form the stabilized clot [26, 27].

7.5.2 Factor XIII Activation

In the final stage of blood coagulation, thrombin activates FXIII by cleavage of an activating peptide on the A-subunit of FXIII (FXIII-A) after Arg37 (Fig. 7.4). Following the cleavage, the activation peptide is released, and the active site is exposed. Therefore, the transglutaminase cross-linked fibrin fibrils and increases clot's mechanical strength (Please refer to Chap. 13) [28].

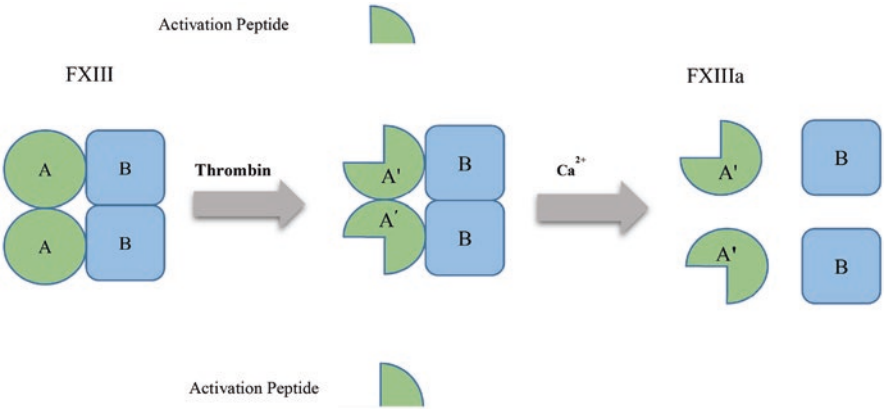

Fig. 7.4 Factor (F) XIII activation. Thrombin cleaves FXIII-A subunit (Arg37). Following the cleavage, the activation peptide is released and the active site is exposed. FXIII-A and FXIII-B subunits are separated from each other in the presence of $Ca^{++,}$ and therefore FXIII became activated

7.5.3 Factor V and Factor VIII Activation

A small amount of thrombin which is generated on the tissue factor-bearing cell acts as a positive feedback of coagulation cascade via activation of FV and FVIII. Following the activation of these two factors, the function of FXa and FIXa is enhanced and therefore leads to increased and more sustained thrombin generation and FXa formation. Thrombin activates these two factors by cleavage of them which is followed by removal of B domain [29]. The cleavage sites in FV are Arg709, Arg1018 and Arg1545. Following the cleavages the A1–A2 domain ionically binds to A3–C1–C2 (Fig. 7.5a). FVIII is cleaved in Arg740, Arg1649, and Arg 1689 residues (Fig. 7.5b). Then the A1–A2 fragments associated non-covalently with A3–C1–C2. The residues Lys70, Arg73, and Trp76 are important for binding of thrombin to FV and FVIII, whereas Arg101 is important only for binding to FV (Please refer to Chaps. 4 and 8) [30, 31].

7.5.4 Thrombin-Activated Fibrinolysis Inhibitors

Thrombin–thrombomodulin (TM) complex inhibits fibrinolysis through activation of thrombin-activatable fibrinolysis inhibitor (TAFI). The activation of TAFI by thrombin occurs via single cleavage at Arg92 and subsequently release of a glycosylated activation peptide. This process requires high concentration of thrombin and is stimulated about 1250-folds by the TM. Activated TAFI (TAFIa) suppresses fibrinolysis by removing C-terminal lysine residues of fibrin that results in disruption of fibrinolytic proteins binding sites [32].

