Congenital Bleeding Disorders

Diagnosis and Management

Akbar Dorgalaleh
Editor

Second Edition

Springer

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ISBN 978-3-031-43155-5 ISBN 978-3-031-43156-2 (eBook)
https://doi.org/10.1007/978-3-031-43156-2

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The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

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Foreword to the 1st Edition

It is a privilege to be given the opportunity to prepare a brief preface to this magisterial and comprehensive work by Dr. Dorgalaleh and his associates.

Indeed, it is not inappropriate for me to have been invited to provide these few words, as I have been a living witness, in the course of over half a century, of the astonishing evolution of the diagnosis and most particularly, the care of patients suffering from inherited bleeding disorders, both in this country and the world.

Care has ranged, during this period, from the use of whole blood for the hemophilias in the 1950s to the current employment of recombinant products and the promise of a revolutionary bipolar monoclonal antibody in hemophilia A (HA), and the successful implementation of gene therapy in both HA and hemophilia B (HB).

Those among us, who have lived through this astonishing clinical and scientific journey, have witnessed a parade of phases in hemophilia care—the initial dearth of therapeutic options; the crippling effects of chronic joint bleeding; the scourge of blood-borne virus infection, and the affliction of inhibitors, which remains current, although a future resolution is within sight.

A few words about the early origins of these bleeding disorders are needed, in order to provide some historical context for this book.

The mutations giving rise to hemophilia are probably of great antiquity because they occur in at least three Orders of placental mammals: the Ungulata, the Carnivora, and the Primates. Hemophilia has been described in the horse (Nossel 1962), nine breeds of dog (Field 1946), as well as in all races of man. The Orders to which these groups belong may have been distinct from the end of the Cretaceous—say 65 million years ago, and the mutations have probably recurred independently many times in all three since they must be lethal in the wild state (GIC Ingram 1976). The mutation rate has been estimated at about $1–4 \times 10^{-5}$ (WHO 1972).

The earliest references to what may have been human hemophilia are attributed to Jewish records of the second century A.D. A ruling of Rabbi Judah the Patriarch exempts a woman's third son from circumcision if two older brothers had died of bleeding following the operation (Katzenelson 1958).

It is now well-known that Queen Victoria herself (1819–1901) was a carrier of hemophilia B, affecting coagulation factor IX. No evidence of the disorder exists among her antecedents, so we must assume that the mutation occurred at spermatogenesis in her father, Edward, Duke of Kent (McKusick 1965). The disorder only manifested itself for the first time with the birth of her eighth child in 1853, when

Leopold, Duke of Albany was born with hemophilia, which proved fatal when he died of a cerebral hemorrhage at the age of 31.

Leopold's sisters, Beatrice and Alice, were both carriers. Alice married the Grand Duke of Hesse, and two of her daughters, Irene and Alix, had hemophilic sons. Alix, better known as Alexandra (Queen Victoria's grand-daughter), married Tsar Nicholas II of Russia, and their son, probably the most famous hemophiliac in the world, was Alexei, who was born in 1904. Alexei was also the most tragic example of the disorder, who created so much anguish in the Romanov family. It was through his successful treatment of Alexei's extreme pain by hypnosis, when he was 8 years old (probably due to a psoas muscle hemorrhage) that Rasputin, the charismatic monk, gained such a sinister influence upon the entire household of the Tsar.

The last known carrier of Queen Victoria's mutation was Princess Alice, wife of the Earl of Athlone, who represented the British Crown at the celebration in Tehran, of Crown Prince Mohammad Reza Pahlavi's marriage to Princess Fowziyeh, the sister of Malek Farouq, the king of Egypt, in 1941.

Until 1964, no one took any interest in the inherited bleeding disorders, such as the hemophilias, in Iran. These were truly clinical orphans, and those unfortunate children suffering from the severe form of hemophilia A, or B who had survived into adulthood, were already hopeless cripples. In addition, they had little recourse to treatment, as the sole available form of therapy was fresh whole blood, carrying infinitesimal quantities of the relevant coagulation factor.

At the newly established Tehran University Clinical Hematology Department at the 500-bed Pahlavi (now Emam Khomeini) Hospital, the small associated laboratory was only equipped to carry out blood counts and May-Grunwald staining and microscopy of patients' peripheral blood and bone marrow aspirates, at the time.

However, a personal grant of 18,000 Pounds Sterling from the Sir Henry Wellcome Trust enabled me to purchase all the equipment required to set up a modern clinical hematology laboratory.

Whilst awaiting the delivery of all these myriad items of equipment from abroad, I became interested in the investigation of inherited bleeding disorders. Using the Thromboplastin Generation Test of Biggs and Douglas (1953), together with the classic Prothrombin Time test of Armand Quick, it became possible to distinguish hemophilia A from hemophilia B, then known as Christmas Disease in the UK, and to carry out bio-assays of these factors, using a broken 37° water-bath and hand-pulled Pasteur pipettes. Although it was exciting to have been able to actually demonstrate the hemostatic defect in the laboratory for the first time in Iran, this academic exercise was of little benefit to the wretched children affected by these bleeding disorders.

As a result of repeated acute hemarthroses, particularly affecting weight-bearing joints, such as knees and ankles, many of them were bed-ridden due to contractures and muscle-wasting. Some had become drug-addicted because of pain and despair. Mothers felt guilty for being carriers; sisters were in an agony of doubt as to whether or not they were carriers of the genetic disorder and would pass it on to their sons. Indeed, in some cases, wives were ostracized and returned to their families, once the husband learned that his spouse was the cause of the disease. The education of

affected boys is disrupted, resulting in unemployment, and a sense of inadequacy. The cost of these inherited bleeding diatheses to society is enormous, not only because of the premature death of potentially useful members of society, but also because, if left untreated, patients end up hopelessly crippled, and a burden upon their families, and the health facilities of their country.

Dr. Judith Graham Pool's discovery of Cryoprecipitate (Pool 1964), allowed for the preparation of a crude, home-made concentrate of Factor VIII in the Hematology Department laboratories. Bottles of cell-free fresh plasma were snap-frozen in a mixture of dry ice and alcohol, and subsequently thawed slowly at 4 °C. A precipitate at the bottom of the bottles contained most of the FVIII, Fibrinogen, and FXIII from the original crude plasma and was stock-piled in deep freeze cabinets for future use. The cryo-supernatant plasma was also stored for use in hemophilia B, burns, and hypovolemia.

Major orthopedic surgery, mainly arthrodesis of knees was successfully carried out in the late 1960s, in cases of severe hemophilia A by a few intrepid surgeons in Tehran, such as Dr. Sheikh ol-Eslamzadeh or Dr. Gorgi, using only cryoprecipitate to prevent bleeding, and circulating FVIII levels were assayed daily, before and after each infusion for at least 10 days. Soft-tissue surgery, such as pyloroplasty and vagotomy for repeated hematemesis, or pulmonary lobectomy for hydatid cyst causing life-threatening hemoptyses were also undertaken with success by Dr. Kazemi, with similar replacement therapy.

It must be emphasized that in the mid-1960s commercial preparations were not easily available anywhere in the world, with the sole exception of Fraction 1-O, pioneered by Birger and Margareta Blomback at the Karolinska Institute in Stockholm, which was later manufactured on an industrial scale by Kabi. The only alternatives were bovine and porcine FVIII, produced in the UK, which, although they were potent, were dangerously antigenic. Indeed, one of the cases I treated with bovine FVIII, developed both thrombocytopenia and a protein-losing nephropathy, probably caused by an immunogenic reaction to this fraction.

Baruch Blumberg had recently reported what came to be known as "Australian Antigen" or hepatitis B surface antigen (Blumberg et al. 1965), and since hemophilic patients had been repeatedly exposed to plasma, even though there had been no history of overt jaundice, it was felt that they should all be screened for the antigen by the Ouchterlony gel-immunodiffusion technique—the only method available at the time. This was the first application of a test for hepatitis B in Iran, and it was found that this viral infection was common among blood donors (Ala 1970).

Inevitably, seeking treatment for hemophilic patients, drew attention to the appalling state of blood transfusion in Iran. Virtually without exception, blood for transfusion, whether in private hospital practice or in government and university hospitals, was procured through disreputable dealers. Professional blood-sellers exploited the poorest sectors of society, who were prey to malnutrition, anemia and hepatitis, as well as drug-addiction. This was also true of the transfusion services of the Red Lion and Sun Society, the Iranian affiliate of the International Red Cross, currently renamed the Red Crescent Society. Even the military hospitals relied solely upon soldiers—never officers—who were ordered to volunteer, in return for

72 h' leave, ostensibly to allow for their recovery. In addition, modern advances in blood group serology, and proper compatibility testing had made little impact upon the rudimentary, fragmented, and grossly commercialized blood services available at the time. Increasing population density and rapid advances in hospital surgery and medicine, together with the growing expectations of both the expanding middle classes, and highly trained medical practitioners, revealed the glaring inadequacies and dangers of the blood services, and set the scene for fundamental reforms in this vital sector of public health infrastructure.

The unsatisfactory state of blood transfusion services led to the conception of a plan, in 1972, for the establishment of a modern, centralized, national service for blood transfusion, based entirely upon the voluntary, unremunerated donation of blood by healthy members of the public. Implementation of such a program called for a veritable social revolution and a profound change in public attitudes, together with an extensive public education and recruitment campaign.

In 1974, the Iranian National Blood Transfusion Service achieved legal status, and within a relatively short time, a technically advanced service, based entirely upon voluntary blood donation replaced the commercialism and inadequacies of the past (Azizi et al. 2015).

In 1971, the World Federation of Hemophilia (WFH) agreed to hold its VIIth international congress in Tehran—the first time such a meeting had been held outside of Europe or Canada (Ala and Denson 1973). This was a ground-breaking meeting in other ways as well, in that the main thrust of the Congress emphasized the impossibility of providing adequate, comprehensive hemophilia care, without the close support of a safe, modern blood transfusion service, which formed an integral part of the national health services.

Even in the 1970s, it was clear that support for the hemophilic population could not remain confined to doctors and scientists alone. There had to be at least minimal participation by parents of affected children, and the patients themselves. Early efforts to establish a viable Hemophilia Society remained unfulfilled for many years. It is a source of satisfaction that today, a strong Iranian Hemophilia Society (IHS) has been established, which is devoted to the interests of patients and their families, which defends their rights as citizens, and acts as their advocate at both national and international levels. The IHS has not merely confined its efforts to the conventional range of activities typical of similar societies elsewhere in the world: social services, dormitory services for patients from the provinces, support for employment and education, counselling affected families, providing information booklets, etc., it has gone much further by creating the first comprehensive, interdisciplinary hemophilia care center in Iran as well. The Iranian Comprehensive Hemophilia Care Center (ICHCC) in Tehran is officially affiliated to the WFH and was inaugurated in April 2001.

A network of hemophilia centers now exists throughout Iran, often affiliated to regional medical schools.

Registered patients suffering from inherited bleeding disorders are covered by a national insurance scheme under the aegis of the Ministry of Health, such that all

their laboratory investigation expenses, as well as replacement therapy with either plasma-derived or recombinant concentrates, which are administered free of charge.

In sum, the authors of this definitive reference book concerning both common and rare inherited hemostatic disorders, meticulously bring together the clinical signs, symptoms, complications, the phenotypic and genotypic diagnoses in the laboratory, together with all the latest forms of treatment currently available, including physiological background, and molecular studies.

This will be an invaluable source of all the relevant, up-to-date, and exhaustively reviewed evidence for post-graduate students, scientists, and research workers in the field.

(Some sections of this piece have been published in a modified form in: Arch. Iranian Medicine (2016);19(3):229–232)

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Preface

Science is experiencing rapid growth, and hematology, particularly thrombosis and hemostasis, is no exception. Over the past 5 years, a significant number of new studies have been conducted and published worldwide in the area of congenital bleeding disorders. These studies have led to relatively significant changes in laboratory diagnosis and clinical management and expanded understanding of the molecular basis of congenital bleeding disorders.

These growing developments in the field have prompted us to prepare a new edition of the book based on the latest documentation and scientific research. In this edition, all aspects of congenital bleeding disorders, including laboratory diagnosis, clinical manifestations, molecular analysis, and management of patients, have been updated.

In this edition, separate chapters have been dedicated to vitamin K-dependent coagulation factor deficiencies, as well as factor V and factor VIII deficiencies. This edition of the book covers both common congenital bleeding disorders, such as von Willebrand disease, hemophilia A and B, and less common bleeding disorders, including rare coagulation factor deficiencies and inherited platelet function disorders.

Outstanding scientists from around the world have contributed to this book. Most of the recommendations are based on strong evidence. In some cases, where data is scarce, available evidence is used.

The goal of this book is to provide evidence-based recommendations and suggestions for the proper and timely diagnosis and appropriate management of patients with congenital bleeding disorders.

Living with such disorders is difficult for affected patients, but a significant number of researchers and scientists from around the world have worked to improve the quality of life of affected patients.

We hope that this revised edition contributes to the further understanding of these conditions, enabling laboratory scientists and clinicians to achieve better diagnosis and management of patients with congenital bleeding disorders.

Respectfully,

Akbar Dorgalaleh
Tehran, Iran

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Introduction to the 1st Edition

I did visit Iran at least twice before the Islamic Revolution. In 1971, the World Federation of Hemophilia held in Tehran its 7th World Congress and in September 1978 I organized in the same city a Hemophilia Workshop on behalf of the same international organization. During the two visits, I was most impressed by the extraordinary and warm friendship that Iranian people expressed for foreign visitors, as well as by the high level of development in the country of blood transfusion services and the advanced degree of clinical and research knowledge in the field of bleeding disorders. The long, useless, and bloody war with Iraq that lasted for a whole decade in the 1980s devastated Iran and made practically impossible for foreigners to visit the country. In addition, many of the excellent scientists and clinicians left and went abroad. I returned to Tehran only in December 1995, prompted by the collaboration with Flora Peyvandi who, native of Iran, had graduated in Medicine at the University in Milan and then became a post-graduate hematology fellow with me. I noticed several changes in the lifestyle of Iranians compared with my previous experiences in the 1970s, but two positive aspects had remained unchanged: the spirit of friendship and collaboration with us foreigners and the high quality of medical services, including those dealing with inherited bleeding disorders. I remember distinctly that during my visit and clinical seminar at the Imam Khomeini hospital in Tehran my attention was drawn by a map of the whole country that identified with colored flags the patients with different inherited coagulation disorders. Together with my Italian colleague Alessandro Gringeri, we were impressed to notice that the flags representing patients with hemophilia A and B were outnumbered by those identifying patients with recessively inherited coagulation disorders (RICD), with absolute numbers much higher than those that I knew for countries like Italy and the UK had general populations not very different in size from that of the Islamic Republic of Iran. Cognizant that global knowledge not only about the molecular basis but also about the most prevailing symptoms of these disorders was rather limited. Flora, the whole staff of clinicians and scientists of the Angelo Bianchi Bonomi, and I developed a strong collaboration program with Iranian clinicians and scientists with emphasis on RICD. This collaboration led to the publication of an array of manuscripts that contributed significantly to extend our general knowledge on these rare disorders. Needless to say, these studies, that made Flora Peyvandi the main scientific authority in this field, were possible due to the enthusiastic collaboration of Iranian clinicians and scientists, of whom the most

active at that time were Manijeh Lak, Sharifian, and Sirous Zeinali, the latter at the Tehran Pasteur Institute. Not surprisingly, the publications on Iranian patients with RICD attracted the attention and interest of international experts other than those from Milan. For instance, Tuddenham, one of the authors of this book, visited Iran during a Summer period, and with the help of Flora Peyvandi managed to publish a seminal study on the molecular basis of the combined deficiency of coagulation factor V and VIII.

The seeds of the international scientific collaboration that Flora and I put on the fertile soil of Iranian hematologists and pediatricians produced subsequently several additional fruits, and this book is clearly witnessing the role that Iran currently plays in the competitive global arena of hemostasis and thrombosis. At a personal level, it was with great pride that owing to my contributions to the advancement of medical sciences I was honored to receive in Teheran in 2008 the Khwarizmi International Award, a most prestigious international initiative of the Iranian Research Organization for Science and Technology, named after a famous Persian mathematician of the seventh century. Flora and I are continuing to collaborate with Iranian scientists, who for the sake of an example were recently major contributors to the landmark SIPPET study published in 2006. All in all, this excellent book and its contents demonstrate clearly the role prominently achieved by clinicians and scientists of the Islamic Republic in an important field of hematology such as that of inherited bleeding disorders.

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Part I

An Overview of Hemostasis and Congenital Bleeding Disorders

An Overview of Hemostasis

1

Maryam Daneshi, Jamal Rashidpanah, and Fatemeh Narouei

1.1 Introduction

The term hemostasis is derived from Greek roots: "heme" means blood and "stasis" means halt; the word means the halt, or stopping, of blood. Hemostasis is a well-controlled physiological process in which the integrity of the circulatory system is maintained after vascular injury. Several cellular and non-cellular components are involved in this process. These components include the coagulation system, platelets, the vascular, fibrinolysis, kinin, and complement systems, and serine protease inhibitors. Or, to put it differently, hemostasis is composed of the vascular system, and cellular and non-cellular components (Fig. 1.1).

1. Vascular system: Endothelial cells, smooth muscle, and connective tissue
2. Cellular components: Platelets, granulocytes, monocytes, and lymphocytes
3. Non-cellular components: Coagulation factors, serine protease inhibitors, fibrinolysis, complement, and kinin systems

Following vascular damage, these components work together closely to stop bleeding and, later, remove the formed clot from the bloodstream. These hemostatic

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_1

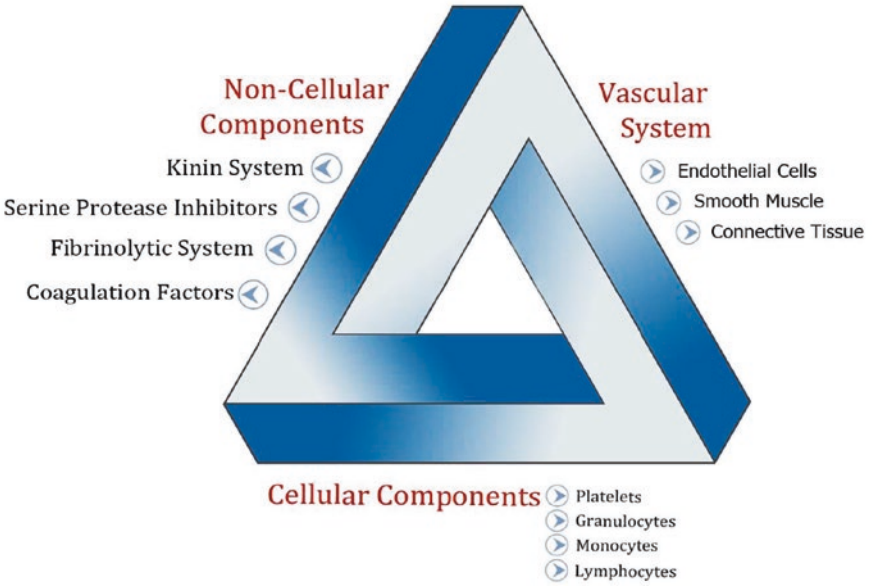

Fig. 1.1 The vascular system, cellular, and non-cellular components maintain hemostasis. Each of the components has several other contributors for circulatory integrity

components also have important roles in other processes, such as wound healing and angiogenesis. The response to vascular injury is prompt and well-controlled to stop the bleeding by:

1. Vasoconstriction
2. Platelet plug formation
3. Blood coagulation [1–3]

Vasoconstriction is the body's first response to vascular injury. This process occurs almost immediately in smooth muscle cells, triggered by the sympathetic nervous system. Direct damage triggers smooth muscles constriction and the release of endothelin-1 from endothelial cells and platelets. This decreases blood flow in the area of injury, thereby reducing blood loss. In the second step, platelets adhere to the sub-endothelium of the vasculature at the site of injury, a process known as adhesion. Platelets change their shape during the process and release their granules, which leads to activation and recruitment of further platelets and induces their aggregation, resulting in formation of a platelet plug. In fact, with vascular injury, sub-endothelial elements, notably collagen types I and III, laminin, and microfibrils, are exposed to circulating blood, causing platelet adhesion, activation, and secretion. Collagen is the main sub-endothelial element that allows platelet adhesion. Adhesion to sub-endothelial collagen is performed by two main platelet glycoproteins (GPs), GPIa/IIa (Integrin α2β1, CD49b/CD29) and GPVI being the principal

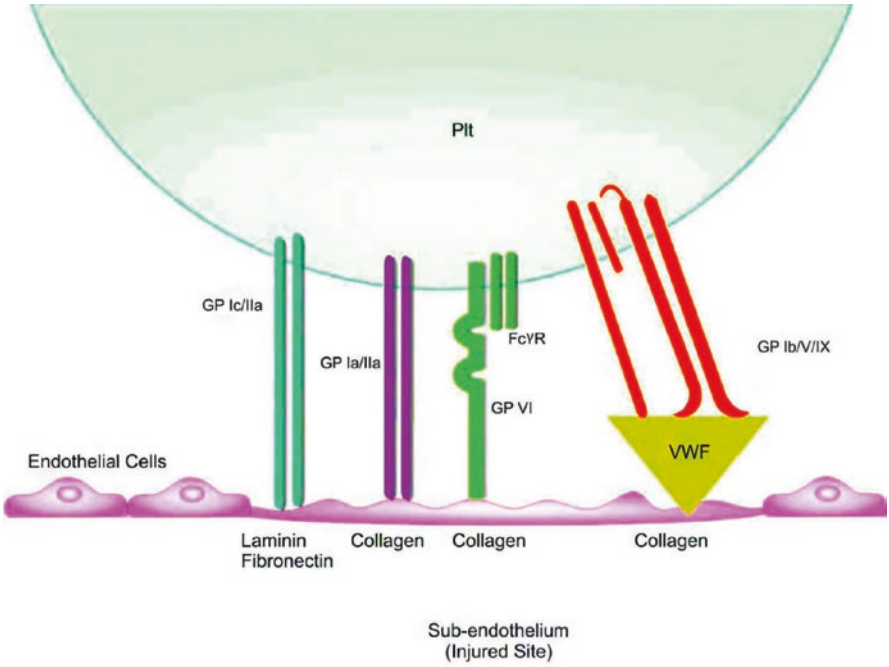

Fig. 1.2 Platelet adhesion, molecular mediations, and sub-endothelial factorsGP-Ic/IIa, GP-Ia/IIa, GP-VI, and GP-Ib/V/IX are the most important GPs that mediate platelet adhesion. GP-Ic/IIa and GP-Ia/IIa directly bind to laminin/fibronectin and collagen, respectively, in the injured site. GP-VI also binds to sub-endothelium collagen directly and FcγR has a main role in signal transduction. GP-Ib/V/IX comprised four chains: GP-Ibα, GP-Ibβ, GP-V, and GP-IX. Its binding to collagen is mediated by VWF. *Plt* platelet, *GP* glycoprotein, *VWF* von Willebrand factor

platelet collagen receptors. GPVI is exclusively expressed by the platelet/megakaryocyte lineage, while GPIa/IIa is not restricted to this lineage. Deficiency of GPIa/IIa and GPVI is extremely rare mild bleeding disorders. The primary adhesion of platelets to sub-endothelium at high shear (high flow rate of blood) is performed by binding GPIb/V/IX (CD42a-d) to von Willebrand factor (VWF) in the sub-endothelial matrix. This interaction helps bind other platelet surface GPs to collagen and other sub-endothelial elements, resulting in firm adhesion of platelets to the injured site [4, 5] (Fig. 1.2).

Activated platelets have a considerable number of agonists that can activate other platelets. These components may be weak platelet stimuli, such as adenosine diphosphate (ADP) and epinephrine, or strong, like thrombin. These agonists lead to further activation and subsequent conformational changes of other platelets. Activation of integrin αIIbβ3 (GPIIb/IIIa) on activated platelets is one of the most important events in the platelet response to vascular injury. In the platelet aggregation process, fibrinogen is attached to integrin αIIbβ3 of neighboring platelets and mediates platelet-platelet interaction [6] (Fig. 1.3).

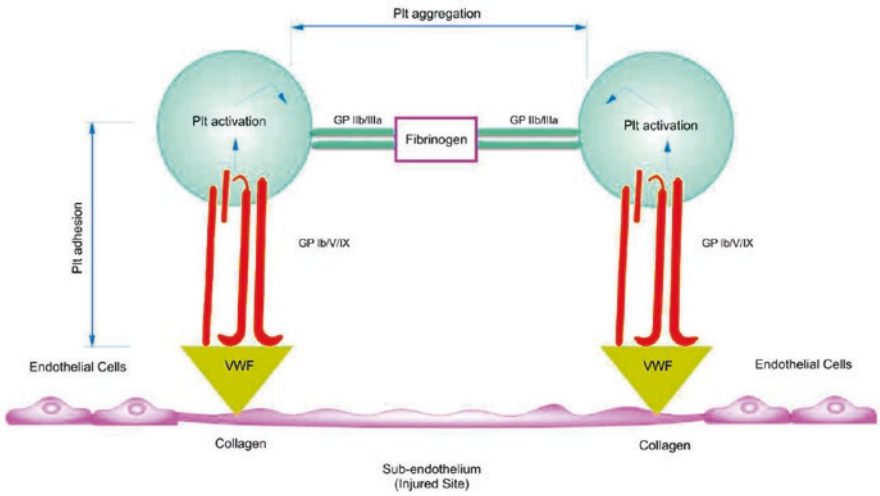

Fig. 1.3 Platelet activation and aggregation and their mediatorsGP-Ib/V/IX and GP-IIb/IIIa are the main GPs that mediate platelet adhesion and aggregation, respectively. Fibrinogen also has an important role in platelet aggregation. *Plt* platelet, *GP* glycoprotein, *VWF* von Willebrand factor

In addition to their primary roles in hemostasis, a considerable number of these components have vital roles in other body processes. Bleeding and thrombotic complications are consequences of abnormalities in hemostasis due to the absence or dysfunction of specific elements.

Hemostasis is categorized as primary or secondary hemostasis [7].

1.2 Platelets

Platelets are non-nucleated cells that have crucial roles in inflammatory processes, tumor angiogenesis, and defense against microbial infection. In normal circumstances, they have no significant interaction with vessel walls, but in vascular injury, they promptly stick to the sub-endothelial extracellular matrix in order to stop the bleeding. This adhesion leads to platelet activation and granule release. Stable platelet adhesion is a dynamic process that includes platelet tethering, rolling, activation, and their firm adhesion [8, 9] (Fig. 1.4).

Sub-endothelial extracellular matrix has several molecules, most being platelet glycoprotein ligands such as laminin, fibronectin, and collagen. Among these adhesive macromolecules, collagen types I and III are most important for platelet adhesion.

Although platelet adhesion is primarily mediated by interaction of the GPIb/V/IX complex with VWF in the sub-endothelial matrix, integrin α2β1 (GPIa/IIa) has a crucial role. Binding of integrin α2β1 to collagen leads to subsequent platelet activation and firm adhesion to sub-endothelium.

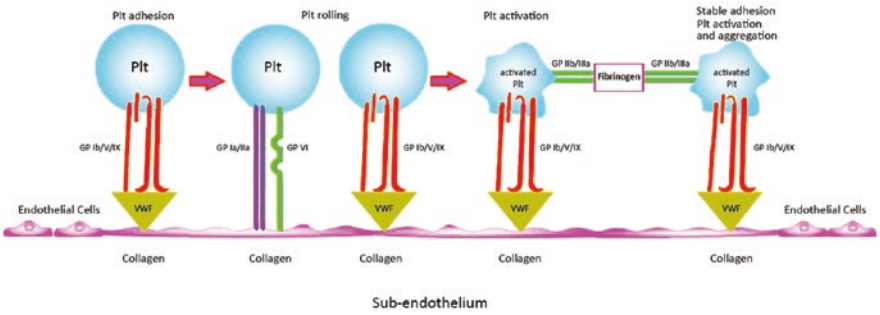

Fig. 1.4 Stable platelet adhesion at the site of an injury is a dynamic process that includes platelet tethering, rolling, activation, and their final firm adhesion. Platelet adhesion is mediated by GP-Ib/V/IX. This GP, along with GPIa/IIa and GP-VI, also has a role in platelet rolling. After platelet activation, a firm clot adheres to the vessel. GP-IIb/IIIa and fibrinogen are the main molecules in platelet aggregation. Plt platelet, GP glycoprotein, VWF von Willebrand factor

Initially, integrin α2β1-collagen interaction results in inducing the collagen-GPVI interaction. Although integrin αIIbβ3 in inactivated form doesn't bind to fibrinogen, upon platelet activation, conformational changes occur in this integrin. Thus, the integrin gains the fibrinogen-binding ability, giving integrin αIIbβ3 roles in both adhesion and aggregation processes. The final stage is firm platelet adhesion to the extracellular matrix. This requires activation of platelets, which shifts integrin αIIbβ3 to its high affinity state. The mechanism of platelet adhesion is different in conditions of low (20–200 s^{-1}) or high shear (300–800 s^{-1}). In low shear, αIIbβ3 and α2β1 integrins interact with fibrinogen and collagen, respectively, directly initiating platelet adhesion. In high shear, initial interaction of the GPIb/V/IX complex with the VWF of the external sub-endothelium is not firmly bound. This initial adhesion is firmed by integrin receptors and their ligands, including α5β1 (GPIc/IIa) and α2β1 (GPIa/IIa) integrins. In addition to integrin αIIbβ3 activation, the platelet adhesion process is accompanied by platelet shape change, degranulation, microparticle release, anionic phospholipid flipping to the outer leaflet of the platelet membrane bi-layer, and CD62P extracellular accessibility. Following platelet adhesion, and with activation of integrin αIIbβ3 and release of platelet agonists, platelet aggregation occurs, followed by formation of a hemostatic plug [10, 11].

1.3 Platelet Surface Glycoproteins

1.3.1 Integrin αIIbβ3 (Glycoprotein IIb/IIIa) (CD41/CD61)

Out of 18 integrin α and 10 integrin β subunits, platelets express four members of β1 including α2β1, α5β1, α6β1, and α8β1 and both β3 subfamilies including αVβ3 and αIIbβ3. α2β1, α5β1, and α6β1 are collagen, fibronectin, and laminin receptors, respectively. Integrin αIIbβ3 is the receptor of several extracellular matrix elements such as collagen, VWF, fibronectin, and vitronectin.

Table 1.1 Platelet glycoprotein properties

Glycoprotein	Alternative name	Number/ cell	Function	Ligand	Prevalence of related defect	Bleeding tendency
GPIa/IIa	1. Integrin α2β1 2. VLA-2 3. CD49b/ CD29	2000–4000	Adhesion	1. Collagen 2. Laminin 3. Vitronectin 4. Tenascin 5. Decorin	Extremely rare	Mild
GPIc/IIa	1. Integrin α5β1 2. VLA-5		Adhesion	1. Fibronectin 2. Denatured collagen 3. Laminin	Extremely rare	Mild
GPIb/V/IX	CD42 a-d	~25,000[a]	1. Platelet-endothelial cell adhesion 2. Platelet-leukocyte adhesion	1.VWF 2. Mac-1 3. P-selectin 4. α-thrombin 5. HMWK 6. FXI 7. FXIIa	Rare	Mild-moderate
GPIIb/IIIa	1. Integrin αIIbβ3 2. CD41/ CD61	40,000–80,000	1. Aggregation 2. Adhesion	1. Fibrinogen 2. VWF	Rare	Moderate-severe
GPIV	GP IIIb, CD36		1. Adhesion 2. Aggregation signaling	1. Collagen 2. Thrombospondin	Extremely rare	Mild
GPVI	–		1. Adhesion	1. Collagen 2. Collagen-related peptide (CRP) 3. Convulxin	Extremely rare	Mild
GPα6/IIa	1. Integrin α6β1 2. VLA-6		Adhesion	1. Laminin 2. Epiligrin	Extremely rare	Mild
–	α8β1 CD29/ CD49h		Adhesion	1. Fibronectin 2. Vitronectin 3. Tenascin 4. Laminin	Extremely rare	Mild
VR (Vitronectin receptor)	1. αVβ3 2. CD51/ CD61	A few hundreds	1. Adhesion 2. Aggregation	1. Collagen 2. Osteopontin 3. Tenascin 4. VWF	Rare	Mild

[a]Approximately 25,000 copies of the first three peptides, including GP Ibα, GP Ibβ, and GP IX, reside in the platelet surface along with half as many copies of GP V

Although β1 family members have a crucial role in adhesion, they have a minor role in platelet aggregation, which they are unable to support independently (Table 1.1).

Integrin αIIbβ3 is the most abundant platelet integrin with 40,000–80,000 copies on unstimulated platelets plus an exposable intracellular pool of this integrin. These are the heterodimer transmembrane proteins that mediate cell adhesion and cell migration by attaching the cell cytoskeleton to the extracellular matrix [12].

Integrin αIIbβ3 is the main platelet glycoprotein required for platelet aggregation; it also has an important role in the final step of adhesion (known as *firm adhesion*). Although platelet aggregation is mediated mainly by integrin αIIbβ3 and its ligands, including VWF and fibrinogen, other molecules such as GPIb and VWF also have a role.

Glanzmann thrombasthenia (GT) is a moderate to severe hemorrhagic disorder, due to mutation in *ITGA2B or ITGB3*, that leads to quantitative or qualitative defects in integrin αIIbβ3, with a defect in platelet aggregation. Symptoms are most prominent in homozygous or compound heterozygous patients. In heterozygotes of GT, about 50–70% of integrin αIIbβ3 is present; that is sufficient for normal aggregation [13].

Integrin αIIbβ3 is present on platelets in inactivated and activated conformations, but, to prevent spontaneous aggregation, it is constrained to the inactivated conformation on circulating platelets. Upon vascular injury, nearly immediate activation of integrin αIIbβ3 is triggered via stimulation of platelet agonists such as thrombin and ADP [14, 15].

1.3.2 Glycoprotein Ib/V/IX (CD42 a–d)

GPIb/V/IX (CD42 a–d) is the major GP initiating adhesion to the sub-endothelial matrix in high shear stress. In fact, the adhesion process at high shear is initiated by interaction between GPIb/V/IX and VWF in the extracellular matrix at the site of vascular injury. This initial interaction arrests platelets at high shear, induces signal transduction, and, finally, integrin-mediated platelet adhesion. The α2β1 (GPIa/IIa), α5β1 (GPIc/IIa), and αIIbβ3 (GPIIb/IIIa) integrins cause firm platelet adhesion.

Afterwards, signal transduction leads to the change of platelet shape, activation, granule secretion and inside-out integrin activation, promoting adhesion and aggregation. During this phase, platelet agonists such as ADP and thrombin are released. These agonists cause activation and recruitment of additional platelets at the site of injury.

The GPIb/V/IX complex consists of several separate subunits: GPIbα (CD42bα), GPIbβ (CD42bβ/CD42c), and GPIX (CD42a), arranged in a 1:2:1 stoichiometry. GPIb-IX is accompanied by GPV (CD42d), likely in a 1:1 stoichiometry, but the association is relatively weak and can be disrupted by nonionic detergents [16].

GPIbα, GPIbβ, GPIX, and GPV are members of the leucine-rich repeat (LRR) family. GPIbα and GPIbβ are linked with each other with disulfide bond(s) while non-covalently associated with GPIX and GPV subunits.

The N-terminal of GPIbα has binding sites for VWF, Mac-1 (CD11b/CD18), P-selectin, α-thrombin, high-molecular-weight kininogen (HMWK), and coagulation factors including factor (F) XI and FXIIa. α-thrombin binding to the N-terminal

of GPIbα leads to platelet activation by thrombin via protease-activated receptor-1 (PAR-1).

GPIbα can mediate platelet-endothelial and platelet-leukocyte adhesion by binding to P-selectin or a type of leukocyte integrin named Mac-1. Platelet-endothelial adhesion also can be mediated by integrin αIIbβ3 on activated platelets, and integrin αVβ3 on activated endothelium via adhesive GPs such as fibrinogen. Qualitative and quantitative defects in GPIbα, GPIbβ, and GPIX cause Bernard-Soulier syndrome (BSS) [17–19].