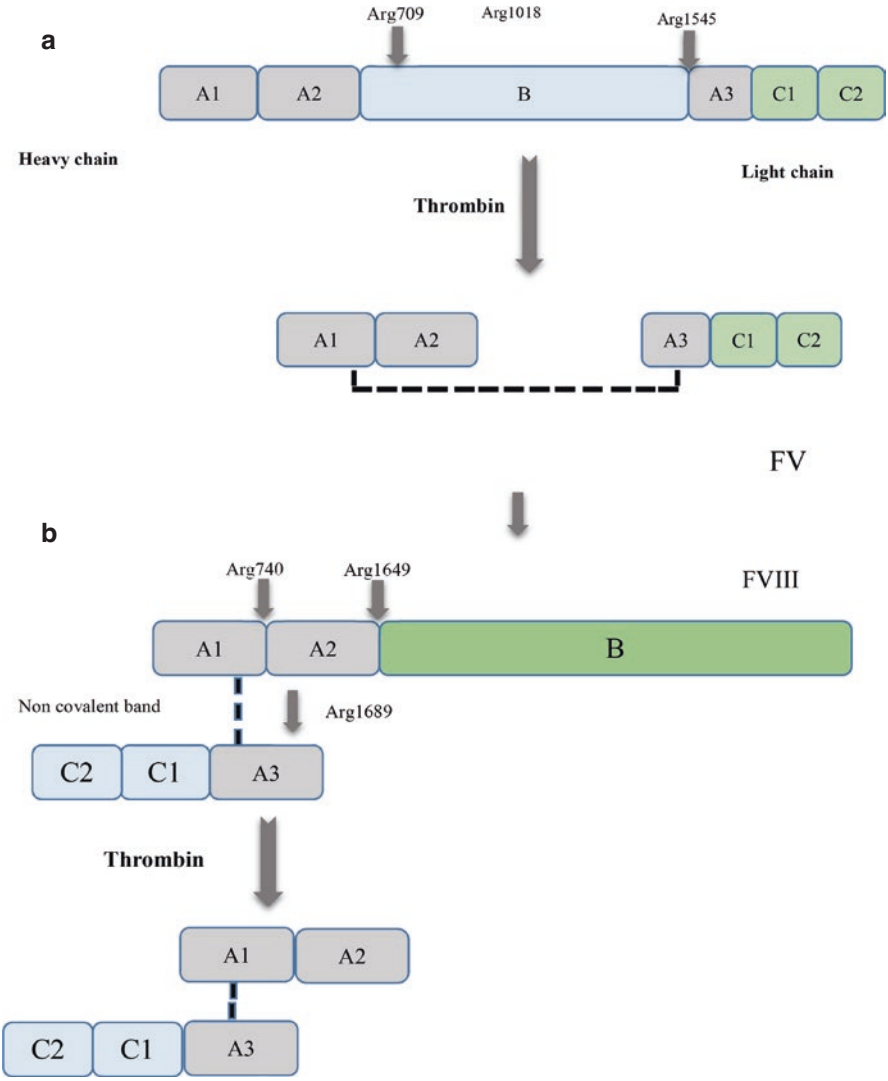

Fig. 7.5 Factor (F) V an FVIII activation. (**a**) FVIII consists of the heavy (A1, A2, and B domains) and light chains (C1, C2, and A3) that noncovalently linked to each other. Thrombin activates FVIII through cleavage between A1-A2, A2-B and at the A3 domains (are shown by arrows). Following the cleavages, the B domain is released and FVIII became activated. (**b**) Thrombin cleaves FV in 3 sites including Arg709, Arg1018 and Arg1545 that leads to FV activation. Activated FV (FVa) consists of 2 chains including heavy (A1 and A2 domains) and light (A3, C1, and C2 domains) chains

7.5.5 Platelet Activation

Thrombin plays an essential role in platelets activation. It causes shape change and release of platelet activators including Adenine di-Phosphate (ADP), serotonin, thromboxane 2, cytokines, and growth factors [33]. Thrombin-mediated effects are preceded by activation of G protein-coupled PAR. Among the PAR, PAR1, 3 and 4 are activated by thrombin, however, PAR1 and PAR4 are the major human platelet thrombin receptors [34].

PAR1 is activated when thrombin cleaves its amino-terminal exodomain to unmask a new receptor amino terminus. Then the new N-terminus serves as a tethered peptide ligand, binds intramolecularly to the receptor and causes receptor activation. PAR1 activation leads to rapid increase in intracellular calcium concentration, while PAR4 induces a more slowly increased and prolonged response in low and high thrombin concentrations, respectively [33].