1.3.3 Integrin α2β1 (Glycoprotein Ia/IIa) (CD49b/CD29)

Integrin α2β1 is one of the main platelet surface collagen receptors that is expressed in different cell types. In fact, platelets have two receptors with definitive roles in platelet-collagen interaction.

Initial adhesion of platelets by GPVI causes platelet activation, and inside-out signaling results in conformational change in integrin α2β1 and an increased affinity of this integrin for collagen from low to high that leads to firm platelet adhesion.

Integrin α2β1 and collagen binding has several consequences, including promoting GPVI and collagen interaction and activation of integrin αIIbβ3. However, in the immune system, ligand binding to αIIbβ3 integrin in platelets inhibits α2β1 integrin-mediated adhesion to collagen.

Integrin α2β1 deficiency is an extremely rare disorder with mild bleeding tendency [20, 21].

1.3.4 Glycoprotein VI

GPVI, a member of the immunoglobulin superfamily, initiates platelet activation and promotes the integrin αIIbβ3 activation required for platelet aggregation at a site of injury. This glycoprotein is currently recognized as a receptor for not only collagen but also a variety of plasma and vascular proteins, including fibrin, fibrinogen, laminin, fibronectin, and galectin-3 [22].

GPVI is present on the platelet surface in a complex with the Fc receptor (FcR) γ-chain; each GPVI monomer being associated with two FcRγ-chains. The FcRγ-chain acts as the signaling arm in this complex. It used to be believed that the extracellular region of GPVI has no affinity for collagen, except in dimeric form (GPVI-Fc2), but recent studies suggest that monomeric GPVI is functional and can bind to ligands including collagen. Dimerization would provide a mechanism to actively bring binding sites on GPVI closer together rather than to form a unique epitope, thereby facilitating activation through the combination of affinity and avidity [23].

Similar to GPIb/V/IX (CD42 a-d), GPVI is critical for initial interaction of platelets, in the blood stream, with extracellular matrix at a vascular injury site under high shear conditions. GPVI deficiency is an extremely rare hemorrhagic disorder

with mild bleeding tendency. One of the most important laboratory findings in these patients is lack of platelet aggregation in response to collagen, with normal response to other agonists, in aggregometry studies. Some patients have a very low response to collagen that is attributed to other collagen receptors on the platelet surface, including integrin α2β1 [24, 25].

1.3.5 Integrin α5β1 (Glycoprotein Ic/IIa)

Integrin α5β1 (glycoprotein Ic/IIa), the major platelet receptor of fibronectin, has a supplementary role in platelet adhesion at a site of vascular injury.

1.3.6 Integrin αVβ3 (CD51/CD61)

Only a few hundred molecules of integrin αVβ3 (CD51/CD61) are present on the platelet surface. The expression of this integrin is not restricted to the megakaryocyte lineage. Similar to other integrins, αVβ3 (CD51/CD61) is present in a low affinity state on the unstimulated platelet surface, while in other cells, this integrin is present in high affinity state. In fact, integrin αVβ3 (CD51/CD61) is present in low affinity (default) state in circulating platelets, while in tissue cells, the default state is high affinity [26–28].

1.4 Platelet Granules and Secretion

Platelet adhesion is accompanied by their activation and the release of procoagulatory mediators such as ADP, thrombin, and prostaglandins. This process leads to thrombus formation that, in addition to platelets, incorporates red blood cells (RBC) and leukocytes. In addition to lysosomes, platelets have two main types of secretory granules: α-granules and dense granules. These granules are formed via partially overlapping, but distinct, membrane trafficking pathways, and serve different physiological roles [29].

In these small non-nucleated cells, more than 300 distinct molecules have been detected in their releasates. α-granules and dense granules are lysosome-related organelles that are restricted to platelets. α-granules, with a frequency of 50–80 per platelet, are the most populous, while only 0–3 lysosomes can be found in each platelet, as can 3–8 dense granules. The diameters of α-granules, dense granules, and lysosomes are 200–500, 200–300, and 200–250 nm, respectively. Platelet granules participate in a variety of bodily functions in addition to hemostasis, namely inflammation, wound healing and angiogenesis, as well as malignancies and antimicrobial host defense, with most functions related to α-granules. The majority of platelet α-granule constituents are synthesized in megakaryocytes, while fibrinogen, FV, albumin, and immunoglobulins are captured and endocytosed by circulating platelets and transported to the α-granules. Platelets serve as circulating reservoirs

Table 1.2 Contents of α-granules

	Contents	
Hemostatic factors	1. Factor V 2. Von Willebrand factor (VWF) 3. Fibrinogen 4. Factor XIII 5. Factor IX 6. Protein S	7. Tissue factor pathway inhibitor (TAFI) 8. Antithrombin 9. Plasminogen 10. Plasminogen activator inhibitor-1(PAI-1)
Growth factors	1. Platelet-derived growth factor (PDGF) 2.Vascular endothelium growth factor (VEGF) 3. Basic fibroblast growth factor (bFGF) 4. Epidermal growth factor [32] 5. Transforming growth factor-β (TGF-β) 6. Stromal cell-derived factor-1 α (SDF-1 α)	
Proteases	1. Matrix metalloproteinase-2 (MMP-2) 2. Matrix metalloproteinase-9 (MMP-9)	
Angiogenic factors	1. Angiogenin 2. Vascular endothelium growth factor (VEGF)	
Anti-angiogenic factors	1. Angiostatin 2. Platelet factor-4 (PF-4)	
Necrotic factors	1. Tumor necrosis factor-α (TNF-α) 2. Tumor necrosis factor-β (TNF-β)	
Chemokines	1. Chemokine (C-X-C motif) ligand-1 (CXCL-1) 2. Chemokine (C-X-C motif) ligand-4 (CXCL-4) 3. Chemokine (C-X-C motif) ligand-5 (CXCL-5) 4. Chemokine (C-X-C motif) ligand-7 (CXCL-7) 5. Chemokine (C-X-C motif) ligand-8 (CXCL-8) 6. Chemokine (C-X-C motif) ligand-12 (CXCL-12)	7. Chemokine (C-C motif) ligand-2 (CCL-2) 8. Chemokine (C-C motif) ligand-3 (CCL-3) 9. Chemokine (C-C motif) ligand-5 (CCL-5)
Granule membrane-specific proteins	1. P-selectin 2. CD63 3. Platelet alpha-granule membrane protein (GMP-33)	

of these components and can rapidly release them at local sites after activation [30, 31] (Table 1.2).

α-granules are immediately exocytosed after platelet activation and enhance hemostasis and inflammatory processes. α-granules express large amounts of P-selectin that are moved to the surface of these cells, after platelet activation, for interaction with neutrophils, monocytes, endothelial cells, and other platelets.

This interaction between P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) leads to tethering and rolling of platelets at the site of vascular injury, ensuring firm adhesion.

In addition to their relatively well-known role in trapping pathogens, platelets have a crucial role in directly killing some pathogens. After *Plasmodium falciparum* infection, activated platelets attach themselves to the infected RBCs and release platelet factor 4 (PF4) from their α-granules. This chemokine inhibits the growth of the pathogen and kills it. β-defensins are other platelet constituents that have direct antimicrobial effects on *Staphylococcus aureus*. PF4 also is able to recruit neutrophils, monocytes, and fibroblasts to the inflammation site. Defensins are not located in the granules mentioned, being either cytosolic molecules or contents of as-yet unidentified granules.

Dense granules, dense bodies, or δ-granules contain adenine nucleotides such as adenosine triphosphate (ATP) and ADP, and serotonin, which are involved in other platelet functions including vasoconstriction, pro-inflammatory cytokine production and inflammation [33] (Table 1.3).

Lysosomes are less frequent than α-granules or dense granules and contain several enzymes involved in the degradation of carbohydrates, proteins, and lipids. These include glycosidases, proteases, and cationic proteins such as β-glucuronidase, elastase, and collagenase with bactericidal activity. These enzymes can help clear pathogens and platelet thrombi, and in extracellular matrix degradation and heparin inactivation [34] (Table 1.4).

When platelets are activated, anionic lipids such as phosphatidylserine are exposed on the platelet surface. FV then is released from the α-granules and bound to the anionic lipids. This FV is activated initially by the small amount of thrombin generated from the initial interaction between tissue factor (TF) and FVII at site of injury. Activated FV (FVa), accompanied with FXa, Ca^{2+}, and anionic lipids, forms the prothrombinase complex. This complex cleaves prothrombin and changes it to thrombin (FIIa). FXa bound to FV is relatively protected from inhibition by plasma inhibitors such as antithrombin (AT). The main consequence is a dramatic increase of thrombin generation on activated platelet surfaces, restricted, however, to the site of vascular injury. In fact, platelets localize the coagulation process to the thrombus and protect coagulation enzymes from inhibition by plasma and platelet inhibitors. In this way, they prevent the occurrence of disseminated intravascular coagulation

Table 1.3 Contents of dense granules

	Contents
Nucleotides	1. Adenosine triphosphate (ATP) 2. Adenosine diphosphate (ADP) 3. Guanosine-5′-triphosphate (GTP) 4. Guanosine diphosphate (GDP)
Amines	1. Serotonin or 5-hydroxytryptamine (5-HT) 2. Histamine
Bivalent cations	1. Calcium (Ca^{2+}) 2. Magnesium

Table 1.4 Contents of lysosomes

	Contents
Acid proteases	1. Carboxypeptidases-A 2. Carboxypeptidases-B 3. Cathepsins-D 4. Cathepsins-E 5. Acid phosphatase 6. Collagenase
Glycohydrolases	1. Heparinase 2. β-N-acetyl-glucosaminidase 3. β-glycerophosphatase 4. β-glucuronidase 5. β-galactosidase 6. α-D-glucosidase
	7. α-L-fucosidase 8. β-D-fucosidase

(DIC). Scott syndrome is a rare inherited platelet function disorder (IPFD), characterized by a deficit in procoagulant activity in platelets and other blood cells, caused by a lack of phosphatidylserine (PS) expression following activation. It results from mutations in ANO6, which encodes the phospholipid scramblase protein, TMEM16F. These issues show the close relationship between activated platelets and coagulation factors [35, 36].

1.5 Endothelium

The endothelium has been described as a barrier between circulatory blood and its surrounding tissues. It is a dynamic organ that can regulate its environment in response to external stresses. Although the endothelium is less than 0.2 μm thick, it includes 6×10^3 endothelial cells that weigh about 1 kilogram in the average person and covers 4000–7000 square meters. Endothelium acts as a blood-compatible surface that maintains blood flow and regulates the blood coagulation system [37].

Some of the endothelium's functions are regulation of vascular tone, cellular adhesion, smooth muscle cell proliferation, and vascular inflammation. Endothelial cells have numerous functions specific to different vascular beds. The main function of the endothelium is regulation of systemic blood flow via changes in vascular diameter. Furthermore, the endothelium acts as a barrier that selectively controls fluid, ion, and macromolecule movement between circulating blood and surrounding tissue. The endothelium regulates recruitment and extravasation of procoagulation leukocytes in response to tissue injury and inflammation. Endothelial cells play a crucial role in the consequent healing process. They also act as a vector of angiogenesis, which is necessary for tissue repair, and for recanalization of obstructive fibrin clots. In concert with smooth muscle cells, endothelial cells also regulate local blood pressure, because these cells are responsive to vasoactive agents. These cells can respond to inflammatory cytokines, and to stresses from

metabolism or hypoxia. Altogether, the endothelium expresses many molecules that regulate platelets and coagulation cascade activation that results in preventing post-vascular-injury thrombosis development [38–40].

Endothelial cells cover arteries, veins, and capillaries. Shear stress, blood oxygenation, and smooth muscle cell density are different among these vessels; endothelial cells respond differently to pro-coagulation signals in different vascular beds. For instance, vasodilation regulation in arteries is faster than veins. These cells adapt their phenotype according to the nature of the surrounding tissue and have abundant phenotypic heterogeneity.

Endothelial cells have an important role in clot development due to their location, and are closely related to the coagulation cascade. Intact endothelial cells express inhibitors to prevent thrombin synthesis and activation. When these cells are activated, they play a role in initiation and development of thrombin production via expression of pro-coagulation factors [41, 42].

The coagulation cascade can be activated via two extrinsic and intrinsic pathways, either of which initiates the cascade by converting FVII to FVIIa and FXII to FXIIa. Activated endothelial cells contribute to the extrinsic pathway, expressing TF in response to vascular injury and inflammation. The TF/FVIIa complex triggers protease-activated receptor-2 (PAR-2) to induce a pro-inflammatory response. The endothelium regulates clot formation by PAR activation. Acute release of endothelial products is mostly mediated by PAR-1. PAR-1 plays an important role in response to pro-coagulation stimuli. It induces activation of Weibel-Palade bodies (WPB), thereby releasing VWF and tissue-type plasminogen activator (t-PA). It also mediates nitric oxide (NO) and prostacyclin (PGI2) production to reduce platelet activation. NO activates guanylate cyclase (GC) in platelets. GC then converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which prevents the release of stored intracellular Ca^{2+} for platelet activation and aggregation. NO is also a potent vasodilator. PGI2, another inhibitor for platelets and vasodilation, is released from intact endothelium. Although NO and PGI2 have different mechanisms of action, they synergize each other's inhibitor effect on platelet activation and aggregation.

Microvascular endothelial cells induce angiogenesis by releasing TF-rich microparticles, which is related to thrombin-mediated PAR-1 activation [43–45].

Although activated endothelial cells are typically related to the extrinsic pathway, they also may play a role in the intrinsic pathway. The mechanism of endothelial cell function in the intrinsic pathway is unclear, but there is a supposition that these cells prevent inhibition of intrinsic pathway factors; for instance, FXIIa can be protected from C1 inhibition activity by endothelial cells [46].

Investigations also have shown that these cells are the primary source of FVIII. Thus, endothelial cells probably are a necessary component of both intrinsic and extrinsic coagulation pathways.

In addition to coagulation, endothelial cells have the main role in primary hemostasis. The interaction between platelets and endothelium is important for platelet activation and regulation. An intact endothelium prevents platelets from adhering, while activated endothelial cells express molecules and receptors that enhance

platelet adhesion to the injured site. WPBs store VWF, P-selectin, angiopoitin-2, t-PA, and endothelin-1 in endothelial cells; these mediate platelet adhesion, leukocyte recruitment, regulation of inflammation, fibrinolysis, and vasoconstriction, respectively. VWF has two main roles in hemostasis: first, it is necessary for platelet adhesion to collagen by GP-Ib/V/IX in injured vascular sites; second, it stabilizes plasma coagulation FVIII [47, 48].

Furthermore, an intact endothelium actively prevents thrombosis by suppressing platelet activation and adhesion. It expresses multiple anticoagulants such as tissue factor pathway inhibitor (TFPI), thrombomodulin, endothelial protein C receptor (EPCR), and heparin-like proteoglycans. There is an ectonucleotidase (CD39) on the endothelial cells surface that converts platelet stimulator ADP into adenosine. One of the most important preventors of coagulation is TFPI, which causes direct inhibition of FXa and the TF/FVIIa/FXa complex. Patients with less than 10% of normal TFPI levels have an increased risk of venous thrombosis and coronary heart disease. Thrombomodulin and EPCR catalyze thrombin-mediated protein C pathway activation. Activated protein C inactivates FVa and FVIIIa, resulting in reduced thrombin formation. Platelet activation is inhibited by prostacyclin and prostaglandin E2 (PGE2), which are released from activated endothelium by vasoactive agents. NO enhances the prostacyclin effect [40, 49–51].

Thrombin is a strong stimulator for platelets. ATIII is a liver-derived plasma protein that forms a complex with thrombin and other serine proteases such as FIXa, FXa, FXIa, and FXIIa, and inhibits them by preventing exposure of the active site of these proteases to their substrates. The activity of ATIII is markedly promoted by heparan sulfates. ATIII binds to heparan sulfates of proteoglycans in the endothelial surface [40].

Endothelial cells are important components that contribute to clot destruction. In the wound healing process, endothelial cells release proteofibrinolytic molecules such as t-PA and urokinase-type plasminogen activator (u-PA), and metalloproteases for clot destruction. These cells also release ADAMTS13 and ADAMTS18 that mediate platelet aggregate dissolution [38, 52, 53].

In general, the repertoire of all these coagulation inhibitors expressed in endothelial cells can vary according to their location in different organs, and even within the vasculature of an organ [54].

Endothelial cells are one of the main parts of blood vessels and can induce angiogenesis after stimulation. For instance, protein C can stimulate angiogenesis in brain endothelium [55].

Endothelial disorders are responsible for inflammation and inappropriate clot formation and can be related to cardiovascular diseases.

1.6 Coagulation Factors

All coagulation factors are synthesized by the liver except for FVIII, which is produced by endothelial cells. VWF is also synthesized in megakaryocytes as well as in endothelial cells.

Initiation of blood coagulation is triggered by vascular injury and exposure of TF to the blood stream, which leads to generation of a small amount of thrombin. This thrombin is sufficient to activate platelets, FV, FVIII, and FXI and to trigger the consolidation pathway leading to sufficient generation of thrombin to convert fibrinogen to fibrin. Thrombin is the key coagulation enzyme, its two major functions in hemostasis being fibrinogen-to-fibrin conversion and activation of platelets.

Extensive revision has been made to the initial theory of the coagulation cascade, by Macfarlane, Davie, and Ratnoff in 1964, which involved a series of enzymatic reactions by which an initial small amount of stimulus was amplified and resulted in generation of a burst of thrombin sufficient for fibrinogen-to-fibrin conversion. Two alternative pathways, namely the intrinsic and extrinsic, have been found to initiate the coagulation cascade.

The coagulation cascade is a precise mechanism leading to clot formation and prevention of blood loss. Under normal conditions, this cascade is counter-balanced by anticoagulant-system mechanisms, exact regulation that prevents aberrant clot formation. In a pathological status, due to hereditary or acquired defects, normal controlling mechanisms of the system may be disrupted, potentially resulting in pathological conditions such as thrombosis [56] (Table 1.5).

The extrinsic pathway: unlike in the intrinsic, an external component of plasma, TF, is the initiator of this pathway. The complex that initiates this pathway is formed of plasma FVIIa and TF from extravascular tissue. Although TF is not present in the bloodstream at high concentration, it is exposed to FVII upon tissue injury and, as cofactor of FVII, induces its activation. This complex cleaves a trace amount of FIX and FX to FIXa and FXa, respectively. FXa, in combination with FVa as its cofactor, forms the prothrombinase complex. This complex converts prothrombin (FII) into thrombin (FIIa), which then converts fibrinogen into fibrin monomers, which are unstable until cross-linked by FXIIIa [11].

The intrinsic or contact activation pathway: this pathway is named *intrinsic* because all components are present in the blood. It is initiated by exposure of several proteins, including FXII, to negatively charged surfaces. FXIIa converts prekallikrein into its activated form, kallikrein. Kallikrein itself converts more amounts of FXII into FXIIa in a positive feedback for FXII activation. FXIIa activates FXI to FXIa. High-molecular-weight kininogen (HMWK) plays a cofactor role for all reactions. HMWK also converts to kininogen and then kinin, causing vasodilation, swelling, erythema, pain, and local hypotension. On the other hand, high amounts of FXIIa, created by kallikrein, play a role as t-PA and plasminogen activator. Therefore, it prevents further clotting in non-injured areas by formation of plasmin. HMWK, kallikrein, and FXIIa are assembled on an anionic, but not necessarily phospholipid, surface. Initiation of plasma clotting via the contact pathway can be induced by providing an anionic surface like kaolin, ellagic acid, celite, or silica.

Thereafter, FXIa with Ca^{2+} as cofactor activates FIX to FIXa. Finally, the intrinsic tenase complex composed of FIXa, FVIIIa, Ca^{2+}, and phospholipid coverts FX into FXa [11, 57].

From this point, the rest of the process continues like that of the extrinsic pathway. This is called the common pathway (Fig. 1.5).

Table 1.5 Characteristics of blood coagulation factors

Coagulation factor	Alternative name	Structure	Function in coagulation cascade	Other functions	Plasma half-life	Hemostatic level	Normal range (%)
Factor I	Fibrinogen	Heterotrimer	Clot formation	Platelet adhesion	72–120 h	20–30%	250–300 mg/dl
Factor II	Prothrombin	Monomer	Serine protease	Anticoagulant activity	60–70 h	20–40%	7–15 mg/dl
Factor III	1. Tissue factor 2. Tissue thromboplastin 3. Tissue phospholipid 4. CD142 5. Morawitz factor	Extra-cellular, transmembrane and intra-cellular zones	Cofactor	Arterial and venous thrombosis Myocardial infarction angiogenesis	–	5–19 pM	0 mg/dl
Factor IV	Calcium (Ca^{2+})	ion	Cofactor	Role in proper function of muscle, nerve and heart	–	–	9–10.5 mg/dl
Factor V	1. Labile factor 2. Proaccelerin 3. Accelerator globulin 4. Owren factor	Single chain protein, consists of 6 domains	Cofactor	Anticoagulant activity	36 h	10%	1 mg/dl
Factor VII	1. Stable factor 2. Proconvertin 3. Serum prothrombin conversion accelerator 4. Autoprothrombin I	FVII: single chain FVIIa: double chain	Serine protease	–	FVII: 4–6 h FVIIa: 2 h	10–15%	0.05 mg/dl
Factor VIII	1. Antihemophilic factor A 2. Antihaemophilic globulin 3. Platelet cofactor I 4. thromboplastinogen	Non-covalent heterodimer	Cofactor	1. Essential blood-clotting protein 2. Association of factor VIIIa with factor IXa to form the intrinsic factor Xase complex 3. Increase the catalytic efficiency for factor Xa generation	17 h	30–60%	0.01 mg/dl

Factor IX	1. Christmas factor 2. Antihemophilic factor B 3. Platelet cofactor II 4. Autoprothrombin II 5. Plasma thromboplastin component	Monomer	Serine protease	Coagulation FX activator	24 h	20–30%	0.5 mg/dl
Factor X	1. Stuart Power factor 2. Autoprothrombin III	Heterodimer of light and heavy chains	Serine protease	Coagulation factor II activator	30–40 h	20%	0.8–1 mg/dl
Factor XI	1. Rosenthal factor 2. Plasma thromboplastin antecedent	Homodimer (4 Apple domains with 1 catalytic domain in each subunit)	Serine protease	1. FIX activator 2. Thrombin generation 3. Anti-fibrinolysis	52 h	15–50%	0.5 mg/dl
Factor XII	Hageman factor	Monomer (SP, fibronectin type I and II, kringle, proline reach, EGF1 and 2 like, catalytic, activating peptide and catalytic activator domains)	Serine protease	Activation of complement classic pathway	60 h	0–2%	3 mg/dl
Factor XIII	1. Fibrin stabilizing factor 2. Laki–Lorand factor 3. Fibrinase 4. Fibrin polymerase 5 Protransglutaminase	Heterodimer (FXIII-A2B2)	Transglutamin-ase	1. Angiogenesis 2. Maintenance of pregnancy 3. Wound healing 4. Bone metabolism 5. Cardio protection	9–12 days	2–5%	53.2–221.3%

(continued)

Table 1.5 (continued)

Coagulation factor	Alternative name	Structure	Function in coagulation cascade	Other functions	Plasma half-life	Hemostatic level	Normal range (%)
HMWK	1. Fitzgerald factor 2. Fleaujeac factor 3. Williams factor 4. Reid factor	Monomer	Cofactor	1. Fibrinogen adherence to phospholipid surfaces 2. Inducing PGI2 and NO generation from endothelium 3. Role in kinin system	9–10 h	0–2%	8 mg/dl
PKK	Fletcher factor	Dimer (SP, catalytic serine protease, activating peptide, hydrolytic and 4 apple domains)	Serine protease	1. Inflammation 2. Pain 3.Smooth muscle cell dilatation 4. Vasodilation	35 h	0–2%	5 mg/dl
VWF	Von Willebrand factor	Monomer (D1, D2, D′, D3, A1, A2, A3, D4, B1, B2, B3, C1, C2 and CK domains)	Cofactor of FVIII Platelet adhesion, platelet aggregation, stabilization of circulating FVIII	1. Inflammation 2. Angiogenesis	12.4 h	10 mg/ml	1 mg/dl

**Mg* milligram, *dl* deciliter, *pM* picomole, *SP* signal peptide, *EGF* epidermal growth factor, *PGI2* prostaglandin I2, *NO* nitric oxide, *HMWK* high molecular weight kininogen, *PKK* prekallikrein

Recent studies have demonstrated that these pathways do not work as discrete tracks but are intertwined in the process of hemostasis [58].

More recent concepts suggest that coagulation is composed of three phases: initiation, amplification, and propagation.

(1) **Initiation phase** starts with TF exposure to coagulation factors caused by endothelium injury or activation. Endothelial cells can express TF in response to inflammatory stimuli such as exposure to bacterial lipopolysaccharide (LPS) in sepsis, adhesion molecules, inflammatory cytokines, and oxidized low density lipoprotein (LDL).

Because there is no human model of TF deficiency, and the murine model exhibits embryonic lethality in homozygous TF knockout mice, it seems that lack of TF is incompatible with life [59].

About 1–2% of FVII is present in blood in activated form. TF forms a catalytic complex with FVIIa (TF/FVIIa complex), the extrinsic tenase complex. This complex is placed on the phospholipid surface of platelets, converting FIX and FX into FIXa and FXa, respectively. Then FXa generates small amounts of FIIa (thrombin). The length of the initiation phase depends on the concentration of TF/FVIIa complex and tissue factor pathway inhibitor (TFPI). TFPI acts as FXa and TF/FVIIa complex neutralizer. In this phase, only a limited amount of thrombin is produced before the extrinsic tenase complex is inactivated by TFPI.

The initiation phase is characterized by localization of the process to TF-expressing cells and generation of thrombin in picomolar amounts. This thrombin activates platelets, FV (released from α-granules), and FVIII (causing it to dissociate from VWF). FVa and FVIIIa will bind to platelet membranes and act as receptors for FXa and FIXa, respectively.

(2) In the **amplification phase**, FIX forms intrinsic tenase complex in its activated form with FVIIIa (FIXa/FVIIIa). This complex is optimally formed on the membrane surface provided by platelets in the presence of Ca^{2+}. The intrinsic tenase complex activates FX 50–100 times greater than the extrinsic tenase complex, necessary for amplifying the coagulation process. The efficacy of intrinsic tenase complex (FIXa/FVIIIa) and prothrombinase complex (FXa/FVa) multiplies with their co-localization on phospholipid membranes in the presence of Ca^{2+}. Generated thrombin in the initiation phase activates more FV and FVIII, which play a cofactor role in the prothrombinase complex. Thus, FIX activates more FX and prothrombin activation is accelerated by FX [60]. Finally, thrombin is generated adequately to form a stable clot.

(3) In the **propagation phase**, activated platelets accumulate at the injury site to provide phospholipid surface for localization of the coagulation process. This leads to optimal thrombin generation that converts fibrinogen to fibrin. Soluble fibrin monomers convert to stable polymer by covalent bonds. These bonds are generated by FXIII that has been activated by thrombin. FXIII, along with fibrinogen, also regulates clot size by controlling the volume of RBCs trapped within the thrombus. Thrombin also activates thrombin-activatable fibrinolysis inhibitor (TAFI), which acts as clot protector against fibrinolysis [57, 61–64].

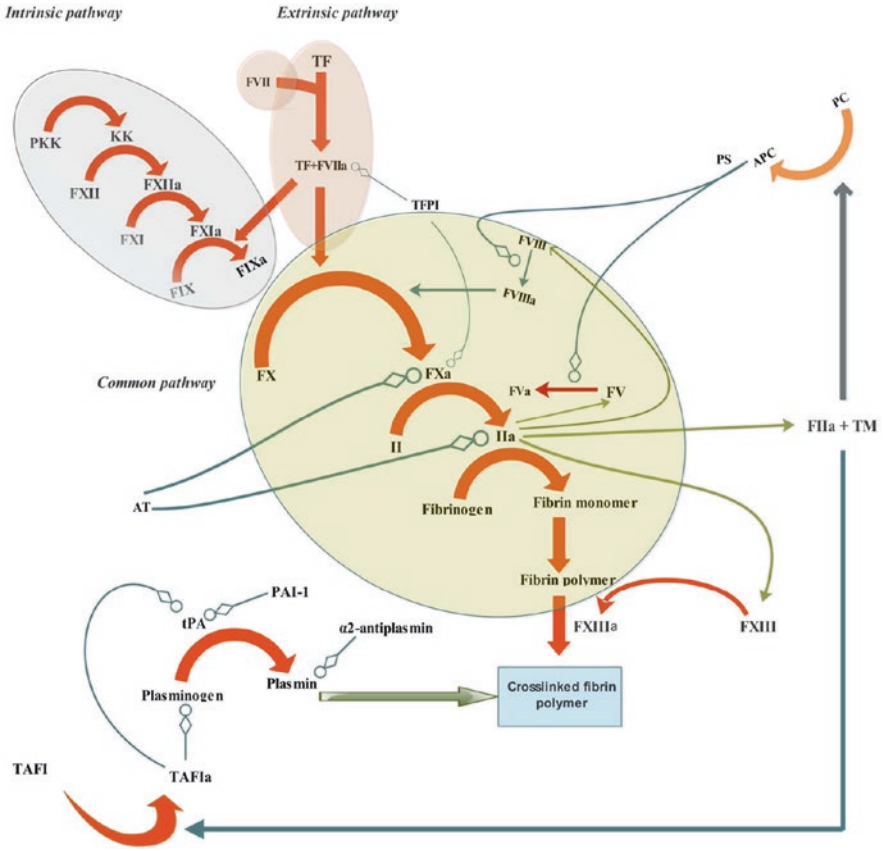

Fig. 1.5 Coagulation cascade and fibrinolysis system interaction

1.7 RBC Contribution in Hemostasis

The incorporation of RBCs in thrombi was thought to occur by passive trapping during the process of fibrin formation; more recently, RBCs are recognized as an important and active part of the thrombotic process. Multiple biochemical properties of RBCs influence the clotting process, including interactions with platelets, von Willebrand factor, and fibrinogen. RBCs are entrained in the fibrin matrix with no bond to FXIIIa; instead, fibrin crosslinking secondarily traps RBCs [65]. In addition to modulating fibrinolytic activity, RBC presence changes the structure of the fibrin mesh in a concentration-dependent manner. The more RBCs are trapped in the fibrin clot, the less the clot porosity. During clot formation, entrained RBCs are compressed into shapes called polyhedrocytes, which appear between fibrin layers as "building blocks" [66–68].

1.8 Anticoagulation Mechanisms

Fibrin clot formation is the end of the coagulation process. Natural anticoagulants are necessary to limit clot formation at the injured site. One of the most important anticoagulants is AT, the main inhibitor of thrombin. It is a vitamin-K-independent serine protease synthetized by the liver. AT mainly inhibits thrombin and FXa, but it can also inhibit FIXa and FVIIa. This inhibitory effect is enhanced in the presence of heparin. Heparan sulphate is also an AT-activity enhancer located on endothelial cell surfaces. Heparin cofactor II is another vitamin-K-independent serine protease that specifically inhibits thrombin. Its inhibitory activity is enhanced in the presence of dermatan sulphate. α_2-macroglobulin and α_1-antitrypsin are other thrombin inhibitors. Protein C is a vitamin-K-dependent serine protease activated by thrombin. Activated protein C (APC) is a potent anticoagulant, which degrades FVa and FVIIIa using protein S as cofactor. This is an important anticoagulant axis, and is highlighted by the most prevalent familial risk of thrombosis due to a mutation in FV (Arg506Gln/ FV Leiden), which slows FVa inactivation by APC [69].

Protein C inhibitor, α_2-macroglobulin, and α_1-antitrypsin limit APC activity. Thrombomodulin (TM) is a transmembrane receptor on endothelial cell surfaces that binds to thrombin. Protein C activation is increased in the presence of TM-thrombin complex [70, 71].

EPCR is another transmembrane receptor on endothelial cell surfaces that binds protein C, leading to more APC generation. In addition to these anticoagulants, TFPI is the main inhibitor of the tissue factor pathway. Its carrier in plasma and in platelet α-granules is FV, which positions the TFPI in proximity to the clot via phospholipid binding [72]. TFPI has little direct effect on FVa, but it binds to FXa at first and generates FXa-TFPI complex. This then binds to TF-FVa complex and generates a complex that inactivates both FV and FX [73]. Furthermore, there is a plasma enzyme named protein-Z-dependent protease inhibitor (ZPI) that plays a role in FIXa, FXa, and FXIa inhibition [74, 75]. The fibrinolysis system is one of the most important mechanisms that degrades formed clots.

1.9 Fibrinolysis System

Once the vascular leak has been sealed and tissue repair is underway, the fibrinolysis system is required to degrade the clot formed by hemostatic mechanisms. Plasminogen is the main enzyme of this system. It is not able to degrade a fibrin clot, but it has an affinity to fibrin, allowing it to bind to the clot. Plasminogen converts to its activated form, named plasmin, by two activators: t-PA and u-PA. T-PA, secreted locally from endothelial cells, initiates fibrinolysis. Plasmin, a serine protease, cleaves the formed fibrin and also produces more t-PA and u-PA. Plasminogen activators have more effect on Lys-plasminogen. Plasmin converts Glu-plasminogen to Lys-plasminogen, which has more affinity to plasminogen activators. In fact, plasmin enhances its own production by positive feedback. Although u-PA is an

important plasminogen-activating enzyme that exists in plasma, t-PA is considered the principal inducer of fibrinolysis [76].

T-PA has little effect on plasminogen in the absence of fibrin. Both t-PA and plasminogen bind to fibrin. Generation of the t-PA/plasminogen/fibrin complex is necessary to produce plasmin. Therefore, fibrin simultaneously acts as cofactor for plasminogen activation and substrate for plasmin [77]. The cofactor role of fibrin for plasminogen maintains the localization of plasmin production and prevents systemic hemorrhagic effects. In addition to acting on the fibrin clot, plasmin cleaves numerous proteins including FXa, FVa, and FXIIIa [11].

Fibrinolysis is limited by plasminogen activator inhibitors (PAI), which prevent plasminogen-to-plasmin conversion by t-PA and u-PA inhibition, and by plasmin inhibitors such as α_2-antiplasmin and α_2-macroglubulin, and TAFI, which inhibits the plasmin effect on fibrin. Thrombin is a weak TAFI activator, but TM-thrombin complex enhances this activity about 1300 times. TAFI can also be activated by plasmin but to a lesser extent than by the TAFI-thrombomodulin complex. TAFI removes lysine and arginine residues from the fibrin C terminal. These residues are necessary for plasminogen and t-PA binding, thus TAFI causes decreased plasminogen activation by t-PA. It also decreases Glu-plasminogen conversion to Lys-plasminogen by plasmin. TAFI also increases direct inhibition of plasmin by α_2-antiplasmin [78–82].

References

1. Osaki T, Ichinose A. Current views of activating and regulatory mechanisms of blood coagulation. Nihon Rinsho Japanese J Clin Med. 2014;72(7):1206–11.
2. Davie EW. A brief historical review of the waterfall/cascade of blood coagulation. J Biol Chem. 2003;278(51):50819–32.
3. Butenas S, Mann K. Blood coagulation. Biochem Mosc. 2002;67(1):3–12.
4. Kasirer-Friede A, Shattil SJ. Regulation of platelet adhesion receptors. Platelets in thrombotic and non-thrombotic disorders. Springer; 2017. p. 69–84.
5. Feghhi S, Munday AD, Tooley WW, Rajsekar S, Fura AM, Kulman JD, et al. Glycoprotein Ib-IX-V complex transmits cytoskeletal forces that enhance platelet adhesion. Biophys J. 2016;111(3):601–8.
6. Bennett JS. Regulation of integrins in platelets. Pept Sci. 2015;104(4):323–33.
7. Versteeg HH, Heemskerk JW, Levi M, Reitsma PH. New fundamentals in hemostasis. Physiol Rev. 2013;93(1):327–58.
8. Gremmel T, Frelinger AL III, Michelson AD. Platelet physiology. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2016.
9. Sorrentino S, Studt J-D, Medalia O, Sapra KT. Roll, adhere, spread and contract: structural mechanics of platelet function. Eur J Cell Biol. 2015;94(3–4):129–38.
10. Goto S, Hasebe T, Takagi S. Platelets: small in size but essential in the regulation of vascular homeostasis–translation from basic science to clinical medicine. Circ J. 2015;79(9):1871–81.
11. Pryzdial EL, Lee FM, Lin BH, Carter RL, Tegegn TZ, Belletrutti MJ. Blood coagulation dissected. Transfus Apher Sci. 2018;57(4):449–57.
12. Dhar H, Santosh A. Glanzmann's thrombasthenia: a review of literature. J South Asian Feder Obs Gynaecol. 2019;11(2):134–7.
13. Mohan G, Malayala SV, Mehta P, Balla M. A comprehensive review of congenital platelet disorders, thrombocytopenias and thrombocytopathies. Cureus. 2020;12(10):e11275.

14. Coller B. α II bβ3: structure and function. J Thromb Haemost. 2015;13:S17–25.
15. Bledzka K, Qin J, Plow EF. Integrin αIIbβ3. Platelets. 2019:227–41.
16. Quach ME, Li R. Structure-function of platelet glycoprotein Ib-IX. J Thromb Haemost. 2020;18(12):3131–41.
17. López JA. The platelet glycoprotein Ib-IX-V complex. Platelets in thrombotic and non-thrombotic disorders. Springer; 2017. p. 85–97.
18. Li R, Emsley J. The organizing principle of the platelet glycoprotein Ib–IX–V complex. J Thromb Haemost. 2013;11(4):605–14.
19. Almomani MH, Mangla A. Bernard Soulier syndrome. 2020.
20. Madamanchi A, Santoro SA, Zutter MM. α2β1 Integrin. I Domain Integrins. 2014;819:41–60.
21. Chastney MR, Conway JR, Ivaska J. Integrin adhesion complexes. Curr Biol. 2021;31(10):R536–R42.
22. Perrella G, Nagy M, Watson SP, Heemskerk JW. Platelet GPVI (Glycoprotein VI) and thrombotic complications in the venous system. Arterioscler Thromb Vasc Biol. 2021;41(11):2681–92.
23. Clark JC, Damaskinaki F-N, Cheung YFH, Slater A, Watson SP. Structure-function relationship of the platelet glycoprotein VI (GPVI) receptor: does it matter if it is a dimer or monomer? Platelets. 2021;32(6):724–32.
24. Arman M, Krauel K. Human platelet IgG Fc receptor Fcγ RIIA in immunity and thrombosis. J Thromb Haemost. 2015;13(6):893–908.
25. Gawaz M, Vogel S, Pfannenberg C, Pichler B, Langer H, Bigalke B. Implications of glycoprotein VI for theranostics. Thromb Haemost. 2014;112(07):26–31.
26. Kunicki TJ. Platelet membrane glycoproteins and their function: an overview. Blut. 1989;59(1):30–4.
27. Nurden AT, editor. Platelet membrane glycoproteins: a historical review. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2014.
28. George JN. Platelet membrane glycoproteins. Springer Science & Business Media; 2013.
29. Flaumenhaft R, Sharda A. Platelet secretion. In: Platelets. Elsevier; 2019. p. 349–70.
30. Koseoglu S, Flaumenhaft R. Advances in platelet granule biology. Curr Opin Hematol. 2013;20(5):464–71.
31. Heijnen H, Van Der Sluijs P. Platelet secretory behaviour: as diverse as the granules… or not? J Thromb Haemost. 2015;13(12):2141–51.
32. Lang T, Johanning K, Metzler H, Piepenbrock S, Solomon C, Rahe-Meyer N, et al. The effects of fibrinogen levels on thromboelastometric variables in the presence of thrombocytopenia. Anesth Analg. 2009;108(3):751–8.
33. Sadeghian MH, Keramati MR, Badiei Z, Ravarian M, Ayatollahi H, Rafatpanah H, et al. Alloimmunization among transfusion-dependent thalassemia patients. Asian J Transfus Sci. 2009;3(2):95.
34. Yadav S, Storrie B. The cellular basis of platelet secretion: emerging structure/function relationships. Platelets. 2017;28(2):108–18.
35. Golebiewska EM, Poole AW. Platelet secretion: From haemostasis to wound healing and beyond. Blood Rev. 2015;29(3):153–62.
36. Millington-Burgess SL, Harper MT. Gene of the issue: ANO6 and Scott syndrome. Platelets. 2020;31(7):964–7.
37. Yau JW, Teoh H, Verma S. Endothelial cell control of thrombosis. BMC Cardiovasc Disord. 2015;15(1):1–11.
38. van Hinsbergh VW. Endothelium—role in regulation of coagulation and inflammation. In: Seminars in immunopathology. Springer; 2012.
39. Monahan-Earley R, Dvorak AM, Aird WC. Evolutionary origins of the blood vascular system and endothelium. J Thromb Haemost. 2013;11:46–66.
40. Neubauer K, Zieger B. Endothelial cells and coagulation. Cell Tissue Res. 2022;387(3):1–8.
41. Dekker RJ, van Thienen JV, Rohlena J, de Jager SC, Elderkamp YW, Seppen J, et al. Endothelial KLF2 links local arterial shear stress levels to the expression of vascular tone-regulating genes. Am J Pathol. 2005;167(2):609–18.

42. Aird WC. Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. Circ Res. 2007;100(2):174–90.
43. Bode M, Mackman N. Protective and pathological roles of tissue factor in the heart. Hamostaseologie. 2015;35(01):37–46.
44. Borissoff JI, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. N Engl J Med. 2011;364(18):1746–60.
45. Fetalvero KM, Martin KA, Hwa J. Cardioprotective prostacyclin signaling in vascular smooth muscle. Prostaglandins Other Lipid Mediat. 2007;82(1–4):109–18.
46. Schousboe I. Binding of activated Factor XII to endothelial cells affects its inactivation by the C1-esterase inhibitor. Eur J Biochem. 2003;270(1):111–8.
47. Do H, Healey JF, Waller EK, Lollar P. Expression of factor VIII by murine liver sinusoidal endothelial cells. J Biol Chem. 1999;274(28):19587–92.
48. Dubois C, Panicot-Dubois L, Gainor JF, Furie BC, Furie B. Thrombin-initiated platelet activation in vivo is vWF independent during thrombus formation in a laser injury model. J Clin Invest. 2007;117(4):953–60.
49. Esmon CT, Esmon NL. The link between vascular features and thrombosis. Annu Rev Physiol. 2011;73:503–14.
50. Wood JP, Ellery PE, Maroney SA, Mast AE. Biology of tissue factor pathway inhibitor. Blood. 2014;123(19):2934–43.
51. Dahm A, van Hylckama VA, Bendz B, Rosendaal F, Bertina RM, Sandset PM. Low levels of tissue factor pathway inhibitor (TFPI) increase the risk of venous thrombosis. Blood. 2003;101(11):4387–92.
52. Collen D, Lijnen HR. The tissue-type plasminogen activator story. Arterioscler Thromb Vasc Biol. 2009;29(8):1151–5.
53. Chung DW, Fujikawa K. Processing of von Willebrand factor by ADAMTS-13. Biochemistry. 2002;41(37):11065–70.
54. Aird WC. Endothelium and hemostasis. Endothelial Cells Health Dis. 2005:450–63.
55. Petraglia AL, Marky AH, Walker C, Thiyagarajan M, Zlokovic BV. Activated protein C is neuroprotective and mediates new blood vessel formation and neurogenesis after controlled cortical impact. Neurosurgery. 2010;66(1):165–72.
56. Adams RL, Bird RJ. coagulation cascade and therapeutics update: relevance to nephrology. Part 1: Overview of coagulation, thrombophilias and history of anticoagulants. Nephrology. 2009;14(5):462–70.
57. Ramanan SV, Rajan J, Rajan S. The coagulation cascade. transfusion practice in clinical. Neurosciences. 2022:257.
58. O'Donnell JS, O'Sullivan JM, Preston RJ. Advances in understanding the molecular mechanisms that maintain normal haemostasis. Br J Haematol. 2019;186(1):24–36.
59. Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Van Vlaenderen L, et al. Role of tissue factor in embryonic blood vessel development. Nature. 1996;383(6595):73–5.
60. Palta S, Saroa R, Palta A. Overview of the coagulation system. Indian J Anaesth. 2014;58(5):515.
61. Mann KG, Brummel-Ziedins K, Orfeo T, Butenas S. Models of blood coagulation. Blood Cell Mol Dis. 2006;36(2):108–17.
62. Grignani G, Maiolo A. Cytokines and hemostasis. Haematologica. 2000;85(9):967–72.
63. Mann KG, Butenas S, Brummel K. The dynamics of thrombin formation. Arterioscler Thromb Vasc Biol. 2003;23(1):17–25.
64. Lasne D, Jude B, Susen S. From normal to pathological hemostasis. Can J Anesth. 2006;53(2):S2–S11.
65. Byrnes JR, Duval C, Wang Y, Hansen CE, Ahn B, Mooberry MJ, et al. Factor XIIIa-dependent retention of red blood cells in clots is mediated by fibrin α-chain crosslinking. Blood. 2015;126(16):1940–8.
66. Weisel J, Litvinov R. Red blood cells: the forgotten player in hemostasis and thrombosis. J Thromb Haemost. 2019;17(2):271–82.
67. Gersh KC, Nagaswami C, Weisel JW. Fibrin network structure and clot mechanical properties are altered by incorporation of erythrocytes. Thromb Haemost. 2009;102(12):1169–75.

68. Gillespie AH, Doctor A. Red blood cell contribution to hemostasis. Front Pediatr. 2021;9:629824.
69. Kujovich JL. Factor V Leiden Thrombophilia. 2018.
70. Pike RN, Buckle AM, le Bonniec BF, Church FC. Control of the coagulation system by serpins: getting by with a little help from glycosaminoglycans. FEBS J. 2005;272(19):4842–51.
71. Rigby AC, Grant MA. Protein S: a conduit between anticoagulation and inflammation. Crit Care Med. 2004;32(5):S336–S41.
72. Bos MH, Camire RM. A bipartite autoinhibitory region within the B-domain suppresses function in factor V. J Biol Chem. 2012;287(31):26342–51.
73. Mast AE. Tissue factor pathway inhibitor: multiple anticoagulant activities for a single protein. Arterioscler Thromb Vasc Biol. 2016;36(1):9–14.
74. Dahm A, Sandset P, Rosendaal F. The association between protein S levels and anticoagulant activity of tissue factor pathway inhibitor type 1. J Thromb Haemost. 2008;6(2):393–5.
75. Corral J, González-Conejero R, Hernández-Espinosa D, Vicente V. Protein Z/Z-dependent protease inhibitor (PZ/ZPI) anticoagulant system and thrombosis. Br J Haematol. 2007;137(2):99–108.
76. Flemmig M, Melzig MF. Serine-proteases as plasminogen activators in terms of fibrinolysis. J Pharm Pharmacol. 2012;64(8):1025–39.
77. Rijken D, Lijnen H. New insights into the molecular mechanisms of the fibrinolytic system. J Thromb Haemost. 2009;7(1):4–13.
78. Dellas C, Loskutoff DJ. Historical analysis of PAI-1 from its discovery to its potential role in cell motility and disease. Thromb Haemost. 2005;93(04):631–40.
79. Ezihe-Ejiofor JA, Hutchinson N. Anticlotting mechanisms 1: physiology and pathology. Contin Educ Anaesth Crit Care Pain. 2013;13(3):87–92.
80. Chapin JC, Hajjar KA. Fibrinolysis and the control of blood coagulation. Blood Rev. 2015;29(1):17–24.
81. Dorgalaleh A, Daneshi M, Rashidpanah J, Roshani YE. An overview of hemostasis. In: Dorgalaleh A, editor. Congenital bleeding disorders. Cham: Springer International Publishing; 2018. p. 3–26.
82. Marar T, Boffa M. Identification of a thrombomodulin interaction site on thrombin-activatable fibrinolysis inhibitor that mediates accelerated activation by thrombin. J Thromb Haemost. 2016;14(4):772–83.

Congenital Bleeding Disorders: Diagnosis and Management

2

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2.1 Congenital Bleeding Disorders

Congenital bleeding disorders (CBDs) are a heterogeneous group of hemorrhagic disorders with highly variable incidence, clinical presentations, and laboratory findings [1, 2]. Although in many of these disorders precise incidence is not clear, they can be as common as von Willebrand disease (VWD), with an incidence of ~1% or be as rare as congenital factor (F) XIII and FII deficiencies with estimated incidence of 1 per two million [3, 4]. Although bleeding tendency is mild in most inherited platelet function disorders (IPFDs or "platelet disorders"), severe life-threatening bleeds such as intracranial hemorrhage (ICH) are common in FXIII deficiency [5, 6]. Severe recurrent bleeds can also be observed in hemophilia A (HA)—the most common severe congenital bleeding disorder—in hemophilia B (HB) and VWD (notably type 3). Timely diagnosis of CBDs is crucial for appropriate management of these disorders [6]. The clinical presentation, family history, and an appropriate laboratory approach are useful for timely diagnosis. Although diagnosis of most

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_2

coagulation factor deficiencies is typically straightforward (except perhaps for FXIII deficiency and some qualitative fibrinogen disorders), diagnosis of most cases of platelet disorders (IPFDs) is sophisticated and requires advanced laboratory tests (except perhaps for Glanzmann thrombasthenia (GT) and Bernard-Soulier syndrome (BSS)) [7]. Timely diagnosis and appropriate management of these disorders can significantly improve the quality of life in these patients. Although most patients with IPFD show mild bleeding tendency, regular prophylaxis is recommended for patients with severe HA and HB. Replacement therapy is a mainstay of treatment in patients with RBDs [7, 8]. Most patients with RBDs require "on-demand therapy," which means treatment of bleeding as soon as possible after onset of the event. Regular primary prophylaxis is typically necessary for patients with severe congenital FXIII deficiency from time of diagnosis (Table 2.1) [8, 9].

In patients with hemophilia, the severity of the bleeding features is directly related to the severity of the FVIII or FIX deficiency. Hemarthrosis ("joint bleeds") is the most common bleeding episode reported in patients with hemophilia. Among patients with RBDs, patients with afibrinogenemia, or deficiencies of FVII, FX, or FXIII, tend to experience more severe bleeding episodes [10]. Patients with FV deficiency generally will present with mucosal bleeding, whereas post-surgery clinical manifestation is the most common clinical feature in patients with FXI deficiency. Timely diagnosis of these disorders is crucial for appropriate management and also for the avoidance of unnecessary treatment. The diagnosis of a congenital disorder is conducted through different first line and secondary laboratory assays [11, 12].

Table 2.1 Common features of congenital bleeding disorders

Congenital bleeding disorder (CBD)			Gene defect	Inheritance	Prevalence	Bleeding tendency
Common bleeding disorders	Von Willebrand disease (VWD)	VWD-type 1	*VWF (12p13.3)*	Autosomal dominant	1 per 1000	Mild
		VWD-type 2		Mainly autosomal dominant[a]	Undetermined	Moderate-severe
		VWD-type 3		Autosomal recessive	1 per one million	Severe
	Hemophilia A (FVIII deficiency)		*FVIII (Xq28)*	X-linked recessive	1 per 10,000 males	Severe
	Hemophilia B (FIX deficiency)		*FIX (Xq27.1)*	X-linked recessive	1 per 50,000 males	Severe

(continued)

Table 2.1 (continued)

Congenital bleeding disorder (CBD)			Gene defect	Inheritance	Prevalence	Bleeding tendency
Rare bleeding disorders (RBD)	FI deficiency	Afibirinogenemia	*FGA, FGB, FGG (4q28)*	Autosomal recessive	1 per one million	Severe
		Hypofibrinogenemia		Mainly autosomal dominant	Undetermined	Mainly asymptomatic
		Dysfibrinogenemia		Mainly autosomal dominant	Undetermined	Mainly asymptomatic
		Hypodysfibrinogenemia		Mainly autosomal dominant	Undetermined	Mild
	FII deficiency		*F2* (11p11–q12)	Autosomal recessive	1 per two million	Moderate-severe
	FV deficiency		*F5* (1q24.2)	Autosomal recessive	1 per one million	Mild
	Combined FV-FVIII deficiency		*LMAN1* (18q21.3–q22) *MCFD2* (2p21–p16.3)	Autosomal recessive	1 per one million	Mild
	Vitamin K-dependent coagulation factor (VKDCF) deficiency		*GGCX* (2p12) *VKORC1* (16p11.2)	Autosomal recessive	1 per one million	Moderate
	FVII deficiency		*F7* (13q34)	Autosomal recessive	1 per 500,000	Moderate-severe
	FX deficiency		*F10* (13q34)	Autosomal recessive	1 per one million	Moderate-severe
	FXI deficiency		*F11* (4q35.2)	Autosomal recessive	1 per one million	Mild
	FXIII deficiency		*F13A1 (6p24–p25)* *F13B (1q31–q32.1)*	Autosomal recessive	1 per two million	Severe

Inherited platelet function disorders (IPFDs)	Glanzmann thrombasthenia (GT)	*ITGA2B&ITGB3 (17q21.31–32)*	Autosomal recessive	1 per one million	Moderate-severe
	Bernard-Soulier syndrome (BSS)	*GP1BA (17p13)* *GP1BB(22q11.21)* *GP9 (3q21)*	Biallelic: Autosomal recessive Monoallelic: Autosomal dominant	1 per one million	Biallelic: Moderate-severe Monoallelic: Mild
	Gray platelet syndrome (GPS)	*NBEAL* *GFI1B* *GATA1*	Mainly autosomal recessive	1 per one million	Mild

CBD Congenital bleeding disorder, *VWD* von Willebrand disease, *RBD* Rare bleeding disorder, *FI* Factor I, *FII* Factor II, *FV* Factor V, *CFV-FVIII* Combined factor V and factor VIII, *FVII* Factor VII, *VKDCF* Vitamin K dependent coagulation factor, *FX* Factor X, *FXI* Factor XI, *FXIII* Factor XIII, *IPFD* Inherited platelet function disorder, *GT* Glanzmann thrombasthenia, *BSS* Bernard-Soulier syndrome, *GPS* Gray platelet syndrome, *GP* glycoprotein

[a]Some of type 2 VWD, such as type 2N VWD, are autosomal recessive

2.2 Clinical Manifestations

The pattern of bleeding is different among patients with CBDs: mucocutaneous bleeding is common in patients with platelet disorders (IPFDs) while deep tissue hemorrhage is more common among patients with congenital coagulation factor deficiencies. Although mucocutaneous bleeds are common in IPFDs, the frequency and severity of bleeding are variable among these patients, even in members of a family, especially in GT and BSS. Although most patients with IPFDs have mild bleeding tendency, GT and BSS are considered severe disorders of this group [6, 13, 14]. In patients with GT, gingival bleeding, purpura, epistaxis, and menorrhagia are common presentations. Purpura (skin hemorrhages) commonly appear after minor trauma or pressure; epistaxis is the most common cause of severe bleeding [15, 16]. The severity of bleeding is more profound in patients with leukocyte adhesion deficiency-III/leukocyte adhesion deficiency-I variant (LAD-III/LAD-Iv) than GT, and severe life-threatening bleeds and recurrent infection are common among these patients. Myelofibrosis is a rare presentation of patients with gray platelet syndrome (GPS) due to a high level of platelet-derived growth factor (PDGF). Oculocutaneous albinism is a key feature of Hermansky-Pudlak syndrome (HPS), while immunodeficiency is a distinguishing feature of HPS-2 [6]. In patients with hemophilia, intensity of bleeding depends on severity of factor deficiency; spontaneous hemorrhages (notably in soft tissues, joints, and muscles) are common among patients with severe hemophilia (FVIII and FIX level <1%). Patients with mild and moderate hemophilia often present with post-trauma or post-surgical bleeding. Hemarthrosis is a common and debilitative manifestation of hemophilia [17–20]. Among patients with RBDs, severe bleeds can be observed among patients with afibrinogenemia, FII, FVII, FX, and FXIII deficiencies, while most of patients with combined FV and FVIII (CFV-FVIII) deficiency are asymptomatic. Patients with FXI deficiency experience post-traumatic or post-surgical bleeding. Patients with FV deficiency generally have a mild bleeding tendency [9, 11, 21]. Umbilical cord bleeding (UCB) is a common clinical presentation of patients with afibrinogenemia (85%) and FXIII deficiency (>80%). Recurrent miscarriage is also common in patients with congenital fibrinogen disorder (CFD) or FXIII deficiency [22–24]. Although successful delivery has been reported, it is believed that homozygous women with FXIII deficiency are generally unable to have successful delivery [25–27]. Intracranial hemorrhage (ICH), with a frequency of ~30%, is more common in patients with congenital

FXIII deficiency than any other CBD. This diathesis is also relatively common in FVII deficiency and has also been reported in afibrinogenemia, FII, and FX deficiencies and rarely in FV deficiency. Neonatal ICH is relatively common in vitamin K-dependent clotting factor (VKDCF) deficiency [5, 28, 29]. Thrombotic and obstetrical complications are common complications of patients with congenital fibrinogen deficiency (CFD), notably dysfibrinogenemia and hypodysfibrinogenemia [23, 30]. These complications can also be observed in patients with FII deficiency [31]. Although heterozygotes of CBDs are generally asymptomatic, severe bleeding has been observed among some heterozygotes, including FXIII and FVII deficiencies [32–34]. Mucocutaneous bleeding including epistaxis and menorrhagia is the typical presentation of patients with VWD. Patients with type 3 VWD can have severe presentations, while type 1 and type 2 are very heterogeneous and bleeding severity is related to functional VWF measured as ristocetin cofactor activity (VWF:RCo). Epistaxis and bruising are the most common presentations of children with VWD, while in adults, hematoma, menorrhagia, and bleeding from minor wounds are the most frequent presentations (Table 2.2) [3, 35–37].

Table 2.2 Clinical features of patients with congenital bleeding disorders

CBD		Common clinical presentations	Less common presentations	Rare clinical presentations
Common bleeding disorders	VWD	Epistaxis (62%) Menorrhagia (60%) Bleeding from minor wounds (36%) Post-extraction bleeding (51%) Hematoma (49%) Gum bleeding (35%) Post-surgical bleeding (28%) Postpartum bleeding (23%)	GI bleeding (14%) Hemarthrosis (8%) Hematuria (7%)	CNS bleeding (1%)
	Hemophilia A (FVIII deficiency)	Hemarthrosis (86%) Post dental extraction bleeding (84%) Hematoma (82%) Post-operative bleeding (76%) Ecchymosis (71%) Oral cavity bleeding (64%) Epistaxis (59%)	Hematuria (12%) GI bleeding (10%)	CNS bleeding (4%) Umbilical cord bleeding (UCB) (2%)
	Hemophilia B (FIX deficiency)	Post dental extraction bleeding (97%) Hemarthrosis (73%) Ecchymosis (57%) Epistaxis (55%)	GI bleeding (4%) Hematuria (12%)	CNS bleeding (5%) Hematoma (4%) Umbilical cord bleeding (UCB) (5%)

(continued)

Table 2.2 (continued)

CBD		Common clinical presentations	Less common presentations	Rare clinical presentations
Rare bleeding disorders (RBD)	Fibrinogen (FI) deficiency[a]	Umbilical cord bleeding (UCB) (85%) Epistaxis (80%) Menorrhagia (70%) Hemarthrosis (54%) Gingival bleeding (70%) Postoperative bleeding (40%)	Thrombotic events (9%)	CNS bleeding (5%)
	FII deficiency	Hematoma (60%) Hemarthrosis (42%) Post dental extraction bleeding (36%) Menorrhagia (20%)	Postoperative bleeding (12%)	GI bleeding (3%) CNS bleeding (1%)
	FV deficiency	Epistaxis (68%) Menorrhagia (50%) Post-operative bleeding (43%) Oral cavity bleeding (31%)	Hemarthrosis (18%) Hematoma (60%) GI bleeding (6%)	Umbilical cord bleeding (UCB) (3%) CNS bleeding (1%)
	Combined FV-FVIII deficiency	Post dental extraction bleeding (88%) Post-partum hemorrhage (83%) Excessive post-major surgical bleeding (75%) Menorrhagia (63%) Post-circumcision hemorrhage (56%) Epistaxis (42.5%) Gum bleeding (29%) Ecchymosis/easy bruising (32%)	Hemarthrosis (18%) GI bleeding (7.5%)	Hematuria (2%) Hematoma (2%) Intracranial hemorrhage (ICH) (1%)
	Vitamin K-dependent coagulation factor (VKDCF) deficiency	ICH (34%) Ecchymosis/easy bruising (21%)	Umbilical cord bleeding (UCB) (17%) Post-trauma/ post-surgical hemorrhage (17%) Epistaxis (17%) Gingival/oral bleeding (12%)	Hemarthrosis (4%)
	FVII deficiency	Epistaxis (60%) Menorrhagia (69%) Easy bruising (36%) Gum bleeding (34%) Hematoma (20%)	Hemarthrosis (19%) GI bleeding (14%) Hematuria (6%)	CNS bleeding (4%)
	FX deficiency	Menorrhagia (71%) Easy bruising (55%) Hematoma (43%) Epistaxis (36%) Hemarthrosis (33%) Gum bleeding (31%)	GI bleeding (12%) Intracranial hemorrhage (ICH) (9%) Hematuria (7%)	Hemarthrosis (2%)
	FXI deficiency	Post-surgical bleeding (66%) Ecchymosis (28%) Epistaxis (24%) GI bleeding (15%)	Menorrhagia (7%)	Gum bleeding (1%) Post dental extraction bleeding (3.5%)
	FXIII deficiency	Umbilical cord bleeding (UCB) (>80%) Hematoma (53%) Prolonged wound bleeding (31%) Intracranial bleeding (~30%) Post-surgical bleeding (19%) Gum bleeding (17%) Epistaxis (14%) Miscarriage (~100%)[b]	GI bleeding (10%) Menorrhagia (10%) Post circumcision bleeding (4%) Delayed post dental extraction bleeding (7%)	Splenic rupture (<1%)

Table 2.2 (continued)

CBD		Common clinical presentations	Less common presentations	Rare clinical presentations
Inherited platelet function disorders (IPFD)	Glanzmann thrombasthenia (GT)	Epistaxis (79%) Menorrhagia (74%) Gingival bleeding (68%) Ecchymosis/easy bruising (43%)	GI bleeding (7.4%) Hematuria (7%) Hematoma (6%)	CNS bleeding (1.8%) Excessive bleeding at time of delivery (0.5%) Umbilical cord bleeding (UCB) (0.3%) Hemarthrosis (0.3%)
	Bernard-Soulier syndrome (BSS)	Post-partum hemorrhage (75%) Epistaxis (64%)	Ecchymosis (7%) Gum bleeding (15.5%) Post-dental extraction bleeding (15%) Menorrhagia (11.3%)	GI bleeding (4.1%) Hematoma (3%) CNS bleeding (1%)
	Gray platelet syndrome (GPS)	Epistaxis (53%) Easy bruising (48%) Menorrhagia (38%) Splenomegaly (70%)	Ecchymosis (7%) Post-surgical bleeding (10%) Post-dental extraction bleeding (6%)	Intracranial hemorrhage (ICH) (1%)

CBD Congenital bleeding disorder, *VWD* von Willebrand disease, *RBD* Rare bleeding disorders, *FI* Factor I, *FII* Factor II, *FV* Factor V, *CFV-FVIII* Combined factor V and factor VIII, FVII, Factor VII, *VKCFD* Vitamin K dependent clotting factor deficiency, *FX* Factor X, *FXI* Factor XI, *FXIII* Factor XIII, *IPFD* Inherited platelet function disorder, *GT* Glanzmann thrombasthenia, *BSS* Bernard-Soulier syndrome, *GPS* Gray platelet syndrome
GI Gastrointestinal bleeding, *CNS* Central nervous system, *UCB* Umbilical cord bleeding, *ICH* Intracranial hemorrhage
[a] Data were presented for afibrinogenemia
[b] Almost all untreated women with severe FXIII deficiency experience miscarriage

2.3 Molecular Basis

Congenital coagulation factor deficiencies are typically due to mutations in genes encoding corresponding coagulation factors except for congenital combined FV-FVIII (CFV-FVIII) deficiency and Vitamin K-dependent coagulation factor (VKDCF) deficiency. CFV-FVIII deficiency is caused by mutation in proteins involving intracellular transport of FV and FVIII. These are *MCFD2* and *LMAN1* genes. In VKDCF deficiency, the congenital defect in coagulation factors is due to mutation in genes encoding enzymes involved in post-translational modification and vitamin K metabolism including *gamma-glutamyl carboxylase (GGCX)* and *vitamin K epoxide reductase (VKOR)*. Most of the RBDs have autosomal recessive manner of inheritance except for some cases with FXI deficiency and congenital fibrinogen deficiency (CFD) (hypofibrinogenemia and dysfibrinogenemia) [9, 38–41]. The inheritance pattern of IPFDs is in an autosomal manner, while in HA and HB, the pattern of inheritance is X-linked recessive [6]. A considerable number of patients with HA and HB have de-novo mutations in *F8* and *F9* genes [42]. Among congenital bleeding disorders (CBDs), recurrent mutations are rare and the most

common gene defect is intron 22 inversion that occurs in ~45% of patients with severe HA. The second most common mutation is intron 1 inversion with a frequency of 1–5%. Screening for these mutations can be the first step in molecular diagnosis of patients with severe HA [43–45]. Such gene defects are rare for other CBDs. According to some studies, FGA IVS 4 + 1 G > T mutation should be the first mutation in the screening algorithm for new individuals with congenital afibrinogenemia of European origin and *FGA* 11-kb deletion is the other common target for molecular diagnosis and the avoidance of diagnostic errors [23, 46]. In Iranian patients with congenital FXIII deficiency, Trp187Arg (c.559 T > C) is the recommended initial screening in the molecular diagnosis [4, 47].

Milder types of HA are mostly due to missense mutations. In patients with congenital fibrinogen deficiency, missense mutations are the most common gene defects and the bleeding severity is more profound in those with missense mutation. Some of these mutations are clustered in specific gene areas, including clustering of missense mutations in the highly conserved βC of FGB. Most cases with CBDs are due to new mutations that are restricted to a specific family or specific geographical area mostly due to founder effect (Fig. 2.1) [48–50].

A wide spectrum of mutations was identified throughout affected genes in CBDs. In RBDs, missense mutations are the most common identified mutations (with a frequency of >50%) [9]. In patients with GT, the disorder is due to homozygous and double heterozygous mutations in *ITGA2B and ITGB3* genes [51, 52]. VWD has autosomal recessive/dominant pattern of inheritance. Although type 1 VWD is an autosomal dominant disorder, in ~30% of patients, an underlying mutation cannot be identified. Type 3 VWD, the most severe type of VWD, is due to homozygous or double heterozygous mutations in the *VWF* gene (Table 2.3) [53–57].

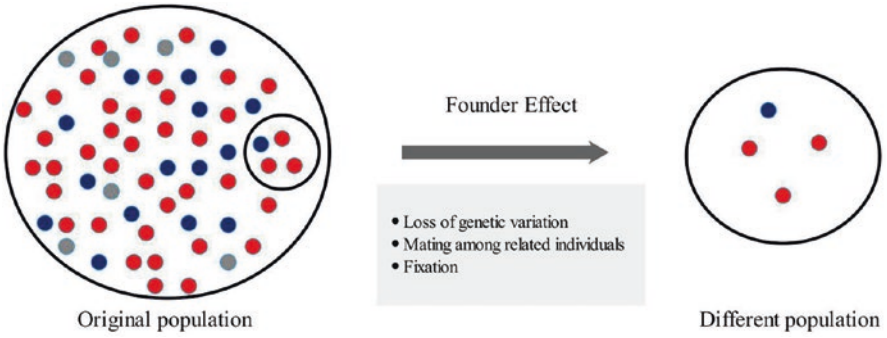

Fig. 2.1 Founder effect. A *founder effect* occurs when a new colony is started by a few members of the original population

Table 2.3 Molecular characteristics of congenital bleeding disorders

Congenital bleeding disorders			Gene defect (location)	Type of mutation	Number of mutations
Common bleeding disorders	Von Willebrand disease (VWD)	VWD-type 1	*VWF (12p13.3)*	Missense: (70%) Splice site: (12%) Small insertion and deletion: (8%) Nonsense: (2%) Promoter change: (8%)	130
		VWD-type 2	*VWF (12p13.3)*	Missense: (89%) Splice site: (2%) Deletion: (3%) Nonsense: (2%) Others: (4%)	Type 2: 160 Type 2A: 75 Type 2B: 25 Type 2N: 30 Type 2M:30
		VWD-type 3	*VWF (12p13.3)*	Missense: (14%) Small insertion: (25%) Small deletion: (15%) Large deletion: (12%) Splicing: (9%) Nonsense: (25%)	120
	Hemophilia A (FVIII deficiency)		*F8 (Xq28)*	Intron 22 inversion: (45%) Intron 1 inversion: (5%) Others: (~50%)	3205
	Hemophilia B (FIX deficiency)		*F9 (Xq27.1)*	Missense + nonsense: (60%) Small insertion/deletion: (19.5%) Large insertion/deletion: (8.3%) Splice site: (8%) Regulatory: (2.3%) Complex rearrangement: (1%)	1283

(continued)

Table 2.3 (continued)

Congenital bleeding disorders			Gene defect (location)	Type of mutation	Number of mutations
Rare bleeding disorders	Fibrinogen (FI) deficiency	Fibrinogen alpha chain mutation (FGA)	*FGA (4q28)*	Missense: (33.96%) Nonsense: (22.64%) Frame shift: (16.03%) Splice site: (7.54%) Insertion: (1.88%) Deletion: (3.77%) Promoter: (0.94%) Undefined: (12.26%)	106
		Fibrinogen beta chain mutation (FGB)	*FGB (4q28)*	Missense: (56.25%) Nonsense: (15.62%) Frameshift: (7.81%) Splice site: (6.25%) Undefined: (12.5%)	64
		Fibrinogen gamma chain mutation (FGG)	*FGG (4q28)*	Missense: (57.89%) Nonsense: (10.52%) Frameshift: (3.50%) Splice site: (14.03%) Undefined: (14.03%)	57
		Undefined	–	Frame shift: (30%) Missense: (20%) Nonsense: (30%) Undefined: (20%)	10
	FII deficiency		*F2 (11p11–q12)*	Missense: (79.5%) Deletion: (8%) Splice site: (3%) Nonsense: (6.5%) Insertion: (3%)	63
	FV deficiency		*F5 (1q24.2)*	Missense: (51.4%) Frameshift: (22.9%) Splice site: (6.5%) Nonsense: (13.9%) Insertion/deletion/ Duplication: (2.5%) Silent: (0.3) Undefined: (2.5%)	323

Table 2.3 (continued)

Congenital bleeding disorders		Gene defect (location)	Type of mutation	Number of mutations
	Combined FV-FVIII deficiency	*LMAN1 (18q21.3–q22)*	Missense: (10.8%) Nonsense: (19.0%) Frameshift: (56.7%) Splice site: (13. 5%)	37
		MCFD2 (2p21–p16.3)	Missense: (42.1%) Nonsense + deletion: (5.3%) Splice site: (21%) Frameshift: (31.6%)	19
	Vitamin K-dependent coagulation factor (VKDCF) deficiency	*GGCX (2p11.2)*	Missense: (~69%) Splice site: (~16.6%) Nonsense: (~9.5%) Deletion: (~4.7%)	42
		VKORC1 (16p11.2)	Missense: (100%)	1
	FVII deficiency	*F7 (13q34)*	Missense: (52.4%) Deletion: (3%) Splice site: (10%) Nonsense: (5.75%) Frameshift: (5.75%) Duplication: (0.3%) Undefined: (22.7%)	330
	FX deficiency	*F10 (13q34)*	Missense: (75%) Deletion: (4.6%) Synonymous: (5.3%) Splice site: (4.2%) Nonsense: (4.2%) Insertion: (~ 0.8%) Frameshift: (~ 0.4%) Duplication: (~ 0.8%) Undefined: (5%)	262
	FXI deficiency	*F11 (4q35.2)*	Missense: (~68.1%) Nonsense: (~12%) Deletion: (~8.1%) Point: (~7.3%) Duplication: (~1.7%) Insertion: (~1.7%) Silent: (~0.8%)	232

(continued)

Table 2.3 (continued)

Congenital bleeding disorders		Gene defect (location)	Type of mutation	Number of mutations
	FXIII deficiency	*F13A1 (6p24–p25)*	Missense: (48.8%) Deletion/insertion: (26.2%) Splice site: (14.5%) Nonsense: (10.5%)	172
		F13B (1q31–q32.1)	Missense: (52%) Splice site: (16%) Frameshift: (32%)	25
Inherited platelet function defects (IPFD)	Glanzmann thrombasthenia (GT)	*ITGA2B (17q21.32)*	Missense + nonsense: (56.27%) Splicing: (17%) Regulatory: (>1%) Deletion + insertion: (30%) Complex rearrangements: (>1%)	217
		ITGB3 (17q21.32)	Missense + nonsense: (63.75%) Splicing: (10%) Deletion + insertion: (25.6%) Complex rearrangements: (>1%)	160
	Bernard-Soulier syndrome (BSS)	*GP1BA (17pter-p12)*	Missense + nonsense: (56.5%) Regulatory: (1.3%) Deletion + insertion: (40.8%) Repeat variations: (1.3%)	76
		GP1BB (22q11.21)	Missense + nonsense: (71.7%) Regulatory: (1.9%) Deletion + insertion: (26.4%)	53
		GP9 (3q21)	Missense + nonsense: (88%) Deletion + insertion: (12%)	42

Table 2.3 (continued)

Congenital bleeding disorders		Gene defect (location)	Type of mutation	Number of mutations
	Gray platelet syndrome (GPS)	*NBEAL2 (3p21.31)*	Missense + nonsense: (60%) Splicing: (13.3%) Deletion + insertion: (26.6%)	45
		GFI1B (9q34.13)	Missense + nonsense: (33.3%) Splicing: (33.3%) Regulatory: (22.2%) Insertion: (11.1%)	9
		GATA1 (Xp11.23)	Missense + nonsense: (73.3%) Splicing: (13.3%) Deletion + insertion: (13.3%)	15

CBD Congenital bleeding disorder, *VWD* von Willebrand disease, *RBD* Rare bleeding disorders, *FI* Factor I, *FII* Factor II, *FV* Factor V, *CFV-FVIII* Combined factor V and factor VIII, FVII, Factor VII, *VKDCF* Vitamin K dependent clotting factor, *FX* Factor X, *FXI* Factor XI, *FXIII* Factor XIII, *IPFD* Inherited platelet function disorder, *GT* Glanzmann thrombasthenia, *BSS* Bernard-Soulier syndrome, *GPS* Gray platelet syndrome, *GP* glycoprotein

2.4 Diagnosis

The diagnosis of congenital bleeding disorders (CBDs) can be made based on clinical presentations, family history and appropriate laboratory approach. A positive family history is an important clue for timely diagnosis of CBDs. Therefore, prior to laboratory assessment, a proper family history should be taken [1, 2, 58]. In CBDs with an autosomal manner of inheritance such as RBDs, IPFDs, and VWD, any family member can be affected, while in HA and HB due to an X-linked manner of inheritance, males are affected. It should be remembered that about one-third of patients with hemophilia have de-novo mutations; therefore, absence of family history should not always lead to ruling out of CBDs [6, 58–60]. In addition to this issue, in some patients with CBDs including GT, BSS, and FX deficiency, even when possessing the same mutation as members of the same family, the bleeding tendency may be variable [9, 52]. The type of bleeding and severity of the disorder can also help in diagnosis. Mucocutaneous hemorrhages are more frequent in patients with platelet disorders (IPFDs), while this type of bleeding is less common in coagulation factor deficiency (hemorrhage in deep tissues is more frequent among these latter patients) [13, 61]. Hemarthrosis is a hallmark of hemophilia, and joints that bleed are often referred to as "target joints." The most common target joints are knees and elbows and less frequently, hip, wrist, and shoulder [62, 63]. Although hemarthrosis is common in hemophilia, it also can be observed in other CBDs including RBDs and especially in type 3 VWD that is accompanied by significant decrease of FVIII (Table 2.4) [9, 64, 65].