7.5.6 Protein C Activation

Thrombin binds to the transmembrane protein TM on the surface of endothelial cells and represents its anticoagulant role in coagulation system. In fact, formation of the thrombin-TM complex results in change of substrate specificity of thrombin from procoagulant to anticoagulant reactions by activation of protein C. Procoagulant activity of thrombin stopped by occupancy of exosite I, while cleavage of protein C (PC) at Arg169 leads to activation of this protein. Activated PC (APC) in association with its cofactor, protein S (PS) inactivates the FV and FVIII and therefore inhibits further thrombin formation. APC also neutralizes the plasminogen activated inhibitor-1 (PAI-1) and therefore inhibits fibrinolysis (Fig. 7.6) (Please refer to Chap. 1) [14, 35].

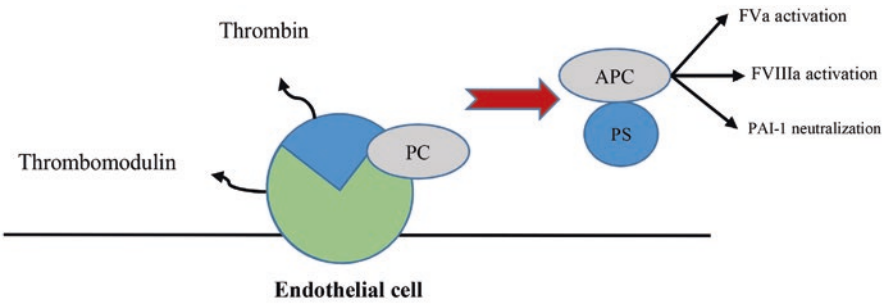

Fig. 7.6 Role of thrombin–thrombomodulin (TM) complex. Thrombin binds to the TM on the endothelial cells and shows its anticoagulant roles. In fact, thrombin–TM complex formation results in activation of protein C (PC). Activated PC (APC) with its cofactor, protein S (PS) inactivates the factor (F) V and FVIII, and therefore inhibits further thrombin formation. In addition, APC inhibits the function of plasminogen activated inhibitor-1 (PAI-1). *PC* protein C, *PS* protein S, *APC* activated protein C, *FVa* activated factor V, *FVIIIa* activated factor VIII, *PAI-1* plasminogen activator inhibitor-1

7.6 Congenital Factor II (Prothrombin) Deficiency

Congenital FII (Prothrombin) deficiency is a very rare autosomal recessive coagulation disorder with estimated incidence of 1 per two million in the general population. The incidence of FII deficiency is higher in regions with high rates of parental consanguinity. Prothrombin deficiency was described by Quick et al. in 1947, while the first case of this disorder was reported by Shapiro in 1969 [5]. Prothrombin deficiency is classified into two main phenotypes including hypoprothrombinemia which is also known as a type I deficiency with low levels of both FII activity and antigen (homozygotes and compound heterozygotes), dysprothrombinemia (type II) characterized by normal or slightly decreased level of FII antigen, but low level of FII activity (homozygotes or heterozygotes), hypo–dys or dys–dys forms (compound heterozygotes) and combined deficiency of prothrombin and other γ-carboxylated coagulation factors [6, 7]. The ratio of type 1 which is also known as a true deficiency to type 2 is 1/2 to 1/3. The complete prothrombin deficiency is incompatible with life. This issue is confirmed in knockout mouse models with experimental prothrombin gene inactivation which results in embryonic lethality. Moreover, based on FII activity, this disorder is classified into three forms. The severe form of diseases is characterized by undetectable FII activity, while the mile and moderate forms of disease are characterized by FII activity >10% and < 10%, respectively. Congenital FII disorders represent approximately 3% of all rare bleeding disorders (RBD) [36, 37].