Table 2.4 Main laboratory features of congenital bleeding disorders

Congenital bleeding disorders				Laboratory features	
Common Bleeding Disorders	VWD	VWD-type 1		VWF:Ag: ↓ to ↓↓ VWF:GPIb binding: ↓ to ↓↓ VWF:CB: ↓ to ↓↓ FVIII:C: N to ↓↓ VWF:RCo: ↓↓	Multimers: Normal pattern but reduced intensity GPIb binding /Ag: > (0.5–0.7) CB/Ag: > (0.5–0.7) FVIII/VWF: > (0.5–0.7) RIPA: N to ↓↓
		VWD-type 2	2 A	VWF:Ag: N to ↓↓ VWF:GPIb binding: ↓↓ to ↓↓↓ VWF:CB: ↓↓ to ↓↓↓ FVIII:C: ↓ to ↓↓ VWF:RCo: ↓↓ to ↓↓↓	Multimers: Loss of high MMW VWF GPIb binding /Ag: < (0.5–0.7) CB/Ag: < (0.5–0.7) FVIII/VWF: > (0.5–0.7) RIPA: ↓ to ↓↓
			2 B	VWF:Ag: N to ↓↓ VWF:GPIb binding: ↓ to ↓↓↓ VWF:CB: ↓ to ↓↓↓ FVIII:C: N to ↓↓ VWF:RCo: ↓↓	Multimers: Loss of high MMW VWF GPIb binding /Ag: < (0.5–0.7) CB/Ag: < (0.5–0.7) FVIII/VWF: > (0.5–0.7) RIPA: ↑
			2N	VWF:Ag: N to ↓↓ VWF:GPIb binding: N to ↓↓ VWF:CB: N to ↓↓ FVIII:C: ↓↓ to ↓↓↓ VWF:RCo: N to ↓↓	Multimers: Normal pattern GPIb binding /Ag: > (0.5–0.7) CB/Ag: > (0.5–0.7) FVIII/VWF: < (0.5–0.7) RIPA: N
			2M	VWF:Ag: N to ↓↓ VWF:GPIb binding: ↓ to ↓↓↓ VWF:CB: ↓ to ↓↓↓ FVIII:C: ↓ to ↓↓ VWF:RCo: ↓↓	Multimers: No loss of HMW VWF but some multimer defects may be present GPIb binding /Ag: < (0.5–0.7) CB/Ag: < (0.5–0.7) FVIII/VWF: > (0.5–0.7) RIPA: ↓
		VWD-type 3		VWF:Ag: ↓↓↓ (absent) VWF:GPIb binding: ↓↓↓ (absent) VWF:CB: ↓↓↓ (absent) FVIII:C: ↓↓↓ VWF:RCo: ↓↓	Multimers: No VWF present GPIb binding /Ag: NA CB/Ag: NA FVIII/VWF: NA RIPA: ↓ to ↓↓
	Hemophilia A			PT: N APTT: ↑↑↑ TT: N BT: N	FVIII: C: ↓↓↓ Mild HA (5–40%), moderato HA (1–5%), and severe HA (<1%) FVIII: Ag: ↓↓↓
	Hemophilia B			PT: N APTT: ↑↑↑ TT: N BT: N	FIX: C: ↓↓↓ Mild HB (5–30%), moderate HB (1–5%), and severe HB (<1%). FIX: Ag: ↓↓↓

Table 2.4 (continued)

Congenital bleeding disorders			Laboratory features	
Rare bleeding disorders (RBD)	Fibrinogen (FI) deficiency	Afibrinogenemia'	PT: ↑↑↑ APTT: ↑↑↑ TT: ↑↑↑ RT: ↑↑↑	Fibrinogen activity: Undetectable Fibrinogen antigen: Undetectable Fibrinogen functional/antigenic ratio: NA
		Hypofibrinogenemia	PT: ↑ to ↑↑ depending on fibrinogen levels APTT: ↑ to ↑↑ depending on fibrinogen levels TT: ↑ to ↑↑ depending on fibrinogen levels RT: ↑ to ↑↑ depending on fibrinogen levels	Fibrinogen activity: <1.5 g/L Fibrinogen antigen: <1.5 g/L Fibrinogen functional/antigenic ratio: > 0.7
		Dysfibrinogenemia	PT: Usually ↑↑ APTT: Usually ↑↑ TT: Usually ↑↑ RT: Usually ↑↑	Fibrinogen activity: <1.5 g/L Fibrinogen antigen:< 1.5 g/L Fibrinogen functional/antigenic ratio: < 0.7
		Hypodysfibrinogenemia	PT: ↑ to ↑↑ depending on fibrinogen levels APTT: ↑ to ↑↑ depending on fibrinogen levels TT: ↑ to ↑↑ depending on fibrinogen levels RT: ↑ to ↑↑ depending on fibrinogen levels	Fibrinogen activity: <1.5 g/L Fibrinogen antigen: <1.5 g/L Fibrinogen functional/antigenic ratio: < 0.7
	FII deficiency		PT: ↑↑ to ↑↑↑ APTT: ↑↑ to ↑↑↑ TT: N BT: N	FII:C: ↓↓↓ Mild (>10%), moderate (<10%) and severe (undetectable) FII:Ag: ↓↓↓
	FV deficiency		PT: ↑↑ to ↑↑↑ APTT: ↑↑ to ↑↑↑ TT: N BT: N	FV:C: ↓↓↓ Mild (>10%), moderate (<10%) and severe (undetectable) FV:Ag: ↓↓↓
	Combined FV-FVIII deficiency		PT: ↑↑ to ↑↑↑ APTT: ↑↑ to ↑↑↑ TT: N BT: N	FV:C: ↓↓ FV:Ag: ↓↓ FVIII: C: ↓↓ FVIII: Ag: ↓↓
	Vitamin K-dependent coagulation factor (VKDCF) deficiency		PT: ↑↑ to ↑↑↑ APTT: ↑↑ to ↑↑↑ TT: N BT: N	FII: C: ↓↓ FII: Ag: ↓↓ FVII: C: ↓↓ FVII:Ag: ↓↓ FIX:C: ↓↓ FIX:Ag: ↓↓ FX:C: ↓↓ FX:Ag: ↓↓
	FVII deficiency		PT: ↑↑ to ↑↑↑ APTT: N TT: N BT: N	FVII:C: ↓↓↓ Mild (>20%), moderate (10–20%) and severe (<10%) FVII:Ag: ↓↓↓
	FX deficiency		PT: ↑↑ to ↑↑↑ APTT: ↑↑ to ↑↑↑ RVVT: ↑↑ BT: N	FX:C: ↓↓↓ Severe (FX: C < 1 U/dL), moderate (FX: C of 1–5 U/dL) and mild (FX: C of 6–10 U/dL) FX:Ag: ↓↓↓
	FXI deficiency		PT: N APTT: ↑↑ TT: N BT: N	FXI:C: ↓↓↓ Severe (1–20 U/dL of FXI: C level), moderate (20–60 U/dL FXI: C levels), and mild (61–80 U/dL FXI: C level) FXI:Ag: ↓↓↓
	FXIII deficiency		PT: N APTT: N TT: N BT: N	Clot solubility test: Abnormal FXIII:C: ↓↓↓ FXIII:Ag: ↓↓↓

(continued)

Table 2.4 (continued)

Congenital bleeding disorders			Laboratory features	
Inherited platelet function defects (IPFD)	Glanzmann thrombasthenia (GT)	Type-I	Plt count: N Plt morphology: N PT: N APTT: N TT: N BT/CT (PFA-100/200): ↑↑	Clot retraction: Absent Platelet integrin αIIbβ3 expression: Undetectable or ↓↓↓ Platelet integrin αIIbβ3 (%) (flow cytometry): < 5 Platelet aggregation: Absent Platelet agglutination: N
		Type-II	Plt count: N Plt morphology: N PT: N APTT: N TT: N BT/CT (PFA-100/200): ↑↑	Clot retraction: ↓ Platelet integrin αIIbβ3 expression: ↓↓↓ Platelet integrin αIIbβ3 (%) (flow cytometry): 5–20 Platelet aggregation: Absent Platelet agglutination: N
		Variant	Plt count: N Plt morphology: N PT: N APTT: N TT: N BT/CT (PFA-100/200): ↑↑	Clot retraction: Variable Platelet integrin αIIbβ3 expression: N Platelet integrin αIIbβ3 (%) (flow cytometry): > 20 Platelet aggregation: Absent/abnormal Platelet agglutination: N
	Bernard-Soulier syndrome (BSS)		Plt count: Thrombocytopenia Plt morphology: Large platelets (monoallelic), giant platelets (biallelic) PT: N APTT: N TT: N BT/CT (PFA-100/200): ↑↑ or ↑↑↑	RIPA: Defective Platelet aggregation: Response to collagen, arachidonic acid, and thromboxane analogues is normal. Decrease in response to low dose, but not high dose of thrombin. Decrease in response to ristocetin and botrocetin. Flow cytometry: GP1b-IX-V (CD42a-d)
	Gray platelet syndrome (GPS)		Plt count: Usually <100 × 109/L Plt morphology: Macrothrombocytopenia, gray and pale platelet PT: N APTT: N TT: N BT: ↑	TEM: ↓↓ α-granules LTA: Normal for ADP, epinephrine, arachidonic acid and ristocetin and defective in response to thrombin and collagen. Vitamin B12 concentration: In half of patients ↑↑↑

CBD Congenital bleeding disorder, *VWD* von Willebrand disease, *RBD* rare bleeding disorders, *FI* Factor I, *FII* Factor II, *FV* Factor V, *CFV-FVIII* combined factor V and factor VIII, *FVII* Factor VII, *VKDCF* Vitamin K dependent clotting factor, *FX* Factor X, *FXI* Factor XI, *FXIII* Factor XIII, *IPFD* inherited platelet function disorder, *GT* Glanzmann thrombasthenia, *BSS* Bernard-Soulier syndrome, *GPS* Gray platelet syndrome, *NA* not applicable, *PT* prothrombin time, *APTT* activated partial thromboplastin time, *TT* thrombin time, *RT* reptilase time, *BT* bleeding time, *Plt* platelet, *CT* closure time, *TEM* thromboelastometry, *LTA* light transmission aggregometry, *ADP* adenosine diphosphate, *PFA* platelet function analysis, *RVVT* Russell's viper venom time, *VWF* CB: von Willebrand Factor collagen binding, *Ag* antigen, *RIPA* Ristocetin-induced platelet aggregation, *GP* glycoprotein

Although a number of IPFDs are considered severe bleeding disorders (including GT and BSS), most patients with IPFDs have mild bleeding tendency and can be undiagnosed during patient's life. In such cases, due to mild phenotype of disorder, both family history and bleeding episodes may be undetectable [6]. Some specific types of hemorrhages can be considered as important diagnostic clues in CBDs. These include umbilical cord bleeding (UCB) that is common in patients with afibrinogenemia and FXIII deficiency. Miscarriage is also common in both of these disorders. Intracranial hemorrhage (ICH) as a severe, life-threatening hemorrhage is more common in FXIII deficiency than any other CBD [24, 66, 67]. Among patients with congenital fibrinogen deficiency (CFD), most patients with hypofibrinogenemia and dysfibrinogenemia are asymptomatic, while hemorrhage is more common in afibrinogenemia. Thrombotic events are relatively common in patients with CFD [23, 37].

In coagulation factor deficiencies that are involved in the common pathway of the coagulation cascade, *both* the prothrombin time and activated partial thromboplastin time (APTT) can be prolonged, based on the severity of the deficiency and the sensitivity of the coagulation reagents [9, 68].

2.5 Diagnosis of Inherited Platelet Function Disorders (IPFDs)

Although in some patients with IPFDs, including GT and BSS, the diagnosis of the disorder is straightforward, the diagnosis of many platelet disorders is complicated and requires precise and sometimes sophisticated laboratory assessments [6]. In routine practice, the rate of misdiagnosis is relatively high, so the platelet physiology subcommittee of the International Society of Thrombosis and Hemostasis (ISTH) has proposed a standard algorithm for proper diagnosis of IPFDs. Based on this guideline, the first step should include blood smear examination, light transmission aggregometry (LTA) with a limited number of agonists, platelet granule release, and flow cytometric analysis of major platelet glycoproteins (Fig. 2.2) [69–72].

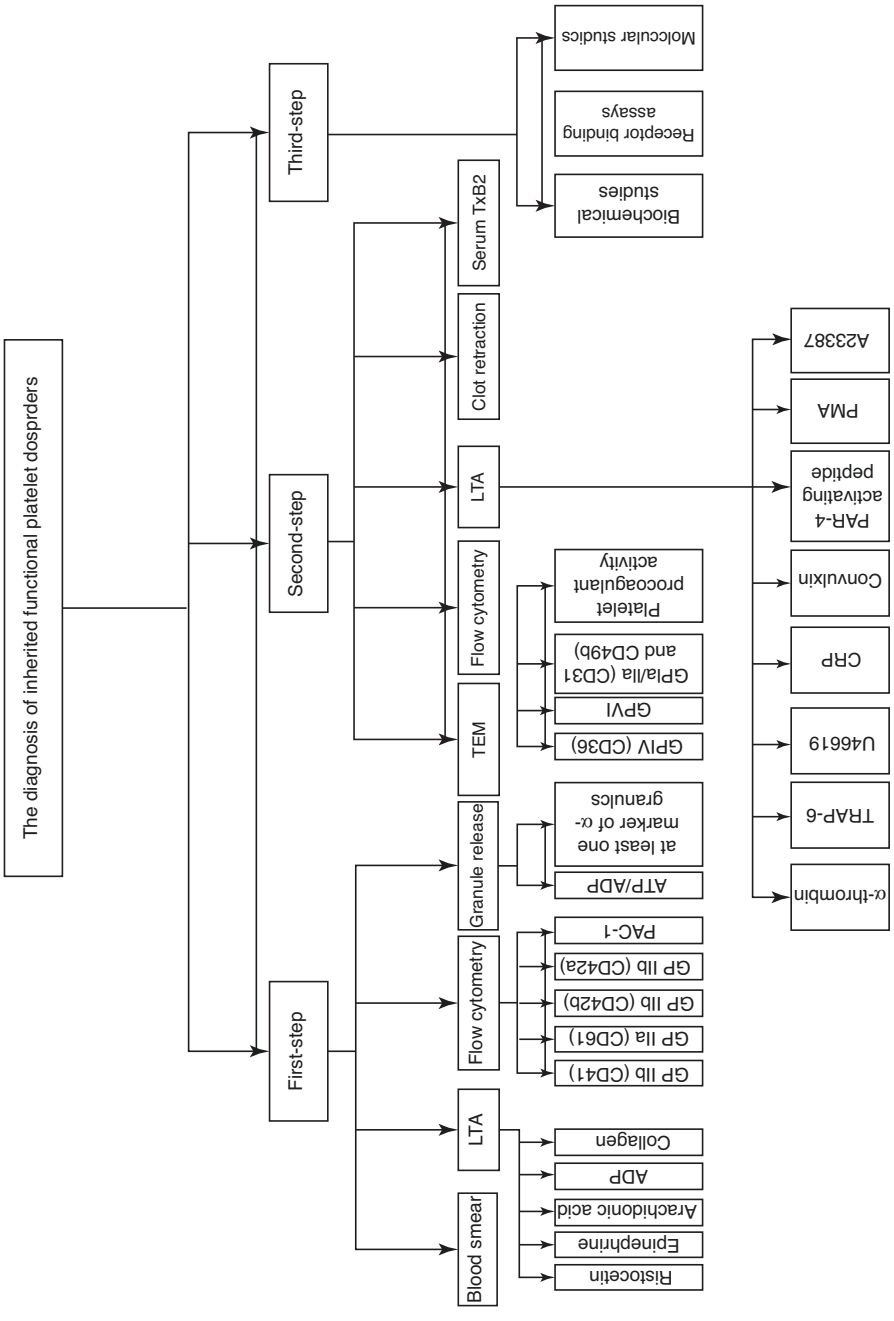

Fig. 2.2 International Society of Thrombosis and Hemostasis (ISTH) suggested algorithm for diagnosis of inherited platelet function [7]

2.6 Diagnosis of Glanzmann Thrombasthenia

GT is a severe inherited platelet disorder (IPFD) that is accompanied by mucocutaneous hemorrhage with impaired aggregation studies with all physiological agonists, while ristocetin induced platelet aggregation (RIPA) is normal. All routine coagulation tests are normal in GT except for functional tests such as PFA-100 or bleeding time. Platelet count and morphology are also normal [52, 73]. Although GT is a rare disorder, it is the most common IPFD to cause severe bleeding episodes. However, the bleeding tendency remains variable among patients. Homozygous and double heterozygous patients may be prone to life-threatening bleeding events, while heterozygous patients are usually asymptomatic. This disorder is due to mutation in *ITGA2B* and *ITGB3* genes, which encode glycoprotein (GP) αIIbβ3. Following platelet activation, GPαIIbβ3 is expressed on the platelet surface. Binding to its ligands (usually fibrinogen) is necessary for platelet aggregation and signal transduction. In GT, the GPαIIbβ3 defect leads to a platelet aggregation disorder. An informative family history and the patient's clinical symptoms are valuable clues to diagnosing the disease. Based on ISTH guideline, diagnosis of GT, similar to other IPFDs, proceeds in three steps (Fig. 2.2) [74, 75]. More than 300 mutations are reported in GT; among which, missense, nonsense, and frameshift are the most common. PCR and DNA sequencing are molecular methods used for diagnosis of this disorder. In general, due to the specific platelet aggregometry pattern in GT and the availability of flow cytometry to confirm the reduction of GPαIIbβ3 on the platelet surface, the diagnosis of the disease is straightforward and there is no need for genetic analysis. However, in cases without the usual laboratory presentation or in cases with the possibility of producing antibodies against GPαIIbβ3 after platelet transfusion, genetic studies become especially important.

Identifying mutations specific to each family is important for family studies and counseling. Patients with mutations that cause type I disease (the severe type of the disease) are more likely to develop antibodies against GPαIIbβ3 following platelet transfusion [52, 73].

2.7 Diagnosis of Bernard-Soulier Syndrome

BSS is a platelet functional defect due to GPIb/V/IX deficiency. This glycoprotein is the major receptor for von Willebrand Factor (VWF) on the platelet surface and has a major role in platelet adhesion to the subendothelium in the injured site. This disorder is recognized by a low platelet count and giant dysfunctional platelets. Bleeding tendency is variable among patients [76]. Four genes, *GPIBA*, *GPIBB*, *GP5*, and *GP9*, are needed to form GPIb/V/IX complex. Different mutations such as insertion/deletion and missense mutations in *GPIBA*, *GPIBB*, and *GP9* genes may lead to BSS, but there is no reported mutation in the *GP5* gene. Most of these mutations decrease GPIb/V/IX expression [77]. The diagnostic approach to BSS typically includes peripheral blood smear assessment, platelet aggregometry with various agonists, flow cytometric analysis for GPIb/V/IX expression on platelets, and molecular studies for disease-causing mutations (Table 2.4) [78].

2.8 Diagnosis of Gray Platelet Syndrome

GPS is a rare inherited disorder of platelet α granules characterized by a bleeding tendency, thrombocytopenia, giant gray platelets, bone marrow myelofibrosis, splenomegaly, and increased plasma vitamin B_{12} [79]. In addition. Due to α granule deficiency, there is a decrease in the contents of these granules, including fibrinogen, FV, VWF, thrombospondin, PF4, and PDGF. Aggregometry studies are normal in response to most agonists except for collagen and thrombin (Table 2.4) [80]. GPS is typically due to a mutation in the *Neurobeachin-like 2 (NBEAL2)* gene. This gene encodes proteins with Beige and Chediak-Higashi (BEACH) domains, which are related to LYST and play a role in vesicular traffic. GPS may also be due to mutation in *GFI1b* or *GATA1* gene [81, 82]. Based on ISTH guidelines, diagnosis of GPS, similar to other IPFDs, is approached in three steps (Fig. 2.2). In patients with GPS, aggregometry studies are variable. Platelets of most patients aggregate normally in response to ADP, arachidonic acid, epinephrine, and ristocetin, but some patients have abnormal responses to collagen and thrombin (Table 2.4) [83].

2.9 Diagnosis of Von Willebrand Disease

VWD is the most common inherited bleeding disorder with a prevalence of 1% in the general population. This disease is due to quantitative or qualitative defects in VWF and is inherited in an autosomal recessive or dominant manner [84]. VWD is a heterogeneous disorder, which is classified into three types based on ISTH guidelines: type I has VWF relative *deficiency*, type II has a *qualitative* defect in VWF, and type III has complete *absence* of VWF. Type II can be further classified into four subtypes: 2A, 2B, 2M, and 2N. Subtype 2A has a reduction of high molecular weight multimers of VWF (and sometimes intermediate). Subtype 2B has *increased* affinity of VWF to platelet GPIb. Subtype 2M has *decreased* affinity of VWF to platelet GPIb. Subtype 2N has a significant decrease of VWF affinity to FVIII (which leads to increased clearance of FVIII from the circulation) [85]. Clinical manifestations and bleeding tendency are widely variable in VWD, ranging from very mild to severe bleeding episodes requiring immediate therapeutic action (Table 2.2) [86–89]. Diagnosis of VWD often is a difficult process requiring sophisticated laboratory assessment. Three main criteria were introduced for diagnosis of VWD. These include a decreased VWF activity level, bleeding symptoms (personal history), and inheritance (family history); among them, the clinical manifestation is

the most important one. When a history of bleeding in a person or family causes suspicion of VWD, a series of screening tests, along with diagnostic and confirmatory tests, are needed to confirm the diagnosis [90–92]. Screening tests may include platelet count, platelet function analysis (e.g., PFA-100 or bleeding time), and APTT. The results of these tests can help in choosing the next diagnostic tests [93]. Due to the limitations and unreliability of screening tests for VWD diagnosis, specific diagnostic tests are required to confirm or rule out the disease. These tests include VWF: Ag, VWF: RCo, FVIII: C, and VWF multimer analysis. There are additional laboratory tests for confirmation of VWD subtypes including VWF: collagen binding (VWF: CB), RIPA, VWF: FVIII binding assay, VWF propeptide assay, and sequencing of the *VWF* gene (Table 2.4).

2.10 Diagnosis of Congenital Factor Deficiency

The diagnosis of most coagulation factor deficiencies is straightforward; however, some disorders including FXIII deficiency and also some qualitative fibrinogen disorders have a more complicated diagnosis [4]. The diagnosis of coagulation disorders should be a multifaceted approach, including the evaluation of the family history, assessment of clinical manifestations, physical examination, laboratory approach, and molecular analysis. Since these disorders are typically inherited as autosomal recessive bleeding disorders or sex-linked recessive (hemophilia) inheritance patterns, evaluation of an informative family history is a key step towards diagnosis. However, the absence of family history does not rule out a congenital disorder. Moreover, certain clinical manifestations are more specifically reported in some types of disorders, such as hemarthrosis in hemophilia, and umbilical cord bleeding (UCB) and recurrent miscarriage in FXIII deficiency and fibrinogen (FI) deficiency. Therefore, taking the full history of clinical manifestations is crucial in the diagnosis of a congenital disorder [11].

The laboratory evaluation is started following the physical examination, taking of the patient history, family history, and evaluation of the clinical manifestations [94]. An informative comprehensive patient bleeding history is highly desirable. The initial step for the diagnosis of these disorders is routine laboratory assays, including prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), and fibrinogen. The results of these screening assays direct more specialized assays, both functional and immunological (Fig. 2.3).

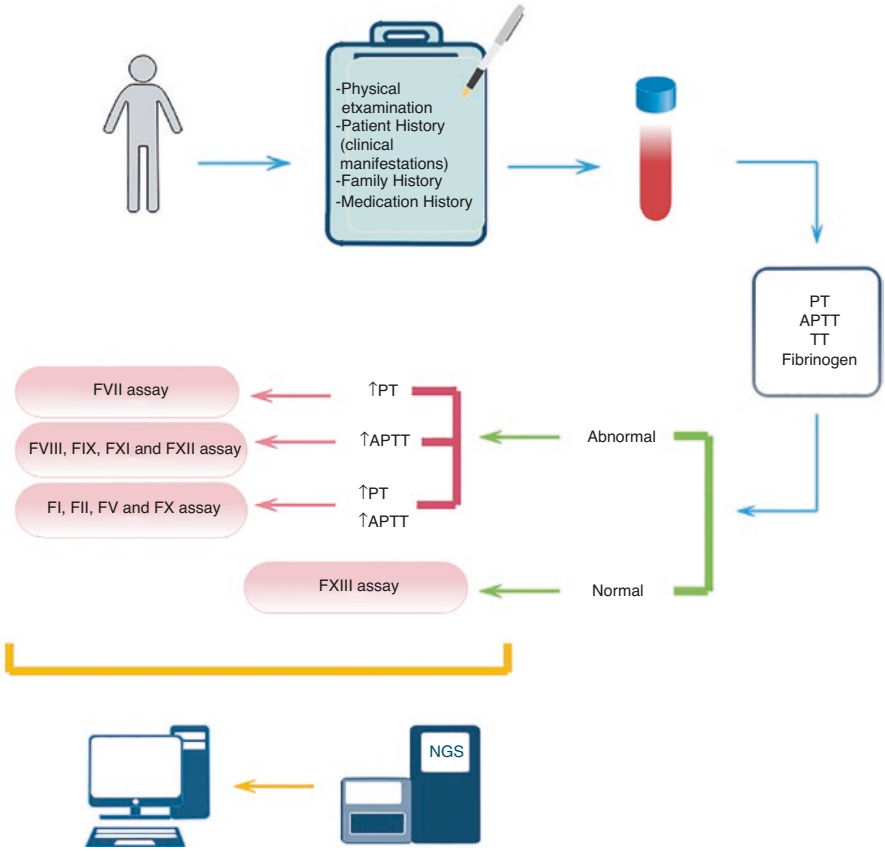

Fig. 2.3 The investigation of patients suspected of having coagulation factors deficiencies [94]. *PT* prothrombin time, *APTT* activated partial thromboplastin time, *TT* thrombin time, *NGS* next-generation sequencing

2.11 Diagnosis of Congenital Fibrinogen Deficiency

Fibrinogen deficiency is suspected if there are prolonged PT, APTT, and a low level of fibrinogen (FI). Although thrombin time (TT) and reptilase time (RT) are typically prolonged in patients with fibrinogen deficiency, they are not often used (Table 2.4). There are different functional fibrinogen assays including the Clauss method, PT-derived fibrinogen assay, and the clottable protein assay. Among them, the Clauss method is the most commonly used assay for the diagnosis of fibrinogen deficiency [95]. The Clauss fibrinogen assay is based on clot formation. In this assay, following the preparation of serial dilutions of standard and patient plasma, thrombin is added and the clotting time is measured. The PT-derived assay is another functional fibrinogen assay that measures the fibrinogen functional level according to the change in light transmission or scatter from a prothrombin time curve. Unlike the Clauss method, high values of fibrin degradation products (FDPs) do not affect the results of this test. Since the PT-derived assay is based on light transmission, lipemic and icteric samples can interfere with the results

of this test. The clottable protein assay is a very accurate method based on the formation of fibrin from fibrinogen. Although this test has high accuracy and repeatability and was used as a reference method for measuring fibrinogen for a long time, today the use of this method is limited due to its time-consuming nature and the need for high skill to perform it [96].

The level of fibrinogen *antigen* is determined by various methods, including enzyme linked immunosorbent assay (ELISA), radial immunodiffusion, immunonephelometry, and immunoturbidimetry. Electrophoresis techniques are also used to identify fibrinogen variants in which the abnormal variants of fibrinogen are identified according to the speed of movement of the bands [23]. The level of fibrinogen antigen is undetectable in afibrinogenemia but decreased in hypofibrinogenemia. In dysfibrinogenemia, the amount of fibrinogen antigen is within the normal range, but in hypodisfibrinogenemia, it is decreased.

Fibrinogen deficiency may be classified into four groups including afibrinogenemia, hypofibrinogenemia, dysfibrinogenemia, and hypodysfibrinogenemia; *functional* assays give different results in each of these disorders. In afibrinogenemia, the fibrinogen level is undetectable, whereas in hypofibrinogenemia, the fibrinogen level is proportionally reduced (<1.5 g/dL). The fibrinogen activity level in dysfibrinogenemia is also decreased (<1.5 g/dL), and there is a discrepancy between the result of the Clauss method and the PT-derived fibrinogen assay. The PT-derived assay, which can overestimate the fibrinogen activity almost five times, correlates to the immunological assay. It should be noted that in the presence of a number of fibrinogen variants such as Longmont and Bordeaux, the Clauss method estimate of the level of fibrinogen activity is typically higher when compared to the PT-derived fibrinogen assay [97, 98].

In order to distinguish dysfibrinogenemia as a *qualitative* disorder from other *quantitative* fibrinogen deficiencies, an immunological assay is recommended. The functional to immunological deficiency ratio may be used to distinguish between qualitative and quantitative deficiencies.

In order to confirm the diagnosis of fibrinogen deficiency, molecular testing is recommended [99]. There are more than 230 reported mutations, which occur in *FGA*, *FGB*, and *FGG* genes and are available at different online databases (such as http://site.geht.org/basefibrinogene/) [100]. For a more precise molecular diagnosis, there is an available algorithm. According to this algorithm, in *quantitative* fibrinogen deficiency, the molecular analysis is initiated with the mutational screening of the exon 4, exon 5, and also the 11 kb deletion of the *FGA* gene and continued by screening the exon 2 of *FGA* and exons 2 and 6 of *FGB*. In *qualitative* fibrinogen deficiency, the genotyping is initiated by screening the exon 2 of *FGA* and exon 8 of *FGG* followed by the exon 5 of *FGA*, exon 2 of *FGB*, and exons 3 and 5 of the *FGG* [101, 102].

2.12 Diagnosis of Prothrombin Deficiency

The diagnosis of FII deficiency is suspected if there is prolongation of the PT and APTT. The point that should be kept in mind is that in isolated prothrombin deficiency, these routine tests are not necessarily always prolonged and in many cases are normal or borderline. Therefore, a normal PT and APTT cannot exclude FII

deficiency [103]. Compared to PT and APTT, other PT-derived tests including Thrombotest and Normotest (hepaplastintest) are more sensitive. Normotest is a test similar to PT, which is used to determine the activity level of extrinsic pathway coagulation factors. The reagents used in this method include tissue factor and bovine plasma absorbed with barium sulfate as a source of fibrinogen and FV. This method is sensitive to changes in the levels of FII, FVII, and FX, while it is not sensitive to decreases of fibrinogen and FV. Normotest is less sensitive to endogenous inhibitors compared to Thrombotest [104–106].

There are different functional assays available, including the one-stage PT-based prothrombin assay, Taipan viper venom assay, tiger snake venom assay, Textarin time, Echis carinatus venom assay, staphylocoagulase, the chromogenic FII assay, and a two-stage FII assay. The one-stage PT-based FII assay is the most common functional assay for diagnosis of prothrombin deficiency [106, 107]. This is based on the ability of dilutions of patient plasma and standard plasma to correct the PT of substrate plasma. In hypoprothrombinemia, the FII activity level is typically <10% in homozygous patients, whereas in patients with heterozygous FII deficiency, the prothrombin level is typically between 40% and 60%. Since the complete absence of prothrombin appears incompatible with life, the FII activity level in patients with homozygote deficiency and severe bleeding tendency is still about 1% [108]. The FII activity level in patients with compound heterozygote deficiency is between 1 and 5%.

Taipan viper venom activates prothrombin directly in the presence of Ca^{2+} and phospholipid. The advantage of this method compared to other one-stage methods is that there is no need for other coagulation factors such as FV, FVII, and FX. Tiger snake venom acts as tissue thromboplastin. This method needs Ca^{2+}, phospholipid, and FV. Textarin is a protein derived from the Australian brown snake and activates prothrombin in the presence of FV, Ca^{2+}, and phospholipid [109–111]. In prothrombin deficiency or in the presence of prothrombin inhibitors, Textarin time is prolonged [112, 113]. Ecarin is a venom derived from Echis carinatus snake. This venom activates prothrombin in the absence of Ca^{2+} and phospholipid. In prothrombin deficiency or in the presence of prothrombin inhibitors, the Echis carinatus venom assay is typically prolonged. It should be noted that the Textarin time and Echis carinatus venom assays are also used to diagnose lupus anticoagulant (LA). In the presence of LA in plasma, the Textarin time is prolonged, but the Echis carinatus venom assay is not changed. Staphylocoagulase is a protein derived from Staphylococcus aureus. This protein makes a complex with prothrombin in the presence of fibrinogen and activates prothrombin to thrombin [113–115]. The chromogenic FII assay is another method for evaluation of prothrombin activity. The basis of the chromogenic assay is the measurement of thrombin activity in the presence of chromogenic substances that interact with the active site of thrombin. After activation, thrombin cleaves the chromogenic substrate, which leads to the formation of color. Measurement of optical density (OD), which is proportional to color intensity, at 405 nm is directly related to prothrombin activity in serum or plasma. In order to distinguish a *qualitative* FII deficiency from a *quantitative* FII deficiency, an immunological assay is necessary. A discrepancy of >15% between FII activity and antigen levels may suggest dysprothrombinemia [108, 116–118].

In the two-stage FII assay, first, prothrombin is converted to thrombin, and in the next step, fibrinogen is converted to fibrin in the presence of thrombin. Finally, the amount of thrombin is measured. One of the disadvantages of the two-stage FII assay is that the formed thrombin is neutralized by antithrombin found in plasma. Therefore, it is recommended to use plasma dilutions; however, dilutions cannot completely eliminate the effect of antithrombin. In addition, sometimes plasma dilution decreases prothrombin to undetectable amounts. Due to these disadvantages, the two-stage FII assay is rarely used [119, 120].

Since borderline confirmatory results may be seen in prothrombin deficiency, molecular testing provides a more precise diagnosis. Most coagulation laboratories evaluate prothrombin 20,210 mutation for molecular diagnosis of patients with prothrombin deficiency, but the molecular diagnosis of prothrombin deficiency is sometimes more complicated. Most of the mutations reported in hypo- and dysprothrombinemias are concentrated in exons 4 through 14. Therefore, in the absence of recurrent mutations, it is recommended to start the mutational screening with these exons. Missense mutations are the most common mutations reported in prothrombin deficiency. Direct sequencing is the most common method for diagnosis of this disorder but in about 5% of patients, the mutations are not detectable by direct sequencing. Of course, next generation sequencing (NGS) solves this problem. In developing countries, due to low resource settings, direct sequencing and NGS may not be practical and linkage analysis is recommended [121–124].

2.13 Diagnosis of Factor V Deficiency

The diagnosis of factor V deficiency may be suspected in patients with a prolonged PT and APTT. In severe FV deficiency, BT may also be prolonged due to a decrease in platelet FV level [125]. There are different functional FV assays such as the one-stage PT-based assay, Lewis and Ware method, EDTA method, and microplate activity assay. Among all these assays, the one-stage PT-based assay is the most commonly used, which is based on the ability of the patient plasma to correct (shorten) the PT of the substrate plasma. Another assay for diagnosis of FV deficiency is the microplate FV activity assay that uses a kinetic microplate reader to monitor the absorbance change during fibrin formation. This assay measures the time, initial rate, extent of the fibrin clot, and measures the 1-stage and 2-stage FV activity [126]. Severe FV deficiency is considered in cases with an undetectable FV level. A detectable FV level <10% is considered moderate FV deficiency, and >10% is considered mild. The diagnosis of FV deficiency commonly relies on the FV activity assay, however, the antigen assay can be used in distinguishing the cross-reacting material positive (CRM^+) patients that may have a dysfunctional protein. There are more than 300 mutations that have been reported, with a large portion of them being missense mutations (~51%). The missense mutations are mostly clustered in the A and C domains, whereas the nonsense mutations are mostly reported in domain B. Since there are no reported recurrent mutations in the *F5* gene, whole *F5* gene direct sequencing and NGS is recommended for genotyping patients. In developing countries, due to low resource settings, linkage analysis or other molecular methods are recommended [126, 127].

2.14 Diagnosis of Factor VII Deficiency

Congenital FVII deficiency is the most common of the rare bleeding disorders (RBD) with a prevalence of 1:500,000. Following clinical examination of the patient and family history investigation, in patients with a prolonged isolated PT, FVII deficiency may be suspected. FVII deficiency may be diagnosed with different functional assays, including the PT-based FVII activity and chromogenic FVII assays [128, 129]. The one-stage PT-based FVII activity relies upon measuring the degree of correction of the PT of FVII deficient-plasma, following addition of patient plasma. Different sources of thromboplastin may be associated with variable results in the FVII functional assays: this is commonly seen in patients with qualitative FVII defects, including FVII Padua (Arg364Gln), FVII Nagoya (Arg364Trp), and FVII Tondabayashi (or Shinjo) (Arg79Gln). Human-derived thromboplastin is recommended for most testing [129].

The activated FVII (FVIIa) assay can be used for monitoring of *recombinant* FVIIa (rFVIIa) treatment. The FVIIa assay may be performed using several methods, including a clotting-based assay using recombinant soluble mutant tissue factor molecule (sTF1–219), and an ELISA. The *antigen* level of FVII can be measured by ELISA and immunoturbidimetric assays [130, 131]. A monoclonal or a specific polyclonal antibody is used for quantitative measurement of plasma FVII. FVIIa is also measured by this method. The FVII *antigen* level measurement is necessary for distinguishing type I deficiency (*quantitative* defect) from type II (*functional* defect) [132].

Various mutations reported in the *F7* gene can be detected through *F7* gene sequencing, including mutations in exons, introns, boundaries, and the promoter regions. About 90% of mutated alleles can be detected through direct sequencing methods, whereas about 10% of gene mutations cannot be identified. NGS should improve this situation [133].

2.15 Diagnosis of Factor VIII and IX Deficiency

The laboratory diagnosis of HA and HB is initiated by routine laboratory assays including PT and APTT, following accurate evaluation of clinical symptoms and patient's family history (Table 2.4). A prolonged APTT may cause suspicion of hemophilia; however, hemophilia should be differentiated from other disorders including vitamin K deficiency and VWD, therefore for an exact diagnosis, specific assays are required [42, 134].

Diagnosis of HA and HB may be done using different functional assays including the one-stage APTT-based FVIII and FIX activity assays ("one-stage") and also chromogenic assays. The one-stage assay is based on the ability of patient's plasma to correct (shorten) the APTT of the substrate plasma (FVIII or FIX deficient plasma) (Fig. 2.4).

The chromogenic is based on a two-stage assay where, following the activation of FVIII or FIX, a chromogen is released, which results in an increase of the absorbance proportional to amount of FVIII or FIX in the plasma. These assays have variable sensitivity and specificity [135].

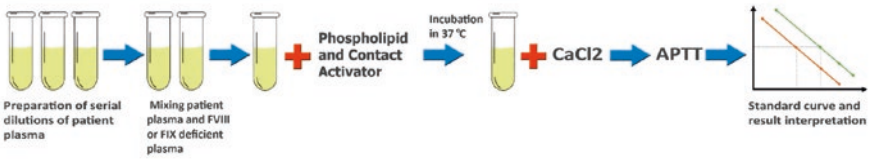

Fig. 2.4 The principles of the one-stage assay based on the activated partial thromboplastin time (APTT)

The one-stage assay is simple, cost-effective, easily automated, and widely used for clinical monitoring. However, it is subject to interference by lupus anticoagulants (LA), lipids, and heparin, and it is sensitive to direct oral anticoagulant drugs. Moreover, the sources of the deficient plasma, the APTT reagent, and phospholipid may also affect the results. The chromogenic assay is not as dependent on phospholipids and typically provides more accurate results when using many of the modified rFVIII and rFIX replacement products. However, even when using the chromogenic assay, the performance with certain modified products (such as bispecific antibody therapy) should be verified before reporting. Following rFVIII replacement therapy, the level of FVIII measured in the chromogenic assay is higher compared to the one-stage assay. Since this difference is within the accepted range, it is not clinically important [136, 137]. However, the difference in the results of these assays using B domain-deleted rFVIII (BDD rFVIII) is clinically important. The activity level of FVIII, following BDD rFVIII administration, in the one-stage assay is estimated to be 20–50% *lower* compared to the chromogenic assay [138].

The chromogenic assays are not affected by LA and, when compared to the one-stage assay, have lower inter-laboratory variability. However, there are discrepancies between these two assays, particularly in mild and moderate hemophilia, which result from the different disease-causing mutations [139]. In case of some mutations in A1, A2, and A3 domains, the activity of FVIII in chromogenic assay is less than half of the one-stage assay. Since in the one-stage assay FVIIIa is produced during the final phase following the addition of Ca^{2+}, the effect of this stability has been minimized. While, in the chromogenic assay, FVIIIa is produced during the incubation of the first phase, and the higher amount of A2 domain dissolution causes a *decrease* in the activity level of FVIII [140–142].

In the presence of some mutations located in the A1, A2, and A3 domains, the FVIII activity measured by the one-stage assay is lower than the chromogenic. Moreover, there may be discrepancies in other categories of non-severe HA, which have mutations in (or close to) the thrombin cleavage sites, or the binding sites of FIX and VWF [143, 144]. Since in the majority of cases with mild HA the FVIII activity level is lower in the one-stage assay than the chromogenic, the diagnosis may be missed when only one assay is used. Therefore, the combined use of both assays for improved diagnosis of HA may be helpful [145, 146]. Although the diagnosis of HA and HB is specifically based on the measurement of the *activity* level of the coagulation factors in plasma, the *antigen* assay is used to assess the concentration of FVIII and differentiate CRM^+ patients. The antigen level of FVIII and FIX can be measured by ELISA.

According to the FVIII activity level, HA is classified as mild (FVIII: C = 5–40%), moderate (1–5%), and severe (<1%). In a similar way, HB is also categorized according to FIX activity as mild (FIX: C: 5–35%), moderate (1–5%), and severe (<1%) [147].

Molecular diagnosis of hemophilia leads to definitive diagnosis of hemophilia carriers, pre-natal diagnosis, and helps with the detection of inhibitors against FVIII and FIX following replacement therapy [148]. It seems that about 40% of molecular defects that may lead to inhibitors are post-treatment [149]. Molecular diagnosis determines the disease-causing mutation in more than 95% of patients [150]. In other cases, they are misdiagnosed due to their similarity with VWD type 2N. Therefore, in all cases with mild to moderate hemophilia that are mutation-negative, molecular testing for VWD type 2N is recommended. In addition, in mutation-negative HA patients, CFV-FVIII deficiency (mutations in *MCFD2* and *LMAN1*) should be assessed [151].

The molecular diagnosis of severe HA begins with an assay for detection of the inversion in intron 22 followed by inversion in intron 1. In the absence of these two genetic defects, the whole *F8* gene including all exons, intron-exon boundaries, and the promoter region should be screened by direct Sanger sequencing. Due to an absence of recurrent mutations in patients with mild or moderate HA and also in patients with HB, whole gene sequencing is recommended [152, 153]. More than 3000 mutations had been detected in the *f8* gene. Missense, nonsense, and splicing mutations are the most common mutations. The most common mutations in the *f9* gene include missense, nonsense, splicing, and frame shift mutations. Deletions are detected by multiplex ligation-dependent probe amplification assays [154]. Hemophilia B Leyden is caused by a gene defect in a small region of the proximal promoter [155]. About 1200 mutations are reported for hemophilia B. For patients with an unidentified disease-causing mutation using Sanger sequencing, NGS is helpful [156]. In developing countries, due to low resource settings, Sanger sequencing is not practical when there is no frequent mutation, especially for long genes like *f8*. So, a cost effective method like linkage analysis is recommended [157].

2.16 Diagnosis of Factor X Deficiency

Diagnosis of FX deficiency is based on clinical examination, family history, routine coagulation tests, and antigen and activity levels of FX. Specific diagnosis of FX deficiency may be suspected following a prolonged PT and APTT due to a prothrombinase formation defect, while thrombin time (TT) is normal. There are four types of FX deficiency including type I (CRM−), type II (CRM+), type III (CRM+ with some dysfunctional protein), and type IV (FX deficiency combined with deficiencies of other coagulation factors). The results of PT and APTT in types III and IV of FX deficiency are variable. The Russell viper venom (RVV) FX assay, which is rarely performed, is another screening test for the diagnosis of FX deficiency [158]. This test is prolonged in deficiency of FI, FII, FV, and FX. Using an FX deficient plasma as substrate, RVV test becomes specific for FX deficiency [159]. The

one-stage PT-based FX activity and chromogenic assays are the functional assays used in diagnosis of FX deficiency [158]. The one-stage PT-based assay compares the correction of the PT of substrate plasma (FX deficient plasma) by dilutions with patient plasma. Chromogenic assay of FX is another method for determination of the FX activity level using a specific chromogenic substrate that is performed based on a two-stage assay. At first RVV and Ca^{2+} activate FX to FXa. Then a specific chromogenic substrate is cleaved by FXa, which shows a yellow color. The intensity of the formed color is proportional to enzymatic activity of FXa. According to the FX activity level, FX deficiency is classified into three clinical groups: severe (FX: C < 1 U/dL), moderate (1–5 U/dL), and mild (6–10 U/dL). Different immunologic methods can be used for assessment of plasma FX antigen levels, such as radioimmunoassay, laser nephelometry, immunodiffusion, antibody neutralization, electroimmunoassay, and ELISA.

There are more than 260 mutations reported in the *F10* gene with the majority of them being missense mutations. FX genotyping is usually via whole gene sequencing of the *F10* gene including all exons, intron–exon boundaries, and the promoter region [160]. In many homozygous mutations of the *F10* gene, such as Glu102Lys, Leu(−32)Pro and Gly114Arg, the FX antigen level is not decreased so patients are asymptomatic or show mild clinical manifestations. In patients with the Glu102Lys mutation, PT and APTT are typically normal. Gly380Arg and Tyr63delAT mutations are reported to be associated with pre-natal intracranial hemorrhage (ICH). In patients with the Gly204Arg mutation, FX antigen is produced but it is not secreted; so, FX antigen is undetectable in the plasma of these patients, and they show the severe type of the disease. Polymerase chain reaction (PCR) and direct sequencing are the most common molecular methods for determination of FX deficiency causing mutations [11, 158].

2.17 Diagnosis of Factor XI Deficiency

Once called "hemophilia C," FXI deficiency is now recognized to have a distinct clinical presentation from HA and HB. The specific diagnosis of FXI deficiency is considered after a prolonged APTT and followed-up by functional and antigenic assays. FXI activity level (FXI:C) may be determined by several methods, including a one-stage APTT-based assay and a chromogenic FXI activity assay. In the one-stage APTT-based FXI activity assay, FXI is measured based on the ability of patient plasma to correct (shorten) the APTT of FXI deficient plasma. In the chromogenic FXI activity assay, the FXI level in the plasma is measured through the ability of FXI to cleave a chromogenic substrate and subsequent release of a chromogen that results in absorbance increase. Another method for FXI activity assessment is FXIIa-inhibited diluted thromboplastin time (FXIIaiDTT), which is based on thrombin generation after FXI activation. Although the measurement of FXI antigen is not routine in most clinical diagnostic laboratories, it is necessary to distinguish between *quantitative* and *qualitative* defects of FXI deficiency. FXI antigen concentration is determined in plasma by immunologic assays such as ELISA.

Congenital FXI deficiency can be divided into CRM− (with combined decrease of antigenic and activity level of FXI) and CRM+ (with normal FXI antigen and decreased FXI activity) [161]. Based on the FXI activity level, congenital FXI deficiency may be classified into three groups including severe (FXI:C of 1–20 U/dL), moderate (20–60 U/dL), and mild (61–80 U/dL). However, FXI levels do not necessarily correlate with bleeding symptoms and spontaneous bleeding is uncommon. Since the bleeding tendency in patients with FXI deficiency is unpredictable, applying a reliable laboratory method leads to proper diagnosis of patients prone to bleeding [162]. A thrombin generation assay (TGA) and thromboelastography/rotational thromboelastometry (TEG/ROTEM) may be used for assessment of bleeding tendency in FXI deficient patients. These assays are also used for monitoring of treatment effectiveness. However, due to the variable level of FXI activity and conflicting results in different studies, the ability of these assays to predict bleeding tendency is debatable [163, 164].

Patients with severe FXI deficiency are typically homozygous or compound heterozygotes for underlying mutations, while those with higher FXI activity level are typically heterozygotes. There are more than 230 mutations in the *F11* gene, with the majority of them being missense mutations (68%) [165]. The prevalence of FXI deficiency is about 1:1,000,000 in the general population, while this disorder is commonly reported in the Jewish population with the prevalence of 1:450 [166, 167]. The FXI deficiency in the Jewish population mostly results from four common types of mutations: type I is a point mutation (G → A substitution), which occurs at the donor splice site of the last intron; type II is a nonsense mutation in exon 5 (Glu117stop); type III is another missense mutation, which occurs in exon 9 (Phe283Leu); and type IV results from a 14 bp deletion in the exon 14/intron N splice site. Type II and III mutations are the most common and account for more than 90% of mutations in the Jewish population. Type II mutations are also reported in Iraqi and Arab populations. The type III mutations Cys88Stop and Cys128Stop are commonly reported in populations with European origin (in French Basques and in the United Kingdom, respectively). These common mutations should be tested based on the population being screened. If the common mutations are not detected, whole *F11* gene sequencing is recommended [163, 168].

2.18 Diagnosis of Contact Coagulation Factor Deficiency

Deficiency in the contact factor system, which includes factor XII (FXII), prekallikrein (PK), and high molecular weight kininogen (HMWK), may be detected by routine coagulation laboratory assays, including the APTT and PT [169, 170]. A prolonged APTT and normal PT in asymptomatic individuals suggest the need for more specific tests (Table 2.2). It should be noted that in the cases of PK deficiency, the increased APTT (>120 s) can be normalized following the prolonged preincubation with APTT reagent but not in plasma deficient in FXII, HMWK, FXI, FIX, FVIII, or plasma containing a lupus anticoagulant (LA) [171, 172].

The specific diagnostic assays for contact factor deficiency are the one-stage APTT-based assay and the chromogenic assay. The former is based on the degree of the correction of the substrate plasma (deficient plasma) by patient plasma, and the latter is based on a two-stage assays cleavage of a chromogenic substrate following

the activation of the factor in question. Although the measurement of contact coagulation factor antigen levels is not routine in most clinical diagnostic laboratories, it is necessary to distinguish between quantitative and qualitative defects. The concentration of contact coagulation factors can be determined by immunologic assays such as ELISA [173, 174].

2.19 Diagnosis of Factor XIII Deficiency

Among all coagulation factor disorders, the diagnosis of FXIII deficiency may be the most complex (Table 2.4). A normal PT and APTT, especially in presence of delayed bleeding episodes, is the hallmark of FXIII deficiency. Diagnosis of FXIII deficiency is suspected following clinical manifestation and family history assessment, and normal results for routine coagulation tests including BT, PT, APTT, thrombin time (TT), and platelet count [175].

The first-line screening test for diagnosis of FXIII deficiency is the FXIII activity assay. However, in a considerable number of countries, the clot solubility test (CST) is the only diagnostic test [4, 176]. The CST typically detects only samples with less than 5% FXIII activity. The misdiagnosis of many patients emphasizes the need to use the more specific assays for accurate diagnosis of FXIII deficiency. If the CST must be used, it should be optimized to increase the sensitivity [177]. The sensitivity of the test depends on various factors such as the solubilizing reagents (acetic acid 2%, urea 5 M, or monochloroacetic acid 1%), the fibrinogen level, and the activating agents (calcium, thrombin, or a combination of both). The *sensitivity* of the acetic acid-based method is twice as high as the methods based on urea and monochloroacetic acid, while its *specificity* is lower [178]. In the 5 M urea method, hypofibrinogenemia and dysfibrinogenemia may cause false positive results; so applying some coagulation tests, for fibrinogen assessment, is recommended including TT, RT, or measurement of the antigen and activity level of fibrinogen [179]. Use of two different CST assays, including 2% acetic acid/thrombin (sensitivity to 10% FXIII level) or 5 M urea/Cacl2 (sensitivity from <0.5 to 5% FXIII level) in parallel is recommended [4, 180]. In summary, although the positive results of CST indicate FXIII deficiency, the negative results of this test do not rule out the possibility of FXIII deficiency (Fig. 2.5).

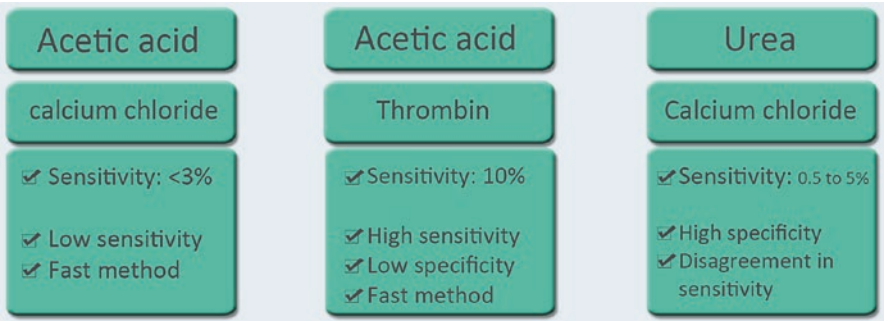

Fig. 2.5 Comparison of different methods of clot solubility testing [4]

Attempts have been made to provide semi-quantitative CST. One of the modifications made is to use fibrinogen without FXIII, with different dilutions of plasma as a substrate. Another modification is adding increasing amounts of FXIII inhibitor (iodoacetate or FXIII-A specific antibody) to the patient's undiluted plasma and then measuring the amount of inhibitor needed to neutralize FXIII activity [4, 11]. However, CST with these modifications is not widely used due to the complexity and high costs [181].

The FXIII activity assay is useful for detecting all types of FXIII deficiency. This assay may be performed by several methods, including the ammonia release assay, amine incorporation assay, and the isopeptidase assay. The functional assays are not able to differentiate between a low FXIII activity level due to the inherited deficiency or due to the presence the autoantibodies. Lipemic plasma or plasma with a high amount of ammonia may cause overestimation of FXIII activity level. So, it is recommended to use blank plasma. In contrast, icteric plasma leads to an underestimation of FXIII activity level [24].

Plasma FXIII is a heterotetramer consisting of two identical A subunits (containing the catalyst sites) and two identical B subunits (containing the fibrinogen binding sites). In order to classify the FXIII defect (FXIII-A or FXIII-B), following the confirmation of FXIII deficiency by the FXIII *activity* assay, the FXIII *antigen* assay may be performed as follows [178]:

1. Measurement of FXIII A2B2 antigen in plasma
2. In the presence of low concentration of FXIII A2B2, both subunits (FXIIIA2 and FXIIIB2) antigen are measured
3. Measurement of FXIIIA2 in platelets

There are different methods for measurement of the FXIII antigen level including electroimmunoassay (EIA), radioimmunoassay (RIA), latex-enhanced immunoprecipitation assay, and ELISA. Due to the difficulty and low sensitivity of EIA and RIA, ELISA is usually used as a sensitive and reliable method [24, 182, 183]. According to instructions of Clinical and Laboratory Standards Institute (CLSI), three things should be considered for FXIII antigen measurement:

1. The interference of free FXIII-B with FXIII A2B2 antigen assay should be avoided.
2. In FXIII subunits measurement, both free and complex antigen forms should react with antibodies to the same extent.
3. The interference of fibrinogen concentration should be avoided in FXIII antigen level measurement [184].

A low FXIII level in plasma is not necessarily due to a congenital FXIII deficiency, but can be related to the presence of an autoantibody against FXIII (autoimmune FXIII deficiency). There are two types of autoantibodies including neutralizing (which *inhibits FXIII activation*) and non-neutralizing (which *accelerates FXIIIa elimination* from plasma). The former is recognized using mixing studies, and the

latter is evaluated using a binding assay [185]. Neutralizing antibodies against FXIII-A causes significant decrease in FXIII activity level, while the antigen level of FXIII-A and FXIII A2B2 may be normal or slightly decreased. Non-neutralizing antibodies cause significant decrease in both activity and antigen levels [186].

The Bethesda assay (most often used to quantitate FVIII inhibitors) may also be used to approximate the amount of FXIII inhibitor. The Nijmegen modification of the Bethesda assay provides more sensitivity and specificity [186].

Congenital FXIII deficiency is typically due to different mutations in the *F13A* gene. The most common molecular defects in this subunit are missense mutations. Other molecular defects include nonsense and insertion/deletion mutations. One hundred and seventy-two mutations are recognized in the *F13A* gene. Less than 5% of cases of congenital FXIII deficiency are due to molecular defects in the *F13B* gene. Twenty-five mutations had been reported in *F13B* gene, most of them are missense mutations [55, 187]. Although gene sequencing is the most reliable molecular method, it is costly and is not able to detect mutations in 5% of cases. However, NGS may improve this technique [47]. In addition, due to the small size of the *F13B* gene (compared to the FXIII-A gene), and low number of reported mutations, whole gene sequencing is more practical for diagnosis of FXIII-B deficiency [188].

2.20 Treatment

Due to the variability in severity of bleeding symptoms in patients with congenital bleeding disorders (CBDs), management of these disorders is highly variable. A considerable number of patients with platelet defects (IPFDs) never experience significant bleeding episodes, and therefore, most of them never require medical intervention [6]. On the other hand, in some severe bleeding disorders such as FXIII deficiency, regular primary prophylaxis is required from the time of diagnosis. This is due to the high rate of life-threatening episodes such as intracranial hemorrhage (ICH) in these patients [5]. In patients with hemophilia, two main treatment strategies are used: *on-demand* treatment, which means stopping bleeding as soon as possible after onset of hemorrhage, and *prophylaxis*. On-demand therapy is the main therapeutic option in a considerable number of countries most often due to limited resources and economic challenges. Prophylaxis treatment remains the treatment of choice for patients with severe hemophilia, particularly in children [189, 190]. In rare bleeding disorders (RBDs), except for FXIII deficiency, on-demand treatment is typically used; however, in some cases with severe life-threatening bleeds—especially those with severe FVII deficiency, FX deficiency, and afibrinogenemia—regular secondary prophylaxis could be considered [11, 96]. In patients with VWD, on-demand therapy is the traditional treatment of choice; however, long-term prophylaxis should be considered for those patients with type 3 VWD who manifest recurrent hemarthrosis, recurrent GI bleeding or frequent epistaxis (Table 2.5) [191].

Table 2.5 Therapeutic options in congenital bleeding disorders

Congenital bleeding disorder		Kind of treatment	Replacement therapy	Non-replacement therapy
Common bleeding disorders	VWD	On-demand[a]	FFP Cryoprecipitate FVIII/VWF concentrate rVWF	Platelet transfusion Desmopressin (DDAVP)
	Hemophilia A	Prophylaxis	FVIII (plasma-derived) rFVIII Extended half-life FVIII	Concizumab (anti-TFPI) Fitusiran (siRNA) Emicizumab
	Hemophilia B	Prophylaxis	FIX (plasma-derived) rFIX Extended half-life FIX	Concizumab (anti-TFPI) Fitusiran (siRNA)
Rare bleeding disorders (RBD)	FI deficiency	On-demand	FFP Cryoprecipitate Fibrinogen (FI) (plasma derived)	NA
	FII deficiency	On-demand	PCC FFP	NA
	FV deficiency	On-demand	FFP FV (plasma-derived) (in clinical study)	Platelet transfusion
	Combined FV-FVIII deficiency	On-demand	FFP rFVIII	Desmopressin (DDAVP)
	Vitamin K-dependent coagulation factor (VKDCF) deficiency	On-demand	Vitamin K PCC FFP	NA
	FVII deficiency	On-demand	rFVIIa PCC FFP FVII (plasma-derived)	NA
	FX deficiency	On-demand	PCC FX (plasma-derived)	NA
	FXI deficiency	On-demand	FXI (plasma-derived)	NA
	FXIII deficiency	Prophylaxis	rFXIII A subunit FXIII (plasma-derived) FFP Cryoprecipitate	NA

Table 2.5 (continued)

Congenital bleeding disorder		Kind of treatment	Replacement therapy	Non-replacement therapy
Inherited platelet function defects (IPFD)	Glanzmann thrombasthenia (GT)	On-demand	Platelet transfusion	Tranexamic acid Epsilon-aminocaproic acid (EACA) Topical thrombin Rfvii
	Bernard-Soulier syndrome (BSS)	On-demand	Platelet transfusion	Tranexamic acid Epsilon-aminocaproic acid (EACA) rFVII
	Gray platelet syndrome (GPS)	On-demand	Platelet transfusion	Tranexamic acid Epsilon-aminocaproic acid (EACA) Desmopressin (DDAVP)

CBD Congenital bleeding disorder, *VWD* von Willebrand disease, *RBD* rare bleeding disorders, *FI* Factor I, *FII* Factor II, *FV* Factor V, *CFV-FVIII* Combined factor V and factor VIII, FVII, Factor VII, *VKDCF* Vitamin K dependent clotting factor, *FX* Factor X, *FXI* Factor XI, *FXIII* Factor XIII, *IPFD* Inherited platelet function disorder, *GT* Glanzmann thrombasthenia, *BSS* Bernard-Soulier syndrome, *GPS* Gray platelet syndrome, *NA* Not applicable, *FFP* fresh frozen plasma, *EHL* extended half-life, *TFPI* tissue factor pathway inhibitor, *PCC* prothrombin complex concentrate, *r* recombinant, *Pd* plasma derived

[a]Desmopressin cannot be used for type 3 VWD and in most cases with type 2 VWD

Different therapeutic choices are available for patients with CBDs, including traditional choices such as fresh frozen plasma (FFP), cryoprecipitate and platelet concentrate, and more recently factor concentrate and recombinant products. FFP can be used for the vast majority of coagulation factor deficiencies, but the risk of blood-borne disease transmission is an important obstacle. In addition, administration of a sufficient quantity of FFP may not be possible with some deficiencies and in some patients. Despite this limitation, in countries with limited resources, FFP may be the only therapeutic choice; in such areas, the virus-inactivated form of product may be beneficial. Cryoprecipitate is another therapeutic choice that can be used for patients with congenital fibrinogen deficiency (CFD), FXIII deficiency, VWD, and hemophilia A (but *not* hemophilia B) [104, 192]. Due to the limited availability of virus-inactivated cryoprecipitate, this product is not recommended in these patients except in emergencies. In CBDs, plasma-derived factor concentrates are available for patients with deficiencies of fibrinogen (FI), FVII, FVIII, FIX, FX, FXIII, for VWD, and more recently for FV deficiency (part of a clinical study). Recombinant products are available for deficiencies of FVII, FVIII, FIX, FXIII, and in VWD [193]. In general, management of bleeding depends on the severity of disease, type of bleeding episode, and the minimal residual activity in a patient's plasma [11].

References

1. Rick ME, Walsh CE, Key NS. Congenital bleeding disorders. ASH Educ Program Book. 2003;2003(1):559–74.
2. Brown DL. Congenital bleeding disorders. Curr Probl Pediatr Adolesc Health Care. 2005;35(2):38–62.
3. Sadler JE, Budde U, Eikenboom J, Favaloro E, Hill F, Holmberg L, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the subcommittee on von Willebrand Factor. J Thromb Haemost. 2006;4(10):2103–14.
4. Dorgalaleh A, Tabibian S, Hosseini S, Shamsizadeh M. Guidelines for laboratory diagnosis of factor XIII deficiency. Blood Coagul Fibrinolysis. 2016;27(4):361–4.
5. Rafiee Alavi SE, Jalalvand M, Assadollahi V, Tabibian S, Dorgalaleh A. Intracranial hemorrhage: a devastating outcome of congenital bleeding disorders-prevalence, diagnosis, and management, with a special focus on congenital factor XIII deficiency. In: Seminars in thrombosis and hemostasis (vol. 44, no. 03). Thieme Medical Publishers; 2017.
6. Dorgalaleh A, Tabibian S, Shamsizadeh M. Inherited platelet function disorders (IPFDs). Clin Lab. 2017;63(1):1–13.
7. Gresele P. Diagnosis of inherited platelet function disorders: guidance from the SSC of the ISTH. J Thromb Haemost. 2015;13(2):314–22.
8. Naderi M, Dorgalaleh A, Alizadeh S, Tabibian S, Hosseini S, Shamsizadeh M, et al. Clinical manifestations and management of life-threatening bleeding in the largest group of patients with severe factor XIII deficiency. Int J Hematol. 2014;100(5):443–9.
9. Palla R, Peyvandi F, Shapiro AD. Rare bleeding disorders: diagnosis and treatment. Blood J Am Soc Hematol. 2015;125(13):2052–61.
10. Hsieh L, Nugent D. Factor XIII deficiency. Haemophilia. 2008;14(6):1190–200.
11. Dorgalaleh A, Alavi SER, Tabibian S, Soori S, Moradi E, Bamedi T, et al. Diagnosis, clinical manifestations and management of rare bleeding disorders in Iran. Hematology. 2017;22(4):224–30.
12. Wheeler AP, Gailani D. Why factor XI deficiency is a clinical concern. Expert Rev Hematol. 2016;9(7):629–37.
13. Biss T, Blanchette V, Clark D, Wakefield C, James P, Rand M. Use of a quantitative pediatric bleeding questionnaire to assess mucocutaneous bleeding symptoms in children with a platelet function disorder. J Thromb Haemost. 2010;8(6):1416–9.
14. Lowe GC, Lordkipanidze M, Watson SP, Group UGs. Utility of the ISTH bleeding assessment tool in predicting platelet defects in participants with suspected inherited platelet function disorders. J Thromb Haemost. 2013;11(9):1663–8.
15. Toygar HU, Guzeldemir E. Excessive gingival bleeding in two patients with Glanzmann thrombasthenia. J Periodontol. 2007;78(6):1154–8.
16. Poon M-C, Di Minno G, d'Oiron R, Zotz R. New insights into the treatment of Glanzmann thrombasthenia. Transfus Med Rev. 2016;30(2):92–9.
17. Santagostino E, Mancuso M, Tripodi A, Chantarangkul V, Clerici M, Garagiola I, et al. Severe hemophilia with mild bleeding phenotype: molecular characterization and global coagulation profile. J Thromb Haemost. 2010;8(4):737–43.
18. Stieltjes N, Calvez T, Demiguel V, Torchet M, Briquel M, Fressinaud E, et al. Intracranial haemorrhages in French haemophilia patients (1991–2001): clinical presentation, management and prognosis factors for death. Haemophilia. 2005;11(5):452–8.
19. Den Uijl I, Fischer K, Van Der Bom J, Grobbee D, Rosendaal F, Plug I. Clinical outcome of moderate haemophilia compared with severe and mild haemophilia. Haemophilia. 2009;15(1):83–90.
20. Van den Berg H, De Groot P, Fischer K. Phenotypic heterogeneity in severe hemophilia. J Thromb Haemost. 2007;5:151–6.