7.7 Acquired Prothrombin and Thrombin Deficiency

Acquired FII deficiency is a rare disorder, which presents with various clinical manifestations. In acquired FII deficiency, antibodies act directly against FII and cause degradation or activity's blocking of this protein. FII inhibitors occur in different conditions including lupus anticoagulant, hypoprothrombinaemia syndrome, liver disease, use of anti-vitamin-K anticoagulant drugs, vitamin K deficiency, hematological malignancies, nephritic syndromes, and infections. To evaluate the causes of FII inhibitor, an extensive workup is recommended [38]. A severe iatrogenic thrombin deficiency is represented by an over-therapeutical concentration of dabigatran, a specific thrombin inhibitor [39], used for prevention of stroke in patients with atrial fibrillation and in the treatment of venous thromboembolism. There are published data that relate dabigatran concentrations to bleeding risk arising from supratherapeutical blood concentrations of the drug [40].

7.8 Clinical Manifestations

The clinical manifestations among patients with FII deficiency are highly variable. Patients affected by severe FII deficiency, presented with more severe bleeding episodes. The most common bleeding features in patients affected by FII deficiency are

Table 7.1 Clinical manifestations of patients affected by congenital prothrombin deficiency

Bleeding features	Girolami et al. (*n*:26) (%)	Acharya et al. (*n*:16) (%)		Stefano Lancellotti et al. [39] (%)
Umbilical cord bleeding	15.4	Homozygous subject (*n*:10)	Heterozygous subject (*n*:6)	–
Epistaxis	53.8			–
Hematoma and ecchymosis	68	40[b]	83[b]	60
Gingival bleeding	12			–
Post-tooth extraction bleeding	36	–	–	36
Gastrointestinal bleeding	11	13	–	12
Hemarthrosis	44	26	–	42
Hematuria	8	–	–	–
Menorrhagia	100[a]	–	–	20
Post-partum hemorrhage	100[a]	–	–	–
Intracranial hemorrhage	12	20	–	12

[a]These bleeding features were observed in all three women who included in the study.
[b]This study does not report the frequency of mucocutaneous bleeding (ecchymosis, epistaxis, and gingival bleeding) individually.

mucosal bleeding, post-trauma bleeding, and hematoma. Life-threatening episodes including gastrointestinal bleeding and central nervous system (CNS) bleeding were reported in a few patients (Table 7.1) [3, 8]. Heterozygote patients (with plasma thrombin level of 40–60%) are usually asymptomatic and in some occasions present with post-surgical bleeding. Patients with dysprothrombinemia usually show milder bleeding episodes in comparison with true FII deficiency (type I deficiency) [3]. There is no exact correlation between coagulation test results and clinical manifestations in patients with dysprothrombinemia. However, severity of bleeding episodes depends on FII activity, type of FII defect, and FII mutation [3].

Based on different studies, homozygous cases with Arg382His mutation show prothrombin activity lower than 20% with minimal bleeding tendency [41]. Moreover, prothrombin Salakta or Himi are not associated with bleeding tendency [42]. Patients with dysprothrombins Yukuhashi and Scranton have mutation in Na^+ binding loop and do not show hemorrhagic phenotypes [43, 44]. Some cases of FII with mutations of C20209T in 3'UTR present with thrombosis [45].

7.9 Molecular Basis of Congenital Prothrombin Deficiency

Congenital prothrombin deficiency results from different mutations in *F2* gene. These causing mutations are missense mutations (80%), insertion/deletion (10%), nonsense (6%), and splice site mutations (4%) [9].

Approximately 60 variants (46 missense, 3 splicing, 4 regulatory, and 7 frameshift) are accompanied with prothrombin deficiency listed in the Human Gene Mutation Database (HGMD) http://www.hgmd.cf.ac.uk/ac/all.Php [9]. These mutations are shown in Fig. 7.8. Although mutations involve different exons, the prevalence of them is higher in exon 8 to exon 14 [3]. Dysfunctional defects are classified into two groups including activation mechanism defects such as FII Barcelona (Arg271Cys) and Padua (Arg271His) or thrombin's protease activity defects [46].