21. Peyvandi F, Palla R, Menegatti M, Siboni S, Halimeh S, Faeser B, et al. Coagulation factor activity and clinical bleeding severity in rare bleeding disorders: results from the European network of rare bleeding disorders. J Thromb Haemost. 2012;10(4):615–21.
22. De Moerloose P, Neerman-Arbez M. Congenital fibrinogen disorders. In: Seminars in thrombosis and hemostasis. © Thieme Medical Publishers; 2009.
23. De Moerloose P, Casini A, Neerman-Arbez M. Congenital fibrinogen disorders: an update. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2013.
24. Inbal A, Muszbek L. Coagulation factor deficiencies and pregnancy loss. In: Seminars in thrombosis and hemostasis. New York: Thieme Medical Publishers, Inc.; 2003. copyright© 2003 by Thieme medical publishers, Inc., 333 seventh avenue, New York.
25. Anwar R, Miloszewski KJ. Factor XIII deficiency. Br J Haematol. 1999;107(3):468–84.
26. Coopland A, Alkjaersig N, Fletcher AP. Reduction in plasma factor XIII (fibrin stabilizing factor) concentration during pregnancy. J Lab Clin Med. 1969;73(1):144–53.
27. Padmanabhan L, Mhaskar R, Mhaskar A, Ross C. Factor XIII deficiency: a rare cause of repeated abortions. Singap Med J. 2004;45(4):186–7.
28. Dorgalaleh A, Naderi M, Shamsizadeh M. Morbidity and mortality in a large number of Iranian patients with severe congenital factor XIII deficiency. Ann Hematol. 2016;95(3):451–5.
29. Dorgalaleh A, Farshi Y, Haeri K, Ghanbari OB, Ahmadi A. Risk and management of intracerebral hemorrhage in patients with bleeding disorders. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers Inc; 2022.
30. Mosesson MW. Dysfibrinogenemia and thrombosis. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers Inc; 1999. Copyright© 1999 by Thieme Medical Publishers Inc.
31. Girolami A, Ferrari S, Cosi E, Girolami B, Lombardi AM. Congenital prothrombin defects: they are not only associated with bleeding but also with thrombosis: a new classification is needed. Hematology. 2018;23(2):105–10.
32. Cramer TJ, Anderson K, Navaz K, Brown JM, Mosnier LO, von Drygalski A. Heterozygous congenital Factor VII deficiency with the 9729del4 mutation, associated with severe spontaneous intracranial bleeding in an adolescent male. Blood Cell Mol Dis. 2016;57:8–12.
33. Girolami A, Cosi E, Ferrari S, Girolami B, Lombardi AM. Bleeding manifestations in heterozygotes with congenital FVII deficiency: a comparison with unaffected family members during a long observation period. Hematology. 2017;22(6):375–9.
34. Dorgalaleh A, Rashidpanah J. Blood coagulation factor XIII and factor XIII deficiency. Blood Rev. 2016;30(6):461–75.
35. Federici AB, Bucciarelli P, Castaman G, Mazzucconi MG, Morfini M, Rocino A, et al. The bleeding score predicts clinical outcomes and replacement therapy in adults with von Willebrand disease. Blood J Am Soc Hematol. 2014;123(26):4037–44.
36. Makris M. Gastrointestinal bleeding in von Willebrand disease. Thromb Res. 2006;118:S13–S7.
37. Samii A, Norouzi M, Ahmadi A, Dorgalaleh A. Gastrointestinal bleeding in congenital bleeding disorders. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers Inc; 2022.
38. Neerman-Arbez M, Johnson K, Morris MA, McVey J, Peyvandi F, Nichols W, et al. Molecular analysis of the ERGIC-53 gene in 35 families with combined factor V-factor VIII deficiency. Blood J Am Soc Hematol. 1999;93(7):2253–60.
39. Zhang B, McGee B, Yamaoka JS, Guglielmone H, Downes KA, Minoldo S, et al. Combined deficiency of factor V and factor VIII is due to mutations in either LMAN1 or MCFD2. Blood. 2006;107(5):1903–7.
40. Brenner B, Sánchez-Vega B, Wu S-M, Lanir N, Stafford DW, Solera J. A missense mutation in γ-glutamyl carboxylase gene causes combined deficiency of all vitamin K-dependent blood coagulation factors. Blood J Am Soc Hematol. 1998;92(12):4554–9.

41. Oldenburg J, Von Brederlow B, Fregin A, Rost S, Wolz W, Eberl W, et al. Congenital deficiency of vitamin K dependent coagulation factors in two families presents as a genetic defect of the vitamin K-epoxide-reductase-complex. Thromb Haemost. 2000;84(12):937–41.
42. Dorgalaleh A, Dadashizadeh G, Bamedi T. Hemophilia in Iran. Hematology. 2016;21(5):300–10.
43. Bagnall RD, Waseem N, Green PM, Giannelli F. Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia a. Blood J Am Soc Hematol. 2002;99(1):168–74.
44. Jenkins P, Collins P, Goldman E, McCraw A, Riddell A, Lee C, et al. Analysis of intron 22 inversions of the factor VIII gene in severe hemophilia A: implications for genetic counseling. Blood. 1994;84(7):2197–201.
45. Antonarakis SE, Rossiter J, Young M, Horst J, De Moerloose P, Sommer S, et al. Factor VIII gene inversions in severe hemophilia A: results of an international consortium study. Blood. 1995;86(6):2206–12.
46. Asselta R, Duga S, Tenchini ML. The molecular basis of quantitative fibrinogen disorders. J Thromb Haemost. 2006;4(10):2115–29.
47. Dorgalaleh A, Tabibian S, Bamedi T, Tamaddon G, Naderi M, Varmaghani B, et al. Molecular genetic analysis of ten unrelated Iranian patients with congenital factor XIII deficiency. Int J Lab Hematol. 2017;39(2):e33–e6.
48. Vu D, Neerman-Arbez M. Molecular mechanisms accounting for fibrinogen deficiency: from large deletions to intracellular retention of misfolded proteins. J Thromb Haemost. 2007;5:125–31.
49. Dorgalaleh A, Bahraini M, Shams M, Parhizkari F, Dabbagh A, Naderi T, Fallah A, Fazeli A, Ahmadi SE, Samii A, Daneshi M. Molecular basis of rare congenital bleeding disorders. Blood Rev. 2022 Nov;9:101029.
50. Naderi M, Tabibian S, Alizadeh S, Abtahi ZS, Dorgalaleh A. Coagulation Factor XIII-A A614T gene variation is suggestive of founder effect in Iranian patients with severe congenital Factor XIII Deficiency. J Cell Mol Anesth. 2016;1(1):19–22.
51. Bellucci S, Caen J. Molecular basis of Glanzmann's thrombasthenia and current strategies in treatment. Blood Rev. 2002;16(3):193–202.
52. Nurden AT. Glanzmann thrombasthenia. Orphanet J Rare Dis. 2006;1(1):1–8.
53. Favaloro EJ. Von Willebrand disease: local diagnosis and management of a globally distributed bleeding disorder. In: Seminars in thrombosis and hemostasis. © Thieme Medical Publishers; 2011.
54. Favaloro EJ. Diagnosis and classification of von Willebrand disease: a review of the differential utility of various functional von Willebrand factor assays. Blood Coagul Fibrinol. 2011;22(7):553–64.
55. Dorgalaleh A, Bahraini M, Shams M, Parhizkari F, Dabbagh A, Naderi T, et al. Molecular basis of rare congenital bleeding disorders. Blood Rev. 2023;59:101029.
56. Hart DP, Giangrande PL. The molecular basis of hemophilia. Mol Hematology, 4th Edition. 2019:221–34. ISBN: 978-1-119-25287-0.
57. Zolkova J, Sokol J, Simurda T, Vadelova L, Snahnicanova Z, Loderer D, et al. Genetic background of von Willebrand disease: history, current state, and future perspectives. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2020.
58. Martinez M, Graf L, Tsakiris DA. Congenital bleeding disorders. In: Perioperative hemostasis. Berlin, Heidelberg: Springer; 2015. p. 71–87.
59. Levinson B, Lehesjoki A, De La Chapelle A, Gitschier J. Molecular analysis of hemophilia a mutations in the Finnish population. Am J Hum Genet. 1990;46(1):53.
60. Vidaud M, Chabret C, Gazengel C, Grunebaum L, Cazenave J, Goossens M. A de novo intragenic deletion of the potential EGF domain of the factor IX gene in a family with severe hemophilia B. Blood. 1986;68(4):961–3.
61. Cattaneo M. Inherited platelet-based bleeding disorders. J Thromb Haemost. 2003;1(7):1628–36.

62. Kern M, Blanchette V, Stain AM, Einarson TR, Feldman BM. Clinical and cost implications of target joints in Canadian boys with severe hemophilia a. J Pediatr. 2004;145(5):628–34.
63. Manco-Johnson MJ, Abshire TC, Shapiro AD, Riske B, Hacker MR, Kilcoyne R, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. N Engl J Med. 2007;357(6):535–44.
64. Castaman G, Goodeve A, Eikenboom J. Principles of care for the diagnosis and treatment of von Willebrand disease. Haematologica. 2013;98(5):667.
65. Castaman G, Rodeghiero F, Tosetto A, Cappelletti A, Baudo F, Eikenboom J, et al. Hemorrhagic symptoms and bleeding risk in obligatory carriers of type 3 von Willebrand disease: an international, multicenter study. J Thromb Haemost. 2006;4(10):2164–9.
66. Anwar R, Minford A, Gallivan L, Trinh CH, Markham AF. Delayed umbilical bleeding—a presenting feature for factor XIII deficiency: clinical features, genetics, and management. Pediatrics. 2002;109(2):e32.
67. Angles-Cano E, Mathonnet F, Dreyfus M, Claeyssens S, de Mazancourt P. A case of afibrinogenemia associated with A-alpha chain gene compound heterozygosity (HUMFIBRA c.[4110delA]+[3200+ 1G> T]). Blood Coagul Fibrinol. 2007;18(1):73–5.
68. Awasthy N, Aggarwal K, Gupta H, Saluja S. Congenital hypofibrinogenemia. Indian Pediatr. 2004;41(2):185–6.
69. Cattaneo M, Hayward C, Moffat K, Pugliano M, Liu Y, Michelson AD. Results of a worldwide survey on the assessment of platelet function by light transmission aggregometry: a report from the platelet physiology subcommittee of the SSC of the ISTH. J Thromb Haemost. 2009;7(6):1029.
70. Cattaneo M, Cerletti C, Harrison P, Hayward C, Kenny D, Nugent D, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. J Thromb Haemost. 2013;11(6):1183–9.
71. Althaus K, Zieger B, Bakchoul T, Jurk K. Standardization of light transmission aggregometry for diagnosis of platelet disorders: an inter-laboratory external quality assessment. Thromb Haemost. 2019;119(07):1154–61.
72. Le Blanc J, Mullier F, Vayne C, Lordkipanidzé M. Advances in platelet function testing—light transmission aggregometry and beyond. J Clin Med. 2020;9(8):2636.
73. Nair S, Ghosh K, Kulkarni B, Shetty S, Mohanty D. Glanzmann's thrombasthenia: updated. Platelets. 2002;13(7):387–93.
74. Botero JP, Lee K, Branchford BR, Bray PF, Freson K, Lambert MP, et al. Glanzmann thrombasthenia: genetic basis and clinical correlates. Haematologica. 2020;105(4):888.
75. Nurden AT, Pillois X. ITGA2B and ITGB3 gene mutations associated with Glanzmann thrombasthenia. Platelets. 2018;29(1):98–101.
76. Andrews RK, Berndt MC. Bernard–Soulier syndrome: an update. In: eminars in thrombosis and hemostasis. Thieme Medical Publishers; 2013.
77. Orbach-Zinger S, Davis A, Ioscovich A. Bernard–Soulier syndrome. In: Consults in obstetric anesthesiology. Springer; 2018. p. 89–91.
78. Dong J-F, Berndt MC, Schade A, McIntire LV, Andrews RK, López JA. Ristocetin-dependent, but not botrocetin-dependent, binding of von Willebrand factor to the platelet glycoprotein Ib-IX-V complex correlates with shear-dependent interactions. Blood J Am Soc Hematol. 2001;97(1):162–8.
79. Flaumenhaft R, Sharda A. Platelet secretion. In: Platelets. Elsevier; 2019. p. 349–70.
80. Nurden AT, Nurden P. The gray platelet syndrome: clinical spectrum of the disease. Blood Rev. 2007;21(1):21–36.
81. Gunay-Aygun M, Zivony-Elboum Y, Gumruk F, Geiger D, Cetin M, Khayat M, et al. Gray platelet syndrome: natural history of a large patient cohort and locus assignment to chromosome 3p. Blood J Am Soc Hematol. 2010;116(23):4990–5001.
82. Gunay-Aygun M, Falik-Zaccai TC, Vilboux T, Zivony-Elboum Y, Gumruk F, Cetin M, et al. NBEAL2 is mutated in gray platelet syndrome and is required for biogenesis of platelet α-granules. Nat Genet. 2011;43(8):732–4.

83. Rogers HJ, Nakashima MO, Kottke-Marchant K. Hemostasis and thrombosis. In: Hematopathology. Elsevier; 2018. p. 57–105. e4.
84. Federici A. Current and emerging approaches for assessing von Willebrand disease in 2016. Int J Lab Hematol. 2016;38:41–9.
85. Leebeek FW, Eikenboom JC. Von Willebrand's disease. N Engl J Med. 2016;375(21):2067–80.
86. Holm E, Carlsson KS, Lövdahl S, Lail A, Abshire T, Berntorp E. Bleeding-related hospitalization in patients with von Willebrand disease and the impact of prophylaxis: results from national registers in Sweden compared with normal controls and participants in the von Willebrand disease prophylaxis network. Haemophilia. 2018;24(4):628–33.
87. Park JJ, Kim C-H, Lee J-G, Cho H-J. Von-Willebrand disease presenting as intractable epistaxis after nasal polypectomy. Case Rep Otolaryngol. 2014;2014:902071.
88. Liao L-C, Liao S-C, Chang C-H, Shih M-Y, Wang J-D. Gastrointestinal angiodysplasia in two patients with type 3 von Willebrand disease. Blood Coagul Fibrinol. 2019;30(5):243–5.
89. Rassoulzadegan M, Ala F, Jazebi M, Enayat MS, Tabibian S, Shams M, et al. Molecular and clinical profile of type 2 von Willebrand disease in Iran: a thirteen-year experience. Int J Hematol. 2020;111(4):535–43.
90. Neff AT. Current controversies in the diagnosis and management of von Willebrand disease. Ther Adv Hematol. 2015;6(4):209–16.
91. Favaloro EJ, Pasalic L, Curnow J. Laboratory tests used to help diagnose von Willebrand disease: an update. Pathology. 2016;48(4):303–18.
92. Favaloro EJ, Soltani S, McDonald J, Grezchnik E, Easton L, Favaloro JW. Reassessment of ABO blood group, sex, and age on laboratory parameters used to diagnose von Willebrand disorder: potential influence on the diagnosis vs the potential association with risk of thrombosis. Am J Clin Pathol. 2005;124(6):910–7.
93. De Jong A, Eikenboom J. Developments in the diagnostic procedures for von Willebrand disease. J Thromb Haemost. 2016;14(3):449–60.
94. Wagenman BL, Townsend KT, Mathew P, Crookston KP. The laboratory approach to inherited and acquired coagulation factor deficiencies. Clin Lab Med. 2009;29(2):229–52.
95. Dorgalaleh A, Casini A, Rahmani P. Congenital fibrinogen disorders. In: Congenital bleeding disorders. Springer; 2018. p. 163–81.
96. De Moerloose P, Neerman-Arbez M. Treatment of congenital fibrinogen disorders. Expert Opin Biol Ther. 2008;8(7):979–92.
97. Cheresh DA, Berliner SA, Vicente V, Ruggeri ZM. Recognition of distinct adhesive sites on fibrinogen by related integrins on platelets and endothelial cells. Cell. 1989;58(5):945–53.
98. Ikeda Y, Handa M, Kawano K, Kamata T, Murata M, Araki Y, et al. The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. J Clin Invest. 1991;87(4):1234–40.
99. Tabibian S, Shams M, Naderi M, Dorgalaleh A. Prenatal diagnosis in rare bleeding disorders—an unresolved issue? Int J Lab Hematol. 2018;40(3):241–50.
100. Casini A, Blondon M, Lebreton A, Koegel J, Tintillier V, de Maistre E, et al. Natural history of patients with congenital dysfibrinogenemia. Blood J Am Soc Hematol. 2015;125(3):553–61.
101. Neerman-Arbez M, De Moerloose P, Casini A. Laboratory and genetic investigation of mutations accounting for congenital fibrinogen disorders. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2016.
102. Casini A, Undas A, Palla R, Thachil J, De Moerloose P, SoF XIII, et al. Diagnosis and classification of congenital fibrinogen disorders: communication from the SSC of the ISTH. J Thromb Haemost. 2018;16(9):1887–90.
103. Acharya S, Coughlin A, Dimichele DM, Group NARBDS. Rare bleeding disorder registry: deficiencies of factors II, V, VII, X, XIII, fibrinogen and dysfibrinogenemias. J Thromb Haemost. 2004;2(2):248–56.
104. Mumford AD, Ackroyd S, Alikhan R, Bowles L, Chowdary P, Grainger J, et al. Guideline for the diagnosis and management of the rare coagulation disorders: a United Kingdom Haemophilia Centre Doctors' Organization guideline on behalf of the British Committee for Standards in Haematology. Br J Haematol. 2014;167(3):304–26.

105. Huntington JA. How Na+ activates thrombin—a review of the functional and structural data. Biol Chem. 2008;389(8):1025–35.
106. Bode W. The structure of thrombin: a janus-headed proteinase. In: Seminars in thrombosis and hemostasis; 2006. Copyright© 2006 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New….
107. Brown JP, Douglas J. Prothrombin (Factor II) deficiency. In: Consults in obstetric anesthesiology. Springer; 2018. p. 493–4.
108. Farshi Y, Dorgalaleh A, Tabibian S. Congenital Factor II deficiency. In: Congenital bleeding disorders. Springer; 2018. p. 183–99.
109. Wolberg AS. Thrombin generation and fibrin clot structure. Blood Rev. 2007;21(3):131–42.
110. Mullin JL, Gorkun OV, Binnie CG, Lord ST. Recombinant fibrinogen studies reveal that thrombin specificity dictates order of fibrinopeptide release. J Biol Chem. 2000;275(33):25239–46.
111. Standeven KF, Ariëns RA, Whitaker P, Ashcroft AE, Weisel JW, Grant PJ. The effect of dimethylbiguanide on thrombin activity, FXIII activation, fibrin polymerization, and fibrin clot formation. Diabetes. 2002;51(1):189–97.
112. Narayanan S. Multifunctional roles of thrombin. Ann Clin Lab Sci. 1999;29(4):275–80.
113. Myles T, Yun TH, Leung LL. Structural requirements for the activation of human factor VIII by thrombin. Blood J Am Soc Hematol. 2002;100(8):2820–6.
114. Bouma BN, Mosnier LO. Thrombin activatable fibrinolysis inhibitor (TAFI)—how does thrombin regulate fibrinolysis? Ann Med. 2006;38(6):378–88.
115. Coughlin SR. Thrombin signalling and protease-activated receptors. Nature. 2000;407(6801):258–64.
116. Girolami A, Santarossa L, Scarparo P, Candeo N, Girolami B. True congenital prothrombin deficiency due to a 'new'mutation in the pre-propeptide (ARG-39 GLN). Acta Haematol. 2008;120(2):82–6.
117. Acharya SS. Rare bleeding disorders in children: identification and primary care management. Pediatrics. 2013;132(5):882–92.
118. Bajaj SP, Rapaport SI, Barclay S, Herbst KD. Acquired hypoprothrombinemia due to nonneutralizing antibodies to prothrombin: mechanism and management. Blood. 1985;65(6):1538–43.
119. Lancellotti S, De Cristofaro R. Congenital prothrombin deficiency. In: Seminars in thrombosis and hemostasis. © Thieme Medical Publishers; 2009.
120. Miyawaki Y, Suzuki A, Fujita J, Maki A, Okuyama E, Murata M, et al. Thrombosis from a prothrombin mutation conveying antithrombin resistance. N Engl J Med. 2012;366(25):2390–6.
121. Su K, Jin Y, Miao Z, Cheng X, Yang L, Wang M. Phenotypic and genetic analysis of dysprothrombinemia due to a novel homozygous mutation. Hematology. 2017;22(6):380–5.
122. Varga EA, Moll S. Prothrombin 20210 mutation (factor II mutation). Circulation. 2004;110(3):e15–e8.
123. Zivelin A, Rosenberg N, Faier S, Kornbrot N, Peretz H, Mannhalter C, et al. A single genetic origin for the common prothrombotic G20210A polymorphism in the prothrombin gene. Blood J Am Soc Hematol. 1998;92(4):1119–24.
124. Pihusch R, Hiller E, Buchholz T, Rogenhofer N, Hasbargen U, Thaler CJ, et al. Thrombophilic gene mutations and recurrent spontaneous abortion: prothrombin mutation increases the risk in the first trimester. Am J Reprod Immunol. 2001;46(2):124–31.
125. Chandler W. Initial evaluation of hemostasis: reagent and method selection. In: Quality in laboratory hemostasis and thrombosis; 2009. p. 63–71. https://doi.org/10.1002/9781444303575.ch7.
126. Tabibian S, Dorgalaleh A, Camire RM. Congenital factor V deficiency. In: Congenital bleeding disorders. Springer; 2018. p. 201–18.
127. Tabibian S, Shiravand Y, Shams M, Safa M, Gholami MS, Heydari F, et al. A comprehensive overview of coagulation factor V and congenital factor V deficiency. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2019.

128. Shams M, Dorgalaleh A. Congenital Factor VII deficiency. In: Congenital bleeding disorders. Springer; 2018. p. 239–59.
129. Sevenet PO, Kaczor DA, Depasse F. Factor VII Deficiency: from basics to clinical laboratory diagnosis and patient management. Clin Appl Thromb Hemost. 2017;23(7):703–10.
130. Tuddenham EG, Pemberton S, Cooper DN. Inherited factor VII deficiency: genetics and molecular pathology. Thromb Haemost. 1995;74(1):313–21.
131. Mariani G, Bernardi F. Factor VII deficiency. Semin Thromb Hemost. 2009;35(4):400–6.
132. Alexander B, Goldstein R, Landwehr G, Cook CD. Congenital SPCA deficiency: a hitherto unrecognized coagulation defect with hemorrhage rectified by serum and serum fractions. J Clin Invest. 1951;30(6):596–608.
133. McVey JH, Boswell E, Mumford AD, Kemball-Cook G, Tuddenham EG. Factor VII deficiency and the FVII mutation database. Hum Mutat. 2001;17(1):3–17.
134. Motlagh H, Pezeshkpoor B, Dorgalaleh A. Hemophilia B. In: Congenital bleeding disorders. Springer; 2018. p. 139–60.
135. Adcock D, Strandberg K, Shima M, Marlar R. Advantages, disadvantages and optimization of one-stage and chromogenic factor activity assays in haemophilia A and B. Int J Lab Hematol. 2018;40(6):621–9.
136. Kitchen S, Hayward C, Negrier C, Dargaud Y. New developments in laboratory diagnosis and monitoring. Haemophilia. 2010;16:61–6.
137. Trossaert M, Lienhart A, Nougier C, Fretigny M, Sigaud M, Meunier S, et al. Diagnosis and management challenges in patients with mild haemophilia A and discrepant FVIII measurements. Haemophilia. 2014;20(4):550–8.
138. Barrowcliffe TW, Raut S, Sands D, Hubbard AR. Coagulation and chromogenic assays of factor VIII activity: general aspects, standardization, and recommendations. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers, Inc.; 2002. Copyright© 2002 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New ….
139. Castaman G, Eckhardt C, van Velzen A, Linari S, Fijnvandraat K. Emerging issues in diagnosis, biology, and inhibitor risk in mild hemophilia A. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2016.
140. Mazurier C, Gaucher C, Jorieux S, Parquet-Gernez A. Mutations in the FVIII gene in seven families with mild haemophilia A. Br J Haematol. 1997;96(2):426–7.
141. Gruppo R, Brown D, Wilkes M, Navickis R. Increased breakthrough bleeding during prophylaxis with B-domain deleted factor VIII–a robust meta-analytic finding. Haemophilia. 2004;10(5):449–51.
142. Pipe SW, Saenko EL, Eickhorst AN, Kemball-Cook G, Kaufman RJ. Hemophilia a mutations associated with 1-stage/2-stage activity discrepancy disrupt protein-protein interactions within the triplicated a domains of thrombin-activated factor VIIIa. Blood J Am Soc Hematol. 2001;97(3):685–91.
143. Pavlova A, Delev D, Pezeshkpoor B, Müller J, Oldenburg J. Haemophilia a mutations in patients with non-severe phenotype associated with a discrepancy between one-stage and chromogenic factor VIII activity assays. Thromb Haemost. 2014;112(05):851–61.
144. Makris M, Oldenburg J, Mauser-Bunschoten E, Peerlinck K, Castaman G, Fijnvandraat K, et al. The definition, diagnosis and management of mild hemophilia a: communication from the SSC of the ISTH. J Thromb Haemost. 2018;16(12):2530–3.
145. Duncan EM, Rodgers SE, McRae SJ. Diagnostic testing for mild hemophilia a in patients with discrepant one-stage, two-stage, and chromogenic factor VIII: C assays. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2013.
146. Rodgers S, Duncan E. Chromogenic factor VIII assays for improved diagnosis of hemophilia A. In: Hemostasis and thrombosis. Springer; 2017. p. 265–76.
147. Girolami A, Scandellari R, Scapin M, Vettore S. Congenital bleeding disorders of the vitamin K-dependent clotting factors. Vitam Horm. 2008;78:281–374.
148. Chandler WL, Ferrell C, Lee J, Tun T, Kha H. Comparison of three methods for measuring factor VIII levels in plasma. Am J Clin Pathol. 2003;120(1):34–9.

149. Barrowcliffe T. Monitoring haemophilia severity and treatment: new or old laboratory tests? Haemophilia. 2004;10:109–14.
150. Gouw SC, van den Berg HM, Oldenburg J, Astermark J, de Groot PG, Margaglione M, et al. F8 gene mutation type and inhibitor development in patients with severe hemophilia a: systematic review and meta-analysis. Blood J Am Soc Hematol. 2012;119(12):2922–34.
151. Pezeshkpoor B, Pavlova A, Oldenburg J, El-Maarri O. F8 genetic analysis strategies when standard approaches fail. Hamostaseologie. 2014;34(2):167–73.
152. Pezeshkpoor B, Pavlova A, Oldenburg J, El-Maarri O. F8 genetic analysis strategies when standard approaches fail. Haemostaseologie. 2014;34(02):167–73.
153. Jenkins PV, Egan H, Keenan C, O'Shea E, Smith OP, Nolan B, et al. Mutation analysis of haemophilia B in the Irish population: increased prevalence caused by founder effect. Haemophilia. 2008;14(4):717–22.
154. Casaña P, Haya S, Cid AR, Oltra S, Martínez F, Cabrera N, et al. Identification of deletion carriers in hemophilia B: quantitative real-time polymerase chain reaction or multiple ligation probe amplification. Transl Res. 2009;153(3):114–7.
155. Funnell AP, Crossley M. Hemophilia B Leyden and once mysterious cis-regulatory mutations. Trends Genet. 2014;30(1):18–23.
156. Swystun LL, James PD. Genetic diagnosis in hemophilia and von Willebrand disease. Blood Rev. 2017;31(1):47–56.
157. Bowen DJ. Haemophilia a and haemophilia B: molecular insights. Mol Pathol. 2002;55(2):127–44.
158. Brown D, Kouides P. Diagnosis and treatment of inherited factor X deficiency. Haemophilia. 2008;14(6):1176–82.
159. Perry DJ. Factor X and its deficiency states. Haemophilia. 1997;3(3):159–72.
160. Millar DS, Elliston L, Deex P, Krawczak M, Wacey AI, Reynaud J, et al. Molecular analysis of the genotype-phenotype relationship in factor X deficiency. Hum Genet. 2000;106(2):249–57.
161. Mannucci PM, Duga S, Peyvandi F. Recessively inherited coagulation disorders. Blood. 2004;104(5):1243–52.
162. Duga S, Salomon O. Congenital factor XI deficiency: an update. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2013.
163. Shpilberg O, Peretz H, Zivelin A, Yatuv R, Chetrit A, Kulka T, et al. One of the two common mutations causing factor XI deficiency in Ashkenazi jews (type II) is also prevalent in Iraqi jews, who represent the ancient gene pool of jews. Blood. 1995;85(2):429–32.
164. Bolton-Maggs P, Peretz H, Butler R, Mountford R, Keeney S, Zacharski L, et al. A common ancestral mutation (C128X) occurring in 11 non-Jewish families from the UK with factor XI deficiency. J Thromb Haemost. 2004;2(6):918–24.
165. Hancock JF, Wieland K, Pugh RE, Martinowitz U, Schulman S, Kakkar VV, et al. A molecular genetic study of factor XI deficiency. Blood. 1991;77(9):1942–8.
166. Tabatabaei T, Dorgalaleh A. Congenital factor XI deficiency. In: Congenital bleeding disorders. Springer; 2018. p. 291–306.
167. Emsley J, McEwan PA, Gailani D. Structure and function of factor XI. Blood J Am Soc Hematol. 2010;115(13):2569–77.
168. Duga S, Asselta R. Mutations in disguise. J Thromb Haemost. 2011;9(10):1973–6.
169. Van Veen JJ, Laidlaw S, Swanevelder J, Harvey N, Watson C, Kitchen S, et al. Contact factor deficiencies and cardiopulmonary bypass surgery: detection of the defect and monitoring of heparin. Eur J Haematol. 2009;82(3):208–12.
170. Brown JP, Douglas J. Factor XI deficiency. In: Consults in obstetric anesthesiology. Springer; 2018. p. 213–5.
171. Asmis LM, Sulzer I, Furlan M, Lämmle B. Prekallikrein deficiency: the characteristic normalization of the severely prolonged aPTT following increased preincubation time is due to autoactivation of factor XII. Thromb Res. 2002;105(6):463–70.
172. Vardi M, Girolami A, Sexton D, Rolland C, Bernstein JA. Long-term safety outcomes of prekallikrein deficiency: a systematic literature review. J Allergy Clin Immunol. 2018;141(2):AB58.

173. Siegerink B, Rosendaal F, Algra A. Antigen levels of coagulation factor XII, coagulation factor XI and prekallikrein, and the risk of myocardial infarction and ischemic stroke in young women. J Thromb Haemost. 2014;12(5):606–13.
174. Patel N, Conley G, McElroy L, Re-faai M. Isolated prolonged activated partial thromboplastin time and con-tact factor deficiencies: case series and management review. Anesthesiol Open J. 2016;1(1):19–23.
175. Inbal A, Lubetsky A, Krapp T, Caste D, Shaish A, Dickneitte G, et al. Impaired wound healing in factor XIII deficient mice. Thromb Haemost. 2005;94(08):432–7.
176. Dorgalaleh A, Farshi Y, Alizadeh S, Naderi M, Tabibian S, Kazemi A, et al. Challenges in implementation of ISTH diagnostic algorithm for diagnosis and classification of factor XIII deficiency in Iran. J Thromb Haemost. 2015;13(9):1735–6.
177. Karimi M, Peyvandi F, Naderi M, Shapiro A. Factor XIII deficiency diagnosis: challenges and tools. Int J Lab Hematol. 2018;40(1):3–11.
178. Dorgalaleh A, Tabibian S, Hosseini MS, Farshi Y, Roshanzamir F, Naderi M, et al. Diagnosis of factor XIII deficiency. Hematology. 2016;21(7):430–9.
179. Nahrendorf M, Hu K, Frantz S, Jaffer FA, Tung C-H, Hiller K-H, et al. Factor XIII deficiency causes cardiac rupture, impairs wound healing, and aggravates cardiac remodeling in mice with myocardial infarction. Circulation. 2006;113(9):1196–202.
180. Dorgalaleh A, Tabibian S, Assadollahi V, Shamsizadeh M, Zareban I, Soori S, et al. Comparison of 2 methods of clot solubility testing in detection of factor XIII deficiency. Lab Med. 2016;47(4):283–5.
181. Muszbek L, Katona É. Diagnosis and management of congenital and acquired FXIII deficiencies. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2016.
182. Muszbek L, Adany R, Szegedi G, Polgar J, Kavai M. Factor XIII of blood coagulation in human monocytes. Thromb Res. 1985;37(3):401–10.
183. Asahina T, Kobayashi T, Okada Y, Goto J, Terao T. Maternal blood coagulation factor XIII is associated with the development of cytotrophoblastic shell. Placenta. 2000;21(4):388–93.
184. Inbal A, Oldenburg J, Carcao M, Rosholm A, Tehranchi R, Nugent D. Recombinant factor XIII: a safe and novel treatment for congenital factor XIII deficiency. Blood J Am Soc Hematol. 2012;119(22):5111–7.
185. Kohler H, Ichinose A, Seitz R, Ariens R, Muszbek L, et al. Diagnosis and classification of factor XIII deficiencies. J Thromb Haemost. 2011;9(7):1404–6. Wiley Online Library
186. Dorgalaleh A, Tabibian S, Shams M, Tavasoli B, Gheidishahran M, Shamsizadeh M. Laboratory diagnosis of factor XIII deficiency in developing countries: an Iranian experience. Lab Med. 2016;47(3):220–6.
187. Asahina T, Kobayashi T, Takeuchi K, Kanayama N. Congenital blood coagulation factor XIII deficiency and successful deliveries: a review of the literature. Obstet Gynecol Surv. 2007;62(4):255–60.
188. Biswas A, Ivaskevicius V, Seitz R, Thomas A, Oldenburg J. An update of the mutation profile of Factor 13 a and B genes. Blood Rev. 2011;25(5):193–204.
189. Royal S, Schramm W, Berntorp E, Giangrande P, Gringeri A, Ludlam C, et al. Quality-of-life differences between prophylactic and on-demand factor replacement therapy in European haemophilia patients. Haemophilia. 2002;8(1):44–50.
190. Valentino L, Mamonov V, Hellmann A, Quon D, Chybicka A, Schroth P, et al. A randomized comparison of two prophylaxis regimens and a paired comparison of on-demand and prophylaxis treatments in hemophilia a management. J Thromb Haemost. 2012;10(3):359–67.
191. Federici A, Castaman G, Mannucci P, Centers IAoH. Guidelines for the diagnosis and management of von Willebrand disease in Italy. Haemophilia. 2002;8(5):607–21.
192. Mannucci PM. Treatment of von Willebrand's disease. N Engl J Med. 2004;351(7):683–94.
193. Bertolini J, Goss N, Curling J. Production of plasma proteins for therapeutic use. John Wiley & Sons; 2012.

Part II

Common Bleeding Disorders

von Willebrand Disease: An Update on Diagnosis and Treatment

3

Emmanuel J. Favaloro

3.1 Introduction

von Willebrand disease (VWD) is reportedly the most common congenital bleeding disorder and may be inherited in an autosomal dominant or autosomal recessive manner [1]. The disorder is classified into six different types, with quantitative deficiency of von Willebrand factor (VWF) identified in type 1 (partial deficiency) and type 3 (complete deficiency), or qualitative defects in VWF identified in type 2 VWD, which comprises four separate (sub)types [2]. VWD may present as a heterogeneous bleeding disorder with variable bleeding tendency, depending on the VWD type, and the associated VWF deficiency/defect. In some patients, the bleeding symptoms may be so mild as to not easily distinguish these potential sufferers from unaffected individuals. However, in severely affected VWD individuals, life endangering bleeding (e.g., in central nervous system (CNS)) may occur. Mucocutaneous bleeding, including epistaxis and menorrhagia, are more typical presentations of the disorder, but other rare presentations also can be observed [1, 2]. As per the ISTH SSC (International Society on Thrombosis and Hemostasis Scientific Standardization Committee) classification of the disorder, and newer guidelines, affected individuals are characterized according to clinical manifestations and laboratory findings

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_3

[3–5]. The severity of VWD is partially associated to the level and activity of the VWF. Although there are no standardized recommendations, as a practical guide, VWD can be considered "mild" when VWF activity levels are in the range 30–50 U/dL and/or factor VIII (FVIII) coagulant activity (FVIII:C): 40–60 U/dL, "moderate" when VWF activity levels are in the range 10–30 U/dL and/or FVIII:C 20–40 U dL–1, or "severe" when VWF activity levels are <10 U/dL and/or FVIII:C < 20 U/dL [5]. Based on such an approach, type 3 VWD, as well as some cases of type 1, type 2A, type 2M, and type 2N VWD, could be classified as severe forms of VWD [5].

Diagnosis of VWD is a global challenge, since a panel of accurate laboratory tests are required to align to a comprehensive and appropriate clinical assessment, and this approach is not available everywhere [5]. Significant progress has occurred in the laboratory diagnosis of VWD in recent years, but accurate diagnosis remains challenging for many health workers. The difficulty in diagnosis is due to several issues; these include (i) the lack of any definitive cut-offs to determine normal vs abnormal levels of VWF, thereby potentially identifying normal individuals vs VWD; (ii) the effect of different molecular changes in the *VWF* gene on VWF level and activity; (iii) other genetic modifiers and physiological factors that can reduce or increase plasma levels of VWF (e.g., blood group); (iv) the overlap of bleeding symptomology in normal individuals vs those with VWD; and (v) difficulties with performance and interpretation of laboratory tests [6, 7]. On-demand therapy is the current mainstay of treatment for people with VWD; however, long-term prophylaxis can significantly improve the quality of life in patients with severe bleeding. Desmopressin (DDAVP) is considered the main therapeutic option for patients with type 1 and a subset of patients with type 2 VWD with minor bleeding or undergoing minor surgical procedures. However, replacement therapy with VWF/FVIII concentrates represents the main therapeutic option in type 3 and most patients with type 2 VWD, as well as those responsive to desmopressin but with long-term treatment needs or undergoing major surgical procedures [7–10].

3.2 von Willebrand Factor Synthesis, Structure, and Function

These matters were comprehensively reviewed in the original chapter on VWD in the first edition of this book, and so will only be briefly summarized here [1].

3.2.1 von Willebrand Factor Biosynthesis

The biosynthesis of VWF comprises a series of sequential steps that ultimately permit incorporation of the protein in various cellular storage organelles. These steps include protein production, removal of a signal peptide, tail to tail dimerization, heat to head multimerization, N-linked glycosylation, maturation of N-glycan, O-linked glycosylation, formation of tubules, and incorporation to storage organelles [11].

3.2.2 von Willebrand Factor Structure

VWF is mapped to the tip of the short arm of chromosome 12. The *VWF* gene spans 178-kb and consists of 52 exons [12], reflecting a unique gene structure consisting of different repeated sequences [13]. VWF is a multimeric protein with unit molecular mass of 350-kDa, as coded by 9 kb mRNA and consisting of 2813 amino acids, including a 741 amino acid pro-peptide and a 2050 amino acid mature polypeptide [14]. VWF is synthesized as a precursor and processed in endoplasmic reticulum (ER) and Golgi apparatus in endothelial cells. After processing, the protein undergoes dimerization in the ER by disulfide bridging and cleavage into two components, the mature protein and a 97-kDa a pro-peptide. The pro-peptide is secreted and circulates independently of mature VWF [11].

VWF comprises several distinct domains, with some domains containing functional binding sites for FVIII, platelet receptors and for collagen. Defects in VWF may affect different domains, and thus lead to different qualitative forms of VWD. In brief, FVIII binds to the D-domain, and defects in this domain can lead to FVIII binding defects, or namely type 2N VWD. Binding to the main VWF receptor on platelets, glycoprotein Ib (GPIb), occurs in the A1 domain, and so defects in this domain can lead to GPIb binding defects, as captured within 2A, 2B, and 2M VWD. Binding to collagen, a protein housed within the subendothelial matrix, occurs within the A1 and A3 domains, and so defects in these domains can lead to collagen binding defects, as also captured within 2A, 2B, and 2M VWD [1].

3.2.3 Disulfide Bridging and Multimerization of von Willebrand Factor

About 8.3% of VWF is composed of the amino acid cysteine (234 of the 2813 residues), which is some fourfold higher than most other human proteins. In contrast to other domains, the triplicated domain A, where most of the adhesive function of VWF resides, has only six cysteine residues. The cysteine residues are otherwise mostly paired in disulfide bonds in the secreted protein; however, several unpaired cysteines remain and are essential for proper folding and secretion of VWF [15]. In ER, the subunits of pro-VWF undergo dimerization by disulfide bonds in C-terminal cysteine knot (CK) domains. This tail-to-tail dimerization needs only the sequence of the last 150 residues. Tail to tail pro-VWF is transported to Golgi and forms head-to-head dimerization by further disulfide bonding in the D3 domain. The VWF pro-peptide (domains D1 and D2) and also D' are important in multimerization. After multimerization, the multimers are organized into a helical structure that permits the compaction of the protein, and its storage in the Weibel-Palade bodies of endothelial cells [11].

Regulation of the multimeric size of VWF is primarily mediated by the metalloproteinase ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13), which cleaves VWF at a single site (Tyr1605-Met1606) in the A2 domain [16, 17]. It appears that domain D4, at residues from 1874 to 2813, has a role in binding to ADAMTS13 and this acts as the initial step for proteolysis of VWF by ADAMTS13 [18].

3.2.4 Post translational Modifications of von Willebrand Factor

During synthesis, VWF undergoes different post translational modifications including removal of the propeptide by the protease furin, completion of N and O-glycosylation, and sulfation of specific N-oligosaccharides [19]. The N-linked oligosaccharides of VWF differ from other proteins because they contain ABO blood group oligosaccharides [20].

3.2.5 Intracellular Storage and Secretion of VWF

Following synthesis, VWF is stored in α-granules of megakaryocytes/platelets or in Weibel-Palade bodies of endothelium. α-granules can still be formed in the absence of VWF, while the formation of Weibel-Palade bodies depends on the presence of VWF [21]. About 95% of the VWF formed is constitutively secreted as smaller multimers, while the remainder is stored as large multimers, with these having a greater number of active sites for interaction with platelets and vessel wall. Therefore, in an environment devoid of ADAMTS13 (i.e., in thrombotic thrombocytopenic purpura, TTP), the thrombogenic risk due to an accumulation of these larger molecules is high [22, 23]. After synthesis and packaging of VWF in Weibel-Palade bodies, a complex pathway is initiated that leads to secretion of VWF.

3.2.6 Biological Activities of von Willebrand Factor

Following injury to the endothelium, VWF binds to different components of subendothelium, as well as to platelets in order to sequester them to the injury site [24].

3.2.6.1 Interaction of von Willebrand Factor with Extracellular Protein

VWF is capable of binding to different types of collagen, including types I, II, III, IV, V, and VI. Domains A1 and A3 are responsible for interaction with the main components of extracellular matrix (especially collagen), with each domain capable of binding to different types of collagen [25, 26]. The A1 domain, which covers residues 497–716, binds to collagen type VI, while the A3 domain, comprising residues 910–1111, binds to collagen types I and III [27, 28]. Collagen types I and III support VWF-dependent platelet adhesion in high shear rate, whereas collagen type IV mostly supports platelet adhesion at lower shear rates.

3.2.7 Interaction of von Willebrand Factor with Glycoprotein Ibα

The A1 domain is responsible for binding to platelet glycoprotein (GP) Ib, a component of the platelet GPIb-V-IX receptor complex [29]. This interaction plays a significant role in platelet activation, platelet adhesion, and platelet

aggregation, ultimately crucial for thrombus formation. In vivo, the initial platelet adhesion results from interaction between VWF and GpIbα under high fluid shear. In vitro, the bacterial glycopeptide antibiotic ristocetin can bind to VWF in laboratory tests, and thus facilitate platelet GPIbα binding under low shear. This property is exploited in several VWF tests, including ristocetin-induced platelet aggregation (RIPA) and the ristocetin cofactor (VWF:RCo) assay, both of which facilitate the laboratory estimation of VWF binding VWF to GpIb [30]. Botrocetin is a venom derived from the viper Bothrops jararaca, which can also activate platelets via GpIb binding, to facilitate VWF-dependent platelet aggregation in vitro [31]. The botrocetin-induced platelet aggregation (BIPA) is only selectively used in laboratory diagnostics [32]. There are several modern alternatives for the classical VWF:RCo assay, including the VWF GPIb binding assays (i) using recombinant GPIb and inert particles (latex or magnetic) to replace native platelets and GPIb otherwise used in the VWF:RCo, but still also using ristocetin ("VWF:GPIbR"), and (ii) using recombinant GPIb with gain of function variants and inert particles (latex or plastic wells) to replace native platelets and GPIb otherwise used in the VWF:RCo, but without need for use of ristocetin ("VWF:GPIbM") [5, 6].

3.2.8 Stabilization and Transport of Coagulation Factor VIII

In patients affected by hemophilia A, caused by defects in the *F8* gene, the FVIII level is decreased and the VWF level is normal. In contrast, in most of patients with VWD, concomitant reduction of both FVIII and VWF occurs, because the survival of FVIII depends on its interaction with VWF. This binding and protection of FVIII permits its delivery to sites of vascular injury, and after release, FVIII can participate in secondary hemostasis and facilitate the conversion of plasma fibrinogen to fibrin (i.e., clot formation) [33]. In this way, VWF contributes to secondary hemostasis. This FVIII binding activity can be assessed in vitro with a specific test called the VWF FVIII binding assay (VWF:FVIIIB) [5, 6].

3.2.9 Interaction of von Willebrand Factor with Integrin αIIbβ3 (GPIIb/IIIa)

Platelet GPIIb-IIIa, also known as the integrin αIIbβ3, is a surface receptor that, after platelet activation, can bind to different ligands, including fibrinogen, fibronectin, and VWF [34]. In domain C1 of VWF, the tetra peptide motif Arg-Gly-Asp-Ser (located in the C-terminal of the molecule) is a binding site for αIIbβ3 (GPIIb/IIIa). Although the major interaction for platelet adhesion is GbIb binding, inhibition of integrin αIIbβ3 (GPIIb/IIIa) also impairs platelet adhesion [31, 35, 36]. Thus, VWF–integrin αIIbβ3 (GPIIb/IIIa) interaction leads to further platelet adhesion and facilitation of platelet aggregation initiated through the binding of GpIb to VWF [36].

3.3 von Willebrand Disease

VWD, with an estimated incidence of ~1% according to epidemiological studies, is the most common congenital bleeding disorder, and is most often inherited in an autosomal dominant pattern. The disorder was first described by Erik von Willebrand in 1926, who named it "hereditary pseudohemophilia" [37]. VWD is caused by defects and/or deficiencies in VWF level, structure, or function. Patients with VWD present with variable bleeding symptoms, but mostly mucocutaneous. Epistaxis and bruising are the most common presentations in children, while menorrhagia and hematoma are common in adults, with severity and frequency of bleeding dependent on age, gender, and VWD type [2]. Plasma levels of VWF gradually increase with age, and this may ameliorate VWD diagnosis or effects in some cases. VWD is classified into three main types: types 1 and 3 represent partial and complete deficiency of VWF, respectively, while type 2 reflects qualitative defects further characterized into four subgroups. These comprise: (i) type 2A VWD, with loss of high and sometimes intermediate molecular weight VWF multimers; (ii) type 2B VWD, with increased affinity of VWF for GPIb; (iii) type 2M VWD, with defects in VWF function such as platelet adhesion but a relatively normal pattern of (i.e., no substantial decrease in high molecular weight) VWF multimers; and (iv) type 2N, with markedly decreased VWF binding affinity to FVIII (Table 3.1) [1, 3, 38].

Table 3.1 Types of von Willebrand disease (VWD)

VWD type		Definition	Prevalence	Inheritance
Type 1		Partial (quantitative) deficiency of VWF	1 in 1000 individuals (40–80% of all VWD cases)	AD
Type 2	2A	Qualitative defect with loss of high and sometimes intermediate molecular weight VWF multimers	5–10% of all VWD cases[a]	AD or AR
	2B	Qualitative defect with increased affinity to platelet GPIb	<5% of all VWD cases[a]	AD
	2M	Qualitative defect with decreased platelet adhesion property but relatively normal pattern of VWF multimers	5–10% of all VWD cases[a]	AD
	2N	Qualitative defect with markedly decreased affinity of VWF to factor VIII	<5% of all VWD cases[a]	AR
Type 3		Complete deficiency of VWF	1 in one million (<1% of all VWD cases[b])	AR

VWD von Willebrand disease, *VWF* von Willebrand factor, *AD* Autosomal dominant, *AR* Autosomal recessive

[a]Overall incidence of all type 2 VWD, with types 2A, 2M, 2B and 2N, represents ~10–20% of all VWD cases, with the incidence of each subtype dependent on the geography, the local study population, and ability of laboratories to appropriately classify VWD cases using comprehensive test panels

[b]Relative incidence is higher in developing countries due to consanguinity and higher relative presentations to clinicians due to severe bleeding symptoms

Type 3 VWD, with an estimated incidence of 1 per one million in the general population in developed countries, is the rarest and the most severe form of disorder, while type 1 is the most commonly diagnosed form of VWD. However, in some developing countries, the reverse may be true, with type 3 VWD more often reported than type 1; this is due to consanguinity, where both parents may have heterozygous type 1 VWD, and also because of higher relative presentations to clinicians due to the severity of bleeding symptoms [39–41].

Types 1 and 2 VWD represent highly heterogeneous bleeding disorders, with bleeding tendency related to the circulating level of functional VWF and type of VWF defect. It is always important to determine whether the bleeding tendency is "life-long" or of recent onset, since the latter potentially indicates an acquired VWF abnormality rather than inherited VWD [42].

Diagnosis of VWD is a challenge worldwide, especially in mildest forms of the disease (where the boundary between normal and abnormal phenotypes is not clearly defined [4]), and in complex types, where misdiagnosis is common and can occur due to use of inferior methods or tests or ineffective test panels [5]. Correct diagnosis and classification of VWD is critical to correct patient management (Table 3.2).

3.3.1 Type 1 von Willebrand Disease

Type 1 is inherited in autosomal dominant manner with variable penetrance and a highly variable phenotype. The latest guidelines on the diagnosis of VWD [5, 43] identify type 1 VWD where VWF levels are <30 U/dL with concordance of functional VWF identified using a VWF GPIb binding assay (VWF:RCo, VWF:GPIbR, or VWF:GPIbM) (i.e., ratio of functional VWF/VWF:Ag >0.7). This is since most of such patients would be identified with a genetic variant in *VWF*. These guidelines [5, 43, 44] also recommend assigning a diagnosis of type 1 VWD where VWF levels are between 30 and 50 U/dL with concordance of functional VWF/VWF:Ag (i.e., >0.7) in those with a significant history of bleeding. The recent ASH ISTH NHF WFH guidelines categorize patients with levels between 30 and 50 U/dL as type 1 VWD only if they have a positive bleeding history, whereas previous guidelines may have classified them as having "low VWF as a risk factor for bleeding." [5] A considerable number of factors are responsible for the highly variable clinical and laboratory phenotype in type 1 VWD. In some patients with type 1 VWD, a variant cannot be detected in the *VWF* gene. Genetic modifiers and physiological factors are major factors that can reduce plasma level of VWF, with one well-known genetic modifier outside the *VWF* gene being ABO blood group [45]. Plasma VWF level are ~25% lower in individuals with O blood group than non-O blood group. The severity of bleeding episodes in type 1 VWD largely depends on severity of the plasma VWF deficiency; thus, risk of severe bleeds is higher in patients with lower plasma levels of VWF. Plasma FVIII level is also reduced in parallel with VWF, and its deficiency can compound bleeding risk in VWD. Since type 1 VWD phenotypically defines an inherited bleeding disorder with partial quantitative VWF deficiency, both VWF:Ag and VWF activity fall in parallel; a functional abnormality of VWF

Table 3.2 Recommended nomenclature from the ISTH VWF SSC for VWF test parameters

Abbreviation for assay	Description of assay	Comments
VWF:Ag	von Willebrand factor antigen	All assays that provide a quantitative level of VWF protein, be it by ELISA, LIA, CLIA, or other methodology
VWF:CB	von Willebrand factor collagen binding capacity	All assays that provide a quantitative level of VWF—collagen binding capacity, be it by ELISA, CLIA, or other methodology
VWF:RCo	von Willebrand factor ristocetin cofactor activity	Historically, this referred to assays that used ristocetin to facilitate VWF binding to GPIb, where the only such assay type available was that based on platelet agglutination. This changed following the advent of non-platelet based methods, which incorporated rGPIb; the updated recommendations now place these new assays into the new category of VWF:GPIbR
VWF:GPIbR	All assays that are based on the ristocetin-induced binding of von Willebrand factor to a recombinant wild type GPIb fragment	Essentially, these are VWF:RCo-like assays that do not use platelets, and which currently comprise several IL Werfen assays, as performed by either latex agglutination or by CLIA technology. These assays generate test results very similar to those generated using classical VWF:RCo assays utilizing platelets
VWF:GPIbM	All assays that are based on the spontaneous binding of von Willebrand factor to gain-of-function mutated GPIb	Essentially, a GPIb binding assay that does not utilize platelets or ristocetin, and which currently comprises the Siemens Innovance VWF Ac assay (by latex agglutination), as well as non-commercialized ELISA based assays. These assays essentially generate test results similar to those using VWF:GPIbR or classical VWF:RCo assays, despite not using ristocetin in the assay
VWF:Ab	All assays that are based on the binding of a monoclonal antibody (MAB) to a VWF A1 domain epitope	Essentially a VWF binding assay that utilizes a monoclonal antibody; this currently comprises the IL Werfen "VWF Activity" assay (LIA), as well as ELISA based assays. VWF:Ab assays do not use ristocetin. VWF:Ab assays provide results that may or may not match VWF:GPIbM, VWF:GPIbR or classical VWF:RCo assays
VWF:FVIIIB	von Willebrand factor: factor VIII binding capacity	All assays that provide a quantitative level of VWF—factor VIII binding capacity, irrespective of specific methodology. Generally performed by ELISA
RIPA	Ristocetin-induced platelet aggregation	To assess for ristocetin threshold responses that trigger agglutination/aggregation, patient platelet-rich plasma (PRP) is challenged with varying concentrations of ristocetin

CLIA chemiluminescent immuno-assay, *ELISA* enzyme linked immunosorbent assay, *GPIb* (platelet) glycoprotein Ib, *LIA* latex immuno-assay, *ISTH* International Society on Thrombosis and Hemostasis, *rGPIb* recombinant (platelet) glycoprotein Ib, *VWF* von Willebrand factor, *SSC* Scientific and Standardisation Committee; International Society on Thrombosis and Haemostasis (ISTH) Scientific Standardisation Committee (SSC)

(i.e., type 2 VWD) can essentially be excluded if all VWF activity/VWF:Ag ratios are around unity (i.e., > 0.7) [46]. With the use of more sensitive assays, a considerable number of patients with historical diagnosis of type 1 VWD based on older assays such as VWF:RCo may be shown to mild abnormalities of multimer structure or distribution [47]. Increased susceptibility of VWF to proteolytic cleavage may also contribute to bleeding severity in type 1 VWD. The Tyr1584Cys variant, for example, increases the susceptibility of VWF to cleavage by ADAMTS13. Desmopressin is generally suitable as the therapeutic choice in most patients with type 1 VWD, especially for short duration or minor treatments [47].

3.3.2 Type 2 von Willebrand Disease

Type 2 VWD is characterized by qualitative defects in structure and function of VWF and is further classified into one of four types: 2A, 2B, 2M, and 2N. Classically, type 2A has been considered the most common form, while 2N and 2B represent the rarest forms. However, the relative frequency of type 2 VWD depends in part on the geography in which the diagnosis is made, as well as the diagnostic test repertoire. For example, type 2N VWD is relatively more frequent in some parts of France and Italy, and type 2B diagnosis requires performance of RIPA, which if omitted may lead to diagnosis of such patients as 2A VWD [48, 49]. Type 2M VWD has classically been considered a rare type of VWD, but it is likely that many cases of historically defined 2A VWD are instead 2M VWD, not appropriately diagnosed due to limited testing.

Type 2 VWD is mostly transmitted in an autosomal dominant manner, except for type 2N, which has an autosomal recessive pattern of inheritance. Type 2 VWD is less common than type 1 VWD, and represents ~20% of all cases of VWD. Type 2 VWD is usually attributable to variants that either impair specific functional domains of VWF or which affect VWF multimer assembly or proteolysis. Type 2 VWD is diagnostically the most challenging form of VWD [50]. The hallmark of this type of disorder is low functional VWF/VWF:Ag ratio (<0.7). Functional VWF was mostly assessed historically as VWF:RCo; however, newer assays, including VWF:GPIbR, VWF:GPIbM, and VWF collagen binding (VWF:CB) are increasingly contributing to better more accurate diagnosis. Lack of large multimers is evident in types 2A and 2B VWD, with loss of intermediate multimers also often seen in type 2A VWD. Impaired RIPA in platelet-rich plasma (PRP) or in whole blood is evident in types 2A and 2M VWD, whereas an increased RIPA responsiveness is seen in 2B VWD [32].

3.3.2.1 Type 2A von Willebrand Disease

Type 2A is classically considered the most frequent type 2 VWD, comprising up to 5–10% of all cases with VWD and typically >40% of all type 2 VWD. Type 2A VWD represents a loss of high and intermediate multimers of VWF, due either to impaired VWF multimerization or increased susceptibility of multimers to degradation with ADAMTS13. The loss of high molecular weight multimers (HMWM) and

often also intermediate molecular weight multimers (IMWM) leads to diminished activity of the binding domains for GPIb, collagen, and probably also GPIIb/IIIa [49].

3.3.2.2 Type 2B von Willebrand Disease

In type 2B VWD, gain-of function variants, usually in the A2 domain of VWF, cause an increased affinity of VWF for platelet GpIb. This is often identified in laboratory testing by elevated RIPA responsiveness. In vivo, the platelet-VWF complex is removed from plasma circulation, often leading to loss of HMWM and also (mild) thrombocytopenia. Additional features of 2B VWD include slightly decreased to normal levels of VWF:Ag and FVIII, and relatively decreased levels of VWF activity (i.e., ratios of VWF:RCo/Ag, VWF:GPIbR/Ag, VWF:GPIbM/Ag, and VWF:CB/Ag are all <0.7). However, not all cases present with this "classic" 2B VWD presentation, and occasionally, VWF multimeric pattern and functional VWF/VWF:Ag ratios may be normal than those with ("atypical") type 2B. In 2B VWD patients in their second and third trimester of pregnancy, the pregnancy associated increased level of gain of function VWF may worsen the thrombocytopenia. A reduction in platelet counts may also be observed if desmopressin is administrated in patients with 2B VWD, and thus, desmopressin is often considered contraindicated in 2B VWD [51].

3.3.2.3 Type 2M von Willebrand Disease

Type 2M is mostly due to variants in the A1 domain of VWF, leading to conformational changes in VWF protein and decreased binding affinity to GpIb. VWF multimeric pattern is essentially normal, but platelet-dependent VWF activities are decreased. Rarely, 2M VWD variants can occur in the A3 domain, and thus also affecting collagen binding. Generally, type 2M VWD cases are identified by low VWF GPIb binding/Ag ratios (i.e., VWF:RCo/Ag, VWF:GPIbR/Ag or VWF:GPIbM/Ag <0.7), but without lack of HMWM or with normal VWF:CB/Ag ratio. Misdiagnosis is an important and largely under-recognized issue in type 2M VWD and a considerable number of clinicians or laboratories identify this type "incorrectly" as type 1 or type 2A VWD [4], often due to incomplete testing or an pre-assumption of 2A VWD. Patients with type 2M tent to present with mild to moderate bleeding tendency but severe bleeds also may occur [52].

3.3.2.4 Type 2N von Willebrand Disease

Type 2N VWD (VWD "Normandy") was originally described in patients from the Normandy region of France, in 1989, as a variant of VWD caused by defects in the ability of VWF to bind FVIII [53]. This defect causes decreased plasma level of FVIII, thereby phenotypically resembling hemophilia A. However, the inheritance pattern of 2N VWD is autosomal recessive (whereas hemophilia A is sex-linked, being carried on the X-chromosome), and the defect is present on VWF (whereas in hemophilia A, the defect is in FVIII). Symptoms of type 2N VWD are similar to that of (mild) hemophilia A [54]. Phenotypically, the multimeric pattern is normal in type 2N VWD, and VWF:Ag and other functional assays (VWF:RCo, VFW:GPIbR,

VWF:GPIbM, VWF:CB) are also normal, unless the 2N VWD is coinherited with a type 1 VWD. The diagnostic feature of 2N VWD is the level of FVIII, which is decreased due to an increased clearance (i.e., as the VWF does not bind FVIII, the FVIII is easily degraded in circulation). As noted, sometimes 2N VWD may arise as a duplex defect. For example, patients with a 2N VWD variant on one *VWF* gene, and another defect in the other *VWF* gene, may show more complex phenotypes and more severe/complex bleeding patterns. For example, if the second *VWF* gene carries a null variant, essentially mimicking a "heterozygous type 3 VWD," then the resulting phenotype will express with lowered VWF levels [55].

3.3.2.5 Type 3 von Willebrand Disease

Type 3 VWD is the most severe form of VWD, albeit fortunately also the rarest type. By definition, type 3 VWD patients present with "undetectable" plasma and platelet level of VWF and also a low level of FVIII: C (<10 U dL^{-1}). Clinical presentations and bleeding symptoms are similar to those of moderately severe hemophilia A, and a misdiagnosis of hemophilia A is possible if only FVIII is assessed [39].

3.4 Clinical Manifestations

Clinical manifestations and bleeding tendency among patients with VWD are highly variable and range from quite mild conditions to severe bleeding diathesis sufficient to require urgent medical intervention. Mucocutaneous bleeding such as epistaxis and menorrhagia represents the most typical presentation of VWD, with post-dental extraction bleeding the most common post-surgical bleeding event. Since VWF also binds to FVIII and facilitates platelet function, VWD may cause bleeding symptoms that are typical of platelet function disorders or mild to moderately severe hemophilia A or both [3, 56].

In some patients, especially in males, surgery may be the first hemostatic challenge that leads to "abnormal bleeding" and therefore facilitates the diagnosis in previously unrecognized cases.

A wide overlap can be observed between the bleeding diathesis of patients with mild VWD and the normal population; therefore, a proper bleeding history is a critical and crucial component in the diagnosis of VWD and should be done carefully. Women with VWD tend to be more symptomatic than men, because they are subject to increased hemostatic challenges (menstruation and childbirth). In children with mild VWD, the pattern of hemorrhagic symptoms is different from children with more severe congenital bleeding disorders, with life-threatening bleeds such as intracranial hemorrhage (ICH) and umbilical cord bleeding being more rare among young VWD patients. In children with VWD, the most common clinical presentations are bruising and epistaxis; however, as these presentations are also frequent in normal healthy children, VWD diagnosis is challenging [57]. Standard clinical presentations in adult patients with VWD, including menorrhagia and post-surgical bleeding, are not evaluable in the pediatric population. Standard bleeding

assessment tools (BATs) and scoring systems may be useful for correct assessment of bleeding episodes among patients, because otherwise significant bleeding diathesis may be overlooked while minimal bleeding symptoms may be over emphasized [58].

In adults, hematoma, menorrhagia, and bleeding from minor wounds are the most frequent symptoms, depending on VWD type, disease severity and gender. Post-dental extraction and post-surgical bleeds are common and occur in about two-thirds of patients. Gastrointestinal (GI) bleeding is also reported in VWD, predominantly among adults, and this can sometimes be severe [59]. In some patients, especially those with severe VWD, epistaxis can be so severe as to require medical intervention with VWF concentrates or blood transfusion.

Postpartum hemorrhage (PPH) can also be observed among women with VWD, but with possibly lower frequency than "expected" because VWF levels increase markedly during pregnancy. Delayed PPH can occur due to gradual decrease of VWF level to baseline level post-delivery. Prolonged vaginal bleeding following normal vaginal delivery is a common presentation of women with VWD. Menorrhagia (>80 mL of blood loss per menstrual period) is a common and important bleeding symptom of women with VWD, and ~15% of women with menorrhagia have VWD. Therefore, menorrhagia is a sensitive but non-specific presentation of VWD in women, as sometimes accompanied by anemia and iron deficiency. Therefore, careful gynecological assessment of women with VWD is crucial [39, 58].

Bleeding symptoms tend to be milder in type 1 VWD and more severe in types 2 and type 3 VWD. In a recent international study, it was observed that the bleeding phenotype was significantly more severe in type 3 VWD than in type 1 VWD. The study found that epistaxis was the most common presentation in patients with type 3 VWD, followed by hemarthrosis in males and menorrhagia in females. Furthermore, the study estimated that the prevalence of CNS bleeding is more than 20 times higher in type 3 VWD than in type 1 VWD [60]. Since FVIII level is only slightly reduced in most types of VWD, spontaneous hemarthrosis or hematoma are rare in types 1, 2A, and 2B VWD, while in type 3 VWD, the severity of diathesis often resembles that of hemophilia. Severe life-threatening bleeding can occur in type 3 VWD and sometimes in patients with type 2 VWD, but is a rare presentation of type 1 VWD. ICH is a rare presentation of VWD that usually is only reported in type 3 VWD [39].

3.5 Diagnosis of von Willebrand Disease

VWD is diagnosed on clinical features, comprising personal and family history of bleeding or bruising, and confirmed by laboratory testing. As VWD is due to deficiencies or defects in the plasma protein VWF, a large adhesive protein with multiple activities, laboratory testing therefore needs to assess VWF level and activity using a panel of tests [8]. The more comprehensive this panel, the more likely the correct diagnosis or exclusion of VWD; the less comprehensive the assay panel, the more likely the misdiagnosis. The minimum recommended three-test panel

comprises Factor VIII coagulant (FVIII:C), VWF:Ag, and VWF GPIb binding activity (i.e., VWF:RCo, VWF:GPIbR, or VWF:GPIbM); in our laboratory, we use a four-test panel comprising these plus VWF:CB. Additional assays including RIPA, VWF multimers, and VWF:FVIIIB are performed selectively, and where (i) low VWF activity/Ag ratios are evident (i.e., VWF:RCo/Ag, VWF:GPIbR/Ag, VWF:GPIbM/Ag, or VWF:CB/Ag <0.7; here reflexing to RIPA and VWF multimers) or (ii) where FVIII:C/VWF:Ag is low (i.e., <0.7; here potentially reflexing to VWF:FVIIIB). A complete diagnosis of VWD requires the classification of all six types of the disorder. Proper diagnosis and classification of VWD are crucial and have significant clinical consequences, as they can change the treatment strategy. Some therapeutic options, such as desmopressin or DDAVP, are effective for the management of type 2 VWD but are ineffective or potentially harmful for other types of the disorder [56]. The most often used assay for measuring VWF GPIb binding activity is the VWF:RCo, historically measuring the agglutination by VWF of fixed human platelets in the presence of ristocetin on an aggregometer, and more recently on automated analyzers [61]. This assay is now increasingly replaced using alternate assays based on binding of VWF to recombinant GPIb on inert particles (i.e., VWF:GPIgR or VWF:GPIbM) [61–63]. Although all these are VWF:GPIbB assays, VWF: RCo differs from both VWF:GPIbR and VWF:GPIbM [8]. Nevertheless, these assays comprise a single group reflecting assays of GPIb binding (i.e., classical VWF:RCo, as well as methodologies now defined as "VWF:GPIbR" and "VWF:GPIbM"), would be expected to broadly derive similar test results for VWD patients, and are thus essentially recognized to be "interchangeable" in VWD diagnostics [8].

The anticipated test patterns in different types of VWD is summarized in Table 3.3. Type 1 VWD represents a partial quantitative deficiency of (functionally normal) VWF, so there is concordant decrease in VWF measured by any VWF assay (be it VWF:Ag, VWF:CB or GPIb binding), and the ratio of any one VWF assay to any other is close to unity (in practice, >0.7). Type 3 VWD represents a total loss of VWF, and all VWF test results will be close to 0 U/dL, albeit recognizing that lower limit VWF sensitivity issues means that some assays cannot detect to these low levels, and that such low levels of detected VWF precludes generation of assay ratios [6].

In contrast, type 2 VWD represents qualitative VWF defects/deficiencies, such that VWF activity is proportionally decreased below that of VWF:Ag; furthermore, the VWD type can be defined by the type of activity reduced. Type 2A VWD, defining a loss of HMW VWF multimers, patients express a relative reduction of all VWF activities sensitive to this loss (this includes both GPIb binding and VWF:CB assays). In practice, this is expressed as the ratio of VWF activity/VWF:Ag assays being lower than ~0.7. Type 2B VWD defines an increased affinity of VWF for GPIb, which often leads to loss of HMW VWF multimers and similar VWF test patterns to type 2A VWD. Type 2M VWD defines decreased VWF-dependent platelet adhesion without a selective deficiency of HMW VWF multimers. In type 2M VWD, there are specific changes in VWF function related to specific *VWF* variants. In practice, most type 2M variants affect GPIb binding, and less so collagen

Table 3.3 Classification scheme for von Willebrand disease including basic assay test patterns

VWD type	Description	Phenotypic diagnosis	Assay patterns
Low VWF	Decreased levels of VWF, but they do not fall under the category of type 1 VWD, nor is there any identifiable VWF variant	Low levels of VWF, with VWF functional concordance (i.e., ratio of functional VWF/VWF:Ag approximates unity), but the levels is higher than type 1 VWD	VWF:Ag (U/dL): 30–50 VWF:RCo (U/dL): 30–50 VWF:GPIbR (U/dL): 30–50 VWF:GPIbM (U/dL): 30–50 VWF:Ab: 30–50 RIPA: ristocetin concentration, mg/mL ≥0.7 VWF:CB (U/dL): 30–50 FVIII:C (U/dL): 30–60 VWF:GPIbB/VWF:Ag (ratio) >0.7 VWF:CB/VWF:Ag (ratio) >0.7 FVIII:C/VWF:Ag (ratio) >0.7
1	Partial quantitative deficiency of VWF	Low levels of VWF, with VWF functional concordance (i.e., ratio of functional VWF/VWF:Ag approximates unity)	VWF:Ag (U/dL) <30 VWF:RCo (U/dL) <30 VWF:GPIbR (U/dL) <30 VWF:GPIbM (U/dL) <30 VWF:Ab (U/dL) <30 RIPA: ristocetin concentration, mg/mL ≥1.0 VWF:CB (U/dL) <30 FVIII:C (U/dL) <60 VWF:GPIbB/VWF:Ag (ratio) >0.7 VWF:CB/VWF:Ag (ratio) >0.7 FVIII:C/VWF:Ag (ratio) >0.7

2A	Decreased VWF-dependent platelet adhesion and a selective deficiency of high-molecular-weight (HMW) VWF multimers	Loss of HMW VWF. Usually low levels of VWF, with VWF functional discordance	VWF:Ag (U/dL) <50 VWF:RCo (U/dL) <30 VWF:GPIbR (U/dL) <30 VWF:GPIbM (U/dL) <30 VWF:Ab (U/dL) <30 RIPA: ristocetin concentration, mg/mL ≥1.5 VWF:CB (U/dL) <30 FVIII:C (U/dL) <60 VWF:GPIbB/VWF:Ag (ratio) <0.6 VWF:CB/VWF:Ag (ratio) <0.6 FVIII:C/VWF:Ag (ratio) >0.7
2B	Increased affinity of VWF for platelet glycoprotein Ib	Low to normal levels of VWF, typically with VWF functional discordance. Atypical cases may not show this pattern	VWF:Ag (U/dL) <70 VWF:RCo (U/dL) <50 VWF:GPIbR (U/dL) <50 VWF:GPIbM (U/dL) <50 VWF:Ab (U/dL) <50 RIPA: ristocetin concentration, mg/mL <0.7 VWF:CB (U/dL) <50 FVIII:C (U/dL) <80 VWF:GPIbB/VWF:Ag (ratio) <0.6 VWF:CB/VWF:Ag (ratio) <0.6 FVIII:C/VWF:Ag (ratio) >0.7

(continued)

Table 3.3 (continued)

VWD type	Description	Phenotypic diagnosis	Assay patterns
2M[a]	Decreased VWF-dependent platelet adhesion without a selective deficiency of high-molecular-weight (HMW) VWF multimers	Low to normal levels of VWF, usually with VWF functional discordance detected by VWF:GPIb binding/Ag, but relatively normal VWF:CB/Ag. HMW VWF present, but multimers may show other abnormalities	VWF:Ag (U/dL) <50 VWF:RCo (U/dL) <30 VWF:GPIbR (U/dL) <30 VWF:GPIbM (U/dL) <30 VWF:Ab (U/dL) <30 RIPA: ristocetin concentration, mg/mL ≥1.5 VWF:CB (U/dL) <30 FVIII:C (U/dL) <60 VWF:GPIbB/VWF:Ag (ratio) <0.6 VWF:CB/VWF:Ag (ratio) <0.6 FVIII:C/VWF:Ag (ratio) >0.7
2N	Markedly decreased binding affinity for factor VIII	Need to distinguish from hemophilia A. Defined by VWF:FVIIIB assay, with low VWF:FVIIIB/Ag ratios	VWF:Ag (U/dL) <70 VWF:RCo (U/dL) <70 VWF:GPIbR (U/dL) <70 VWF:GPIbM (U/dL) <70 VWF:Ab (U/dL) <70 RIPA: ristocetin concentration, mg/mL ≥0.7 VWF:CB (U/dL) <70 FVIII:C (U/dL) <50 VWF:GPIbB/VWF:Ag (ratio) >0.7 VWF:CB/VWF:Ag (ratio) >0.7 FVIII:C/VWF:Ag (ratio) <0.7

3	Virtually complete deficiency of VWF	Typically defined by VWF levels <2 U/dL[b]	VWF:Ag (U/dL) <2 VWF:RCo (U/dL) <2 VWF:GPIbR (U/dL) <2 VWF:GPIbM (U/dL) <2 VWF:Ab (U/dL) <2 RIPA: ristocetin concentration, mg/mL: No aggregation VWF:CB (U/dL) <2 FVIII:C (U/dL) <10 VWF:GPIbB/VWF:Ag (ratio): NA VWF:CB/VWF:Ag (ratio): NA FVIII:C/VWF:Ag (ratio): NA

VWF von Willebrand facto, *FVIII* factor VIII, *RIPA* Ristocetin-induced platelet aggregation, *VWF:Ag* von Willebrand facto: antigen, *VWF:Ab* monoclonal antibody-based VWF binding assay, *FVIII:C* factor VIII coagulant assay, *VWF:FVIIIB* VWF: Factor VIII binding assay, *VWF:GPIbM* VWF GPIb binding assay using (recombinant) mutated GPIb, *VWF:RCo* VWF ristocetin cofactor assay. *VWF: GPIbR* VWF GPIb binding assay using recombinant GPIb

[a]2M VWD patterns may vary depending on the VWF variant (i.e., whether it affects glycoprotein Ib binding, collagen binding, or both)

[b]Some assays cannot detect levels of VWF <10 U/dL, challenging the diagnosis of type 3 VWD. *NA* not applicable

binding; thus, there is usually a low VWF:GPIb binding activity/VWF:Ag ratio, but the VWF:CB/Ag ratio may be normal. Type 2N VWD defines a decreased binding affinity for FVIII, as identified by the specific test VWF:FVIIIB. Phenotypically, however, these patients present similarly to those with hemophilia A, showing relatively lower FVIII:C to VWF:Ag ratios [8].