Defects in protease activity of thrombin result from:

(1) Amidolytic activity defects for both low and macro-molecular substrates which result from impaired catalytic activity of thrombin. FII Molise (Arg418Trp and Stop codon 174) and FII Vellore (Ala362 [56] Thr) cause this type of defect. (2) Defective interaction of thrombin with macromolecular substrates including fibrinogen, TM, and PAR1, which occurs due to mutations in molecular recognition domain of enzyme or insertion loop. FII Quick (Arg382 (67) Cys and stop codon Gln541 (209)), FII Salakta (Glu466 (146) Ala), FII Himi II (Arg388 (73) His) and Arg382 (67) His lead to this type of defect. (3) Recently, another missense mutation p.Arg340 (35)Trp has recently been discovered in a subject with two heterozygous variants: one is a previously reported pathogenic deletion (c.1814_1815del; p.His562Argfs*13), and the other is the novel missense variant (c.1147C > T; p.Arg340 (35)Trp). The numbering system detailed above is based on the primary prothrombin sequence of the mature protein (Fig. 7.7), whereas the number in parentheses indicates the chymotrypsin numbering system of the active thrombin [13]. The mutational spectrum of prothrombin natural mutation is shown in Fig. 7.8.

Heterozygous prothrombin mutations are found in approximately 2% of US white population, while it is rare in other populations (African Americans, Asian, and Native American) [47]. Some polymorphisms in *F2* gene lead to increased FII

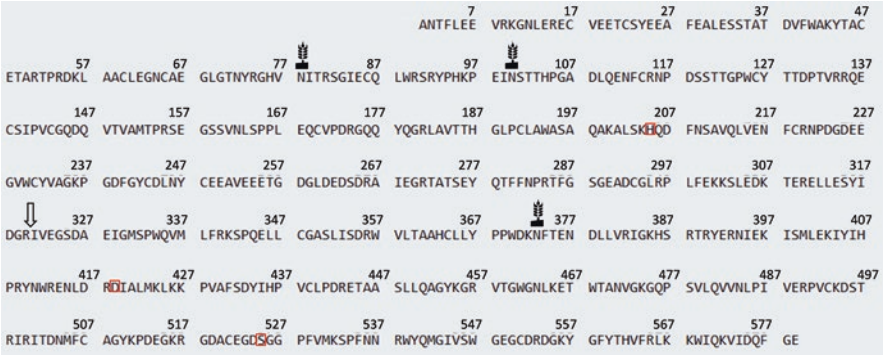

Fig. 7.7 Primary sequence of mature prothrombin. The residues forming the catalytic triad (H-S-D) are shown with a red rectangle at positions 205, 419, and 525, respectively. The putative N-glycosylation sites (N78, N100, and N373) are shown by symbols of black ears. The open arrow shows the factor Xa cleavage site

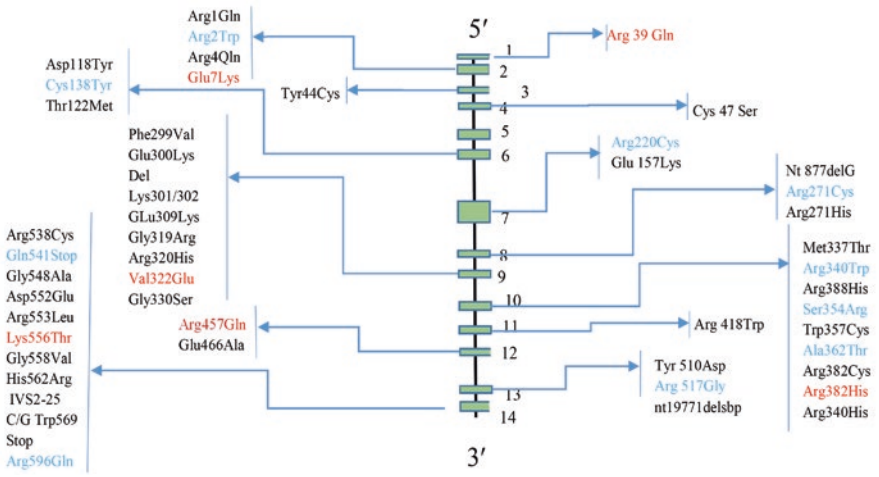

Fig. 7.8 The mutational spectrum of factor II deficiency. Almost all 60 variants (46 missenses, 3 splicing, 4 regulatory, and 7 frameshift) are represented. Exons and introns are shown by boxes and lines, respectively

level. The most common polymorphism of this type is G20210A in the 3′ UTR of gene. This substitution (G to A) is associated with higher prothrombin levels. The prevalence of FII G20210A heterozygotes is higher in Caucasian population. This substitution alters processing, stability, and translocation of prothrombin mRNA. Another polymorphism is A19911G polymorphism located within last intron of prothrombin [48–50]. 19911G allele is associated with mildly elevated plasma prothrombin level (4UdL^{-1} higher than A-allele) and increases the venous thrombosis risk [51]. Of interest, the natural mutations at Arg553 (Arg221A) are characterized by both a reduction of the prothrombin level and at the same time of a paradoxical prothrombotic phenotype [52]. The Arg221A is in fact engaged in the formation of the thrombin domain that interacts with glycosaminoglycans referred to as "exosite II" or "heparin binding site" and thus in the physiological pathway of thrombin inhibition by antithrombin [13]. Thus, any mutation that impairs a correct interaction of thrombin with glycosaminoglycans inhibits at the same time the kinetics of thrombin interaction with antithrombin.

7.10 Laboratory Diagnosis

In general, family history, clinical manifestations, and screening laboratory tests are pivotal for diagnose of FII deficiency. The presence of family history for bleeding disorders can be helpful, but their absence does not exclude the existence of these disorders. FII deficiency is suspected in the presence of prolonged PT and aPTT. For confirmation of FII deficiency, FII activity and antigen level should be performed [53, 54].

7.10.1 Measurement of Factor II Activity

Following the prolonged PT and aPTT, specific assays should be performed. Several assays including PT-based-one stage assay, Tiger snake venom assay, Taipan viper venom assay, Textarin time, Echis carinatus venom assay, and staphylocoagulase assay are available which today the PT-based one stage assay is the most widely used method. Each assay has a special activating substance, which converts prothrombin to thrombin in different ways. In classical one stage assay the presence of FV, phospholipids, and calcium is necessary and tissue thromboplastin acts as an activating agent. Tiger snake venom acts as an activating agent in presence of FV, calcium, and phospholipids while *Echis cainatus* venom activates prothrombin without the need of other factors. Taipan viper venom activates the prothrombin to thrombin in presence of calcium and phospholipids [55, 56].

7.10.1.1 PT- Based One-Stage Assay

PT-based one-stage assay is a specific test which determines the FII activity. This assay is based on PT test and compared the ability of patient's plasma with standard plasma to correct the PT of substrate (prothrombin-depleted plasma). In this assay, equal volume of dilution of patient's plasma and standard's plasma (1 in 5, 1 in 10, 1 in 20, and 1 in 40) are mixed with substrate plasma and are warmed at 37 °C. Then, by adding the equal volume of diluted thromboplastin (recombinant and re-lipidated tissue factor), the clotting time is recorded. For calculation of the FII activity, the clotting time of each dilution of test plasma and standard plasma is plotted against concentration of FII [3, 10].

7.10.1.2 Chromogenic Assay

The chromogenic/florogenic assays can also be used for measurement of thrombin level. In this assay, the amidolytic activity of thrombin in presence of chromogenic substances such as anilides or florogenic peptide including 7-amino-4 methyl coumarin-amides is measured. These chromogenic/florogenic substances interact with thrombin's active side pocket. This method is able to detect any difference between the level of FII in this assay and the level of it in the coagulation assay. Therefore, the result of chromogenic activity is not always the same as coagulation activity. Any difference between this assay and PT-based one stage assay suggests the presence of dysprothrombin which results from mutation of recognition domain of thrombin especially fibrinogen recognition domain [38].

7.11 Determination of Factor II Antigen Level

Enzyme-linked immunosorbent assay (ELISA) is used conventionally for measurement of FII antigen level. In this assay, a specific antibody is coated on the plate. Following the addition of standards and patient's plasma, anti-human prothrombin primary antibody binds to captured protein. Then unbounded antibodies are washed away and the secondary antibody which is conjugated with horseradish peroxidase

(HRP) is added. By adding the substrate, the color change is spectrophotometrically assessed at 450 nm. The concentration of FII in sample is determined by comparing the optical density (OD) of sample via standard curve [10, 57].

7.12 Factor II Inhibitor Assay

For detection of inhibitor against FII, the patient's plasma is mixed with normal pooled plasma (50:50). When the results of PT and aPTT do not normalize, the presence of an inhibitor is suspected. Then inhibitor should be titrated by Bethesda method.

For this method, 2 tubes were prepared for normal plasma and patient's plasma, respectively and 2 tubes were prepared for equal mixture of patient and normal plasma (time-dependent and immediate inhibitors, respectively). APTT and PT should be performed duplicate for all tubes (Please refer to Chap. 8) [58] (Table 7.2).

7.12.1 Bethesda Assay

For inhibitor titration, the Bethesda method is used. The Bethesda unit is defined as amount of inhibitor which neutralized 50% of 1 unit of FII in normal plasma. In this method two dilution series are prepared as follows and incubated at 37 °C for 0 h, 1 h, or 2 h:

1. Dilutions of patient's plasma with an equal volume of normal plasma (normally containing 100% FII).
2. Dilutions of control plasma which contain no inhibitors (equal volume of normal plasma and buffer).

The residual FII is determined based on one-stage PT-assay for each mixture. The dilution with residual FII activity of about 50% is considered for determination of inhibitor strength. For calculation of FII inhibitor, the standard graph of residual FII activity versus inhibitor units is used [10, 57].

Table 7.2 Interpretation of the inhibitor screen for factor II deficiency based on PT and aPTT

Tube	Content	The results of clotting time		
		FII deficiency	Immediate inhibitors	Time-dependent inhibitors
1	Normal plasma	Normal	Normal	Normal
2	Patient's plasma	Prolonged	Prolonged	Prolonged
3	Equal volume of normal and patient's plasma (2 h incubation)	Normal	Prolonged	Prolonged
4	Equal volume of normal and patient's plasma (without incubation)	Normal	Normal	Prolonged

APTT activated partial thromboplastin time, *PT* prothrombin time

7.13 Treatment

Replacement therapy is required in the homozygous cases with bleeding or prior to surgical procedures. The minimum level of FII needed for hemostasis is >10% [59].

As no prothrombin specific concentrate is available for replacement therapy of FII, PCC or FFP are currently used for on-demand therapy and long-term prophylaxis in patients with FII deficiency. Moreover, in cases with mild bleeding, antifibrinolytic agents (tranexamic acid and epsilon-aminocaproic acid) are also recommended (Table 7.3) [10].

Although PCC is a mainstay of therapy in prothrombin, high or repeated doses of PCC have been associated with arterial and venous thrombosis, therefore patients require close monitoring [10]. PCC contains three factors including FII, FIX, and FX. This product is known as three-factor PCC. If the amount of FVII is more than 10%, this product is labeled as a 4-factor PCC (please refer to Chap. 10) [60].

As the incidence of severe bleeding is not high during the neonatal periods, prophylactic replacement therapy is not routinely recommended for this group. On the other hand, prophylactic replacement therapy should be used according to the frequency and type of bleeding [61]. Although it is difficult to make firm recommendations on pregnancy management, it has been suggested that an FII level up of to 25 IU/dL minimizes the bleeding complications during labor and delivery [62].

Table 7.3 Doses and target therapeutic levels in patients with factor II deficiency

Kind of treatment	Recommend level	Dosage of therapeutic level
On demand therapy	>10%	15–25 mL/kg: FFP 20–40 U/kg: PCC
Long term prophylaxis	>10%	20–40 U/kg once a week
Major surgery	>20% (maintaining level)	20–40 IU/kg) before surgery) 10–20 IU/kg every 48 h

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