Including FVIII:C as part of a VWD diagnostic profile has many benefits. Since VWF protects FVIII, lower levels of VWF (i.e., VWD) will also generally mean lower levels of FVIII:C, and so represent additional bleeding risk. In type 3 VWD, for example, levels of FVIII:C are generally <10 U/dL. In type 1 VWD, the FVIII:C is generally proportional to VWF:Ag, and in type 2N VWD, FVIII:C is proportionally lower than VWF:Ag [8].

In summary, the recommended approach to diagnosis or exclusion of VWD can be aided by the use of algorithms, such as shown in Fig. 3.1 using a four-test panel comprising VWF:Ag, a VWF:GPIb binding assay (VWF:RCo, VWF:GPIbR, or VWF:GPIbM), VWF:CB, and FVIII:C. A four-test panel recommendation is made ahead of the more basic three-test panel based on decades of experience, and the high rate of diagnostic errors associated to smaller first line testing panels [6, 55–68]. Which tests within each category are employed by laboratories will depend on local availabilities (i.e., instrumentation and tests). VWF:Ag is mostly performed by latex immunoassay (LIA) procedure and less often by ELISA; however, chemiluminescence immuno-assay methodology is emerging and least problematic (CLIA). VWF:GPIb binding assays may be performed by platelet agglutination (VWF:RCo), latex agglutination (VWF:GPIbR, VWF:GPIbM), CLIA (VWF:GPIbR), or potentially by ELISA (VWF:GPIbM). VWF:CB testing is usually performed by ELISA, and increasingly by CLIA [64, 69]. Also, problems with reproducibility and low level VWF detection with classical VWF:RCo assays may drive usage of more modern VWF:GPIb binding assay alternatives such as VWF:GPIbR or VWF:GPIbM.

Once the methodology has been selected and validated, the recommended basic VWD four-test panel should be able to diagnose or exclude VWD, with additional investigations selected on a case by case basis, dependent on the results from the four-test panel (Fig. 3.1).

The basic caveat always remains that diagnosis or exclusion of VWD requires at the very least repeat testing using a fresh sample, due to preanalytical and also analytical limitations.

If all VWF tests are normal, as confirmed on repeat testing, then the patient either does not have VWD or has a form of VWD that is not able to be defined with current testing. Additional assays to define the bleeding disorder, inclusive of platelet function or additional factor assays, may be required. If all VWF tests are low, confirmed on repeat testing, but all VWF values are concordant (ratios of VWF:GPIb binding/Ag and VWF:CB/Ag both >0.7), then the patient has type 1 VWD if the level of VWF is <30 U/dL (or 30–50 U/dL with appropriate history as per the latest VWD diagnostic guidelines) [5]. In such cases, assessment of severity may be based on absolute VWF level; however, unlike the case for hemophilia [70], there is no available consensus for cut-off values defining severity of VWD.

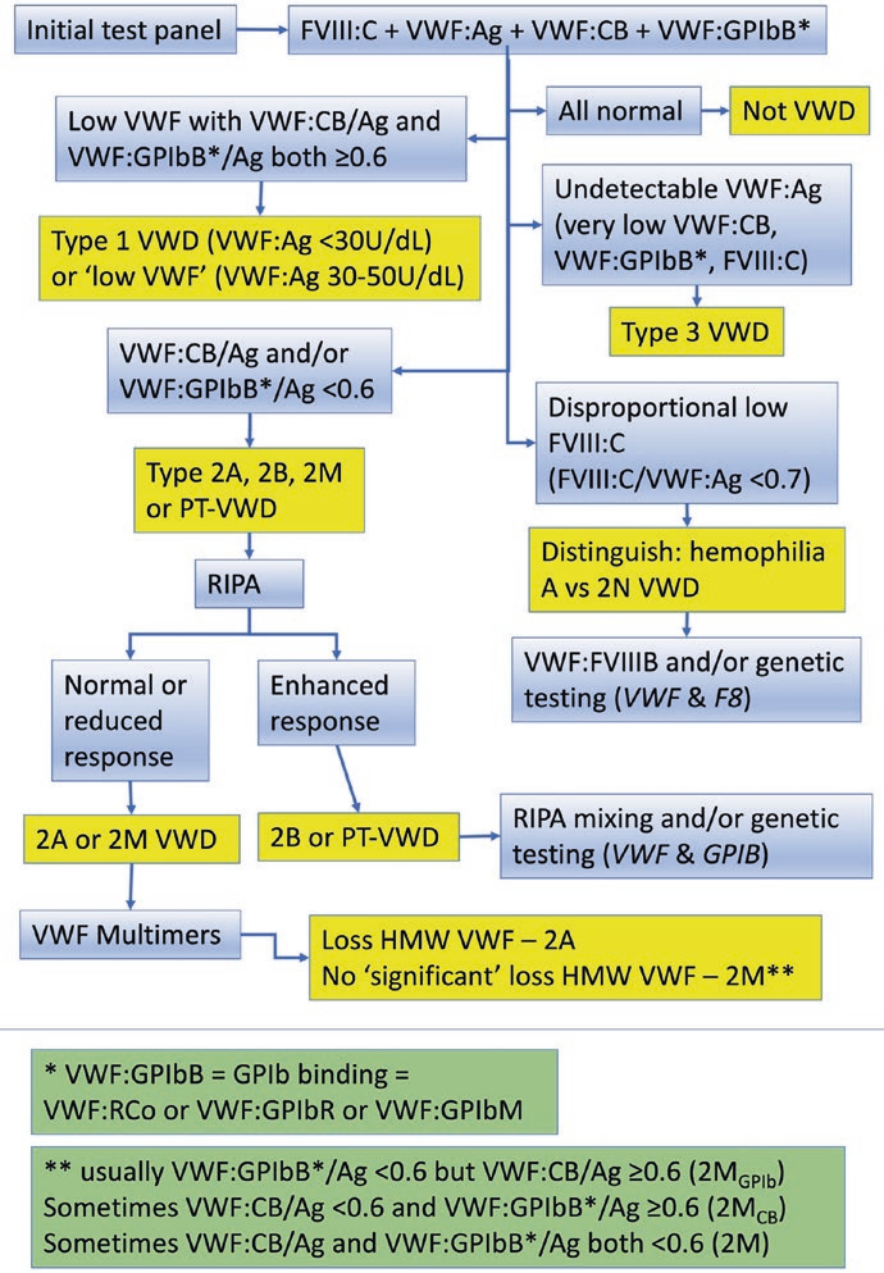

Fig. 3.1 An algorithm for the diagnosis or exclusion of VWD using an initial four-test panel, as used at the Westmead laboratory

If there is a low ratio of VWF:GPIb binding/Ag and/or VWF:CB/Ag, and this pattern is confirmed on repeat testing with a fresh sample, then the patient may have type 2A, 2B, or 2M VWD (or possibly platelet type [PT] VWD). Here, further tests should be undertaken (i.e., RIPA, VWF multimer assessment, depending on test findings and local test availability). In our experience, RIPA analysis is usually more important than multimer analysis; moreover, selection of the right test methodologies for VWF:Ag, VWF:CB and VWF:GPIb binding assays will often enable prediction of the VWF multimer pattern negating any need for its performance. Thus, a low ratio of VWF:GPIb binding/Ag plus a low ratio of VWF:CB/Ag usually points to a loss of HMW VWF, and thus likely 2A, 2B, or platelet type VWD. Instead, a low ratio of VWF:GPIb binding/Ag ***or*** VWF:CB/Ag (but not both), usually discounts a loss of HMW VWF and instead points to a type 2M VWD [8].

If all VWF test results are below the measuring range of the assays used, then this will create problems with clear diagnosis, but usually infers severe type 1 VWD or else type 3 VWD; therapy is similar in both cases, although clinical severity is often worse in type 3 VWD.

Finally, if the ratio of FVIII:C/VWF:Ag is low, this suggests either hemophilia A or 2N VWD. Hemophilia A is more common, and being sex-linked affects males more than females; however, misdiagnoses of both hemophilia A and 2N VWD, where the correct diagnosis was the other, do occur. Thus, testing by performance of a VWF:FVIIIB assay is recommended, and after repeat testing for confirmation, could include genetic analysis of *F8* and/or *VWF* for final definitive verification.

Genetic analysis may also be useful where patients have been defined to be type 2A, 2B, 2M, or platelet type VWD, and is also typically successful when performed on such patients. Genetic analysis is useful in some type 3 VWD investigations, but generally not useful in type 1 VWD [71].

3.6 Molecular Basis of von Willebrand Disease

VWF is encoded by the *VWF* gene, which is located on the short arm of chromosome 12 (12p13.3), spans 180 kb, and consists of 52 exons, of which, exon 50 (40 kb) and exon 28 (1/3 kb) are considered the longest and smallest exons, respectively. The VWF pseudogene 1 (VWFP1), which is located on the long arm of chromosome 22 (22q11–13), spans ~21–29 kb and shows 97% homology with 23–34 exons of the *VWF* gene but encodes no functional transcript. The *VWF* gene is transcribed to an 8.8 kb mRNA, which translates to a 2813-amino acid pre-pro-VWF protein. Pre-pro-VWF comprises a signal peptide (pre) with 22 amino acids, a pro-peptide (pro) with 741 amino acids, and a 2050-amino acid mature protein. Any variant that leads to qualitative and/or quantitative abnormalities in VWF can be associated with VWD. Variants may affect different biosynthetic events, including gene expression (transcription, translation), post-translational processing, dimerization/multimerization mechanisms, proteolytic processing, storage, secretion processing, structure, clearance, and function of VWF. Different variant types are associated with VWD including: (1) those that involve transcription factor binding sites that lead to absent or reduced RNA transcription; (2) splice site mutations

that disrupt the splice donor site and splice acceptor site of each intron, leading to exon skipping and production of shortened RNA and protein; (3) nonsense variants; (4) small deletions; (5) insertions; (6) duplications; (7) large deletions; and (8) missense variants. According to the ISTH-SSC VWF Online database (https://dbs.eahad.org/upcoming-vwf-db/) and many other studies, almost 600 separate variants have currently been reported in patients with VWD, about 80% of them are missense [37]. Patients with acquired von Willebrand syndrome (AVWS) VWD do not have hereditary *VWF* variants [72].

3.7 Treatment of von Willebrand Disease

Management of patients with VWD includes prevention or treatment of bleeding by correction of the hemostatic defects, which may be those of primary hemostasis (due to lack or decrease of VWF) or secondary hemostasis (due to FVIII deficiency). These can be achieved by increasing endogenous VWF (using desmopressin) or in unresponsive/contraindicated patients or long-term need, by infusion of exogenous VWF/FVIII (typically as plasma concentrates, although recombinant FVIII and VWF are available in some locations) [9, 73, 74]. In some developing countries, cryoprecipitate and fresh frozen plasma (FFP) might still be used; however, the risk of virus transmission and the need to transfuse a high volume of product reflect substantive obstacles for their use. Additional adjuvant therapies can also be employed for some situations, including antifibrinolytic therapy with tranexamic acid or epsilon aminocaproic acid, which can improve hemostasis in patients without altering their plasma VWF levels [9, 74].

On-demand therapy (meaning treatment of hemorrhage as soon as possible after onset of bleeding) remains the mainstay of treatment of patients with VWD. However long-term prophylaxis may be necessary for those with severe hemorrhages (e.g., type 3 VWD) to improve their quality of life by reducing annualized bleeding rates [9] and will be increasingly employed [75].

3.7.1 Desmopressin

Desmopressin (1-deamino-8-D-arginine vasopressin) (DDAVP) is synthetic analogue of vasopressin that causes an increase of endogenous plasma FVIII and VWF by facilitating their release from endothelial storage sites. Depending on location, desmopressin may be given intravenously, intranasally, or subcutaneously. Desmopressin is most likely to be useful in patients with baseline FVIII and functional VWF levels of 10–20 U/dL or more. Thus, desmopressin is most useful in management of patients with mild hemophilia or mild VWD (especially type 1 VWD). If required, patients can receive repeated doses of drug in 12 to 24 h intervals; however, repeated doses of desmopressin cause less effective responses, and eventual depletion of FVIII and VWF stores (called tachyphylaxis) [9, 73, 74]. A test dose of desmopressin is recommended before establishment of the magnitude and duration of the drug response in any given patient, since the genotype and phenotype of VWD can affect its effectiveness [76].

Although desmopressin is effective in some patients with type 2 VWD, mostly 2A and 2M, it is ineffective in the majority of these patients, and in patients with type 2B, it is considered contradicted because it may worsen thrombocytopenia and potentially increase the risk of bleeding. Type 3 VWD are unresponsive to desmopressin, due to lack of releasable stores of VWF in these patients [9, 76].

The use of desmopressin can be associated with certain side effects, including hypotension, cardiovascular complications, flushing, and hyponatremia. Hyponatremia can be prevented by limited fluid intake for 24 h after desmopressin administration, while other side effects are mostly due to vasodilating effects that can often be attenuated with intravenous infusion by slowing the rate [74, 76].

For intravenous purpose, 0.3 μg/kg in 50 mL is administrated over 30 min. FVIII:C, VWF:Ag and functional VWF (e.g., VWF:RCo) should be assessed pre-infusion and 1, 2, and/or 4 and 24 h post-infusion. Patients with a sufficient response to desmopressin have a two to five times increase from baseline levels and have FVIII and VWF levels above 50 U/dL at 1 h post-infusion. The levels remain above 30 U/dL at 4 h post-infusion but generally return to baseline levels by 24 h [4, 76, 77].

3.7.2 von Willebrand Factor Concentrates

Transfusion therapy using products containing virus inactivated concentrates of human VWF/FVIII represents the main therapeutic choice in patients unresponsive to desmopressin, or for long-term therapy use. These concentrates can be used for on-demand therapy (to stop bleeding when they occur), can prevent bleeding in surgery, or can be used for long-term secondary prophylaxis [78].

This treatment type reflects the current treatment of choice in patients with type 3 VWD, most patients with type 2 VWD, and a number of patients with type 1 VWD, (Table 3.4) [4, 10].

A number of different VWF/FVIII concentrates are available for use. The quantity of HMWM VWF and FVIII differs between products [1, 77]. It is important to avoid excessive concentrations of FVIII:C with injection of repeated doses in patients with VWD [4, 77]. Thus, the required dose of concentrate to use in patients may differ according to the product, and will also differ according to the situation (e.g., type and severity of bleeding episodes, type of planned surgery.

3.7.3 Recombinant von Willebrand Factor

Recombinant VWF (Vonicog alfa, Vonvendi (USA), Veyvondi (Europe)) is a relatively new product for management of VWD that is only available in some countries. Since the product is not exposed to ADAMTS13, it retains all sizes of VWF multimers including HMWM and ultra-large multimers [83]. Recombinant VWF has been approved by the US Food and Drug Administration (FDA) for on-demand therapy, surgical procedures, and prophylaxis [84]. It has also been approved by the European Medicines Agency (EMA), and multiple studies have demonstrated its safety and efficacy in managing VWD [85–87].

Table 3.4 Therapeutic recommendation for management of patients with von Willebrand disease

Indication	Dose in IU FVIII/kg	Frequency of infusions	Treatment goal	Reference
Mild mucocutaneous bleeding	20	Usually single dose	–	[79]
Spontaneous/ traumatic bleeding	20–40	Usually single dose	–	[79]
Dental extraction	20–40	Single dose plus tranexamic acid	FVIII:C and VWF:RCo > 50 IU/dL	[79]
Delivery	40–50 (RCo (IU/kg))	Daily before delivery and in the postpartum period	50–100 U/dL; maintain levels for 5–10 d	[80]
Indication	**Loading dose**	**Maintenance dose**	**Treatment goal**	
Major surgery/ bleeding[a]	40–60 U/kg	30–60 U/kg	Trough VWF: RCo and FVIII >50 IU/dL for 7–14 d	[81]
Minor surgery/ bleeding[a]	30–60 U/kg	20–40 U/kg every 12–48 h	Trough VWF: RCo and FVIII >50 IU/dL for 3–5 d	[81]
Indication	**Dose of VWF:RCo (IU/kg)**	**Number of infusions**	**Treatment goal (VWF:RCo level)**	
Type 1 VWD major surgery/ bleeding[b]	Loading dose 40, then 40–50	Every 8–12 h for 3 d then daily for up to 7 d	> 50 U/dL; maintain levels for 7–10 d	[82]
Type 1 VWD minor surgery/ bleeding[b]	40–50	1 or 2 doses	> 30 U/dL; maintain levels for 2–4 d	[82]
Type 2 or 3 VWD major surgery/ bleeding[b]	Loading dose 50–60 then 40–60	Every 8–12 h for 3 d then daily for up to 7 d	> 50 U/dL; maintain levels for 7–10 d	[82]
Type 2 or 3 VWD minor surgery/ bleeding[b]	40–50	1 or 2 doses	> 30 U/dL; maintain levels for 2–4 d	[82]

VWF:RCo von Willebrand factor ristocetin cofactor activity, *VWD* von Willebrand disease, *VWF* von Willebrand factor, *FVIII* Factor VIII

[a]NHLBI expert panel

[b]Australian and New Zealand studies

3.7.4 Prophylaxis

Prophylaxis should be considered for patients with type 3 VWD and recurrent hemarthrosis. Patients with recurrent GI bleeding and those with frequent epistaxis can also benefit from prophylaxis. Regular prophylaxis will decrease the number of bleeds and the severity of hemorrhages, prevent arthropathy, and improve quality of life in patients with VWD [77].

3.7.5 Surgery

Surgical procedures represent an important hemostatic challenge in patients with VWD; the majority of patients having major surgery will be managed by replacement VWF/FVIII therapy. Strategies for pre-operative management depends on type of VWD, type of surgery, baseline levels of VWF and FVIII, and therapeutic response to desmopressin [77].

3.7.6 Pregnancy and Delivery

VWF and FVIII levels increase two to three-fold during the second and third trimesters in patients with types 1 and 2 VWD and fall to baseline by 7–21 days postpartum; however, this increase is not observed in type 3 VWD. Also, in type 2 VWD, the increase in VWF level may not be associated with any substantive increase in functional VWF level, or in type 2B VWD, may lead to an increase in "abnormal VWF" that may worsen thrombocytopenia..Thus, management of pregnant women with VWF/FVIII may be required in types 2 and 3 VWD. A rapid decrease in plasma VWF and FVIII can also occur post-partum, and subsequently PPH, including delayed PPH, can also occur [88].

Generally, pregnant patients with VWD should be monitored for functional VWF (e.g., VWF:RCo, VWF:GPIbR, VWF:GPIbM, and VWF:CB) and FVIII:C once during the third trimester and within 10 days of expected date of delivery [89].

Acknowledgments The author acknowledges the contribution of Akbar Dorgalaleh, Yavar Shiravand, and Shadi Tabibian, who provided substantial input into the original chapter, upon which this revision is based. As the revision is built upon the shoulders of others, its incremental value would have been severely diminished without their original contribution.

References

1. Dorgalaleh A, Tabibian S, Shiravand Y, Favaloro EJ. von Willebrand disease. In: Dorgalaleh A, editor. Congenital bleeding disorders: diagnosis and management. Cham, Switzerland: Springer Press; 2018. p. 57–102.
2. James PD, Goodeve AC. von Willebrand disease. Genet Med. 2011;13(5):365–76.
3. Sadler J, Budde U, Eikenboom J, Favaloro E, Hill F, Holmberg L, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. J Thromb Haemost. 2006;4(10):2103–14.
4. Laffan M, Brown S, Collins PW, Cumming A, Hill F, Keeling D, et al. The diagnosis of von Willebrand disease: a guideline from the UK Haemophilia Centre Doctors' Organization. Haemophilia. 2004;10(3):199–217.
5. James PD, Connell NT, Ameer B, Di Paola J, Eikenboom J, Giraud N, Haberichter S, Jacobs-Pratt V, Konkle B, McLintock C, McRae S. ASH ISTH NHF WFH 2021 guidelines on the diagnosis of von Willebrand disease. Blood Adv. 2021;5(1):280–300.
6. Favaloro EJ, Pasalic L. Laboratory diagnosis of von Willebrand Disease (VWD): Geographical Perspectives. Semin Thromb Hemost. 2022;48(6):750–66.

7. Federici AB. Diagnosis of inherited von Willebrand disease: a clinical perspective. Semin Thromb Hemost. 2006;32(6):555–65.
8. Favaloro EJ, Pasalic L, Curnow J. Laboratory tests used to help diagnose von Willebrand disease: an update. Pathology. 2016;48(4):303–18.
9. Sadler J, Mannucci P, Berntorp E, Bochkov N, Boulyjenkov V, Ginsburg D, et al. Impact, diagnosis and treatment of von Willebrand disease. Thromb Haemost. 2000;84(2):160–74.
10. Mannucci PM, Chediak J, Hanna W, Byrnes J, Ledford M, Ewenstein BM, et al. Treatment of von Willebrand disease with a high-purity factor VIII/von Willebrand factor concentrate: a prospective, multicenter study. Blood. 2002;99(2):450–6.
11. Lenting PJ, Christophe OD, Denis CV. von Willebrand factor biosynthesis, secretion, and clearance: connecting the far ends. Blood. 2015;125(13):2019–28.
12. Ginsburg D, Handin RI, Bonthron DT, Donlon TA, Bruns GA, Latt SA, et al. Human von Willebrand factor (vWF): isolation of complementary DNA (cDNA) clones and chromosomal localization. Science. 1985;228:1401–7.
13. Mancuso D, Tuley E, Westfield L, Worrall N, Shelton-Inloes B, Sorace J, et al. Structure of the gene for human von Willebrand factor. J Biol Chem. 1989;264(33):19514–27.
14. Federici AB, Lee CA, Berntorp EE, Lillicrap D, Montgomery RR. Von Willebrand disease: basic and clinical aspects. John Wiley & Sons; 2011.
15. Shapiro S, Nowak A, Wooding C, Birdsey G, Laffan M, McKinnon T. The von Willebrand factor predicted unpaired cysteines are essential for secretion. J Thromb Haemost. 2014;12(2):246–54.
16. Sadler JE. Von Willebrand factor, ADAMTS13, and thrombotic thrombocytopenic purpura. Blood. 2008;112(1):11–8.
17. Zanardelli S, Crawley JT, Chion CKCK, Lam JK, Preston RJ, Lane DA. ADAMTS13 substrate recognition of von Willebrand factor A2 domain. J Biol Chem. 2006;281(3):1555–63.
18. Zanardelli S, Chion AC, Groot E, Lenting PJ, McKinnon TA, Laffan MA, et al. A novel binding site for ADAMTS13 constitutively exposed on the surface of globular VWF. Blood. 2009;114(13):2819–28.
19. Samor B, Michalski JC, Debray H, Mazurier C, Goudemand M, Halbeek H, et al. Primary structure of a new tetraantennary glycan of the N-acetyllactosaminic type isolated from human factor VIII/von Willebrand factor. FEBS J. 1986;158(2):295–8.
20. Canis K, McKinnon TA, Nowak A, Haslam SM, Panico M, Morris HR, et al. Mapping the N-glycome of human von Willebrand factor. Biochem J. 2012;447(2):217–28.
21. Haberichter SL, Merricks EP, Fahs SA, Christopherson PA, Nichols TC, Montgomery RR. Re-establishment of VWF-dependent Weibel-Palade bodies in VWD endothelial cells. Blood. 2005;105(1):145–52.
22. Sporn LA, Marder VJ, Wagner DD. Inducible secretion of large, biologically potent von Willebrand factor multimers. Cell. 1986;46(2):185–90.
23. George JN. Thrombotic Thrombocytopenic Purpura: From 1972 to 2022 and Beyond. Semin Thromb Hemost. 2022;48(8):926–36.
24. Ruggeri ZM, Mendolicchio G. Interaction of von Willebrand factor with platelets and the vessel wall. Hamostaseologie. 2015;35(3):211–24.
25. Roth GJ, Titani K, Hoyer LW, Hickey MJ. Localization of binding sites within human von Willebrand factor for monomeric type III collagen. Biochemistry. 1986;25(26):8357–61.
26. Cruz MA, Yuan H, Lee JR, Wise RJ, Handin RI. Interaction of the von Willebrand factor (vWF) with collagen Localization of the primary collagen-binding site by analysis of recombinant vWF a domain polypeptides. J Biol Chem. 1995;270(18):10822–7.
27. Pareti FI, Niiya K, McPherson J, Ruggeri Z. Isolation and characterization of two domains of human von Willebrand factor that interact with fibrillar collagen types I and III. J Biol Chem. 1987;262(28):13835–41.
28. Hoylaerts MF, Yamamoto H, Nuyts K, Vreys I, Deckmyn H, Vermylen J. von Willebrand factor binds to native collagen VI primarily via its A1 domain. Biochem J. 1997;324(1):185–91.

29. Fujimura Y, Titani K, Holland L, Russell S, Roberts J, Elder J, et al. von Willebrand factor. A reduced and alkylated 52/48-kDa fragment beginning at amino acid residue 449 contains the domain interacting with platelet glycoprotein Ib. J Biol Chem. 1986;261(1):381–5.
30. Scott J, Montgomery R, Retzinger G. Dimeric ristocetin flocculates proteins, binds to platelets, and mediates von Willebrand factor-dependent agglutination of platelets. J Biol Chem. 1991;266(13):8149–55.
31. Sadler JE. Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem. 1998;67(1):395–424.
32. Wohner N, Legendre P, Casari C, Christophe O, Lenting P, Denis C. Shear stress-independent binding of von Willebrand factor-type 2B mutants p. R1306Q & p. V1316M to LRP1 explains their increased clearance. J Thromb Haemost. 2015;13(5):815–20.
33. Terraube V, O'donnell JS, Jenkins PV. Factor VIII and von Willebrand factor interaction: biological, clinical and therapeutic importance. Haemophilia. 2010;16(1):3–13.
34. Plow EF, Pierschbacher MD, Ruoslahti E, Marguerie GA, Ginsberg MH. The effect of Arg-Gly-Asp-containing peptides on fibrinogen and von Willebrand factor binding to platelets. Proc Natl Acad Sci. 1985;82(23):8057–61.
35. Fressinaud E, Baruch D, Girma J-P, Sakariassen KS, Baumgartner HR, Meyer D. von Willebrand factor-mediated platelet adhesion to collagen involves platelet membrane glycoprotein IIb-IIIa as well as glycoprotein Ib. Transl Res. 1988;112(1):58–67.
36. Aoki T, Tomiyama Y, Honda S, Mihara K, Yamanaka T, Okubo M, et al. Association of the antagonism of von Willebrand factor but not fibrinogen by platelet αIIbβ3 antagonists with prolongation of bleeding time. J Thromb Haemost. 2005;3(10):2307–14.
37. Zolkova J, Sokol J, Simurda T, Vadelova L, Snahnicanova Z, Loderer D, Dobrotova M, Ivankova J, Skornova I, Lasabova Z, Kubisz P. Genetic Background of von Willebrand Disease: History, Current State, and Future Perspectives. Semin Thromb Hemost. 2020;46(4):484–500
38. Ginsburg D. von Willebrand disease. Williams hematology. 6th ed. Philadelphia: McGraw-Hill; 2001. p. 1813–28.
39. Eikenboom JC. Congenital von Willebrand disease type 3: clinical manifestations, pathophysiology and molecular biology. Best Pract Res Clin Haematol. 2001;14(2):365–79.
40. Favaloro EJ. Von Willebrand disease: local diagnosis and management of a globally distributed bleeding disorder. Semin Thromb Hemost. 2011;37(5):440–55.
41. Goodeve A, Eikenboom J, Castaman G, Rodeghiero F, Federici AB, Batlle J, et al. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD). Blood. 2007;109(1):112–21.
42. Colonne CK, Reardon B, Curnow J, Favaloro EJ. Why is misdiagnosis of von Willebrand disease still prevalent and how can we overcome it? A focus on clinical considerations and recommendations. J Blood Med. 2021;12:755–68.
43. Nichols WL, Rick ME, Ortel TL, Montgomery RR, Sadler JE, Yawn BP, James AH, Hultin MB, Manco-Johnson MJ, Weinstein M. Clinical and laboratory diagnosis of von Willebrand disease: a synopsis of the 2008 NHLBI/NIH guidelines. Am J Hematol. 2009;84(6):366–70.
44. Sadler JE, Rodeghiero F. Provisional criteria for the diagnosis of VWD type 1: on behalf of the ISTH SSC Subcommittee on von Willebrand factor. J Thromb Haemost. 2005;3(4):775–7.
45. Peake I, Goodeve A. Type 1 von Willebrand disease. J Thromb Haemost. 2007;5(s1):7–11.
46. Quiroga T, Goycoolea M, Belmont S, Panes O, Aranda E, Zuniga P, et al. Quantitative impact of using different criteria for the laboratory diagnosis of type 1 von Willebrand disease. J Thromb Haemost. 2014;12(8):1238–43.
47. Sadler JE. Von Willebrand disease type 1: a diagnosis in search of a disease. Blood. 2003;101(6):2089–93.
48. Mazurier C, Goudemand J, Hilbert L, Caron C, Fressinaud E, Meyer D. Type 2N von Willebrand disease: clinical manifestations, pathophysiology, laboratory diagnosis and molecular biology. Best Pract Res Clin Haematol. 2001;14(2):337–47.

49. Sutherland JJ, O'Brien LA, Lillicrap D, Weaver DF. Molecular modeling of the von Willebrand factor A2 domain and the effects of associated type 2A von Willebrand disease mutations. J Mol Model. 2004;10(4):259–70.
50. Woods AI, Kempfer AC, Paiva J, Sanchez-Luceros A, Bermejo E, Chuit R, et al. Phenotypic Parameters in Genotypically Selected Type 2B von Willebrand Disease Patients: A Large, Single-Center Experience Including a New Novel Mutation. Semin Thromb Hemost. 2017;43(1):92–100.
51. Leebeek FW, Eikenboom JC. Von Willebrand's disease. N Engl J Med. 2016;375(21):2067–80.
52. Castaman G, Federici A, Tosetto A, La Marca S, Stufano F, Mannucci P, et al. Different bleeding risk in type 2A and 2M von Willebrand disease: a 2-year prospective study in 107 patients. J Thromb Haemost. 2012;10(4):632–8.
53. Favaloro EJ, Mohammed S, Koutts J. Identification and prevalence of von Willebrand disease type 2N (Normandy) in Australia. Blood Coagul Fibrinolysis. 2009;20(8):706–14.
54. Perez Botero J, Pruthi RK, Nichols WL, Ashrani AA, Patnaik MM. von Willebrand disease type1/type 2N compound heterozygotes: diagnostic and management challenges. Br J Haematol. 2017;176(6):994–7.
55. Gadisseur A, Berneman Z, Schroyens W, Michiels JJ. Pseudohemophilia of Erik von Willebrand caused by homozygous one nucleotide deletion in exon 18 of the VW-factor gene. World J Hematol. 2013;2(4):99–108.
56. Federici A, Castaman G, Mannucci P. Guidelines for the diagnosis and management of von Willebrand disease in Italy. Haemophilia. 2002;8(5):607–21.
57. Federici AB, Bucciarelli P, Castaman G, Mazzucconi MG, Morfini M, Rocino A, et al. The bleeding score predicts clinical outcomes and replacement therapy in adults with von Willebrand disease. Blood. 2014;123(26):4037–44.
58. Rodeghiero F, Tosetto A, Abshire T, Arnold D, Coller B, James P, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. J Thromb Haemost. 2010;8(9):2063–5.
59. Makris M. Gastrointestinal bleeding in von Willebrand disease. Thromb Res. 2006;118:S13–S7.
60. Tosetto A, Badiee Z, Baghaipour MR, Baronciani L, Battle J, Berntorp E, Bodó I, Budde U, Castaman G, Eikenboom JC, Eshghi P. Bleeding symptoms in patients diagnosed as type 3 von Willebrand disease: results from 3WINTERS-IPS, an international and collaborative cross-sectional study. J Thromb Haemost. 2020;18(9):2145–54.
61. Favaloro EJ. Diagnosing von Willebrand disease: a short history of laboratory milestones and innovations, plus current status, challenges, and solutions. Semin Thromb Hemost. 2014;40(5):551–70.
62. Just S. Laboratory Testing for von Willebrand Disease: The Past, Present, and Future State of Play for von Willebrand Factor Assays that Measure Platelet Binding Activity, with or without Ristocetin. Semin Thromb Hemost. 2017 Feb;43(1):75–91.
63. Bodo I, Eikenboom J, Montgomery R, Patzke J, Schneppenheim R, Di Paola J, von Willebrand P-d, factor activity. Nomenclature and methodology: communication from the SSC of the ISTH. J Thromb Haemost. 2015;13(7):1345–50.
64. Favaloro EJ, Bonar RA, Meiring M, Duncan E, Mohammed S, Sioufi J, et al. Evaluating errors in the laboratory identification of von Willebrand disease in the real world. Thromb Res. 2014;134(2):393–403.
65. Favaloro E, Bonar R, Mohammed S, Arbelaez A, Niemann J, Freney R, et al. Type 2M von Willebrand disease–more often misidentified than correctly identified. Haemophilia. 2016;22(3):e145–55.
66. Favaloro EJ, Bonar R, Kershaw G, Sioufi J, Baker R, Hertzberg M, et al. Reducing errors in identification of von Willebrand disease: the experience of the royal college of pathologists of australasia quality assurance program. Semin Thromb Hemost. 2006;32(5):505–13.

67. Favaloro EJ, Mohammed S. Towards improved diagnosis of von Willebrand disease: comparative evaluations of several automated von Willebrand factor antigen and activity assays. Thromb Res. 2014;134(6):1292–300.
68. Favaloro EJ, Mohammed S. Evaluation of a von Willebrand factor three test panel and chemiluminescent-based assay system for identification of, and therapy monitoring in, von Willebrand disease. Thromb Res. 2016;141:202–11.
69. Favaloro EJ, Dean E, Arunachalam S, Vong R, Mohammed S. Evaluating errors in the laboratory identification of von Willebrand disease using contemporary von Willebrand factor assays. Pathology. 2022;54(3):308–17.
70. Blanchette VS, Srivastava A. Definitions in Hemophilia: Resolved and Unresolved Issues. Semin Thromb Hemost. 2015;41(8):819–25.
71. Favaloro E. Genetic testing for von Willebrand disease: the case against. J Thromb Haemost. 2010;8(1):6–12.
72. Lillicrap D. The molecular genetics of von Willebrand disease. Hematology Meeting Reports (formerly Haematologica Reports); 2009.
73. Mannucci PM. How I treat patients with von Willebrand disease. Blood. 2001;97(7):1915–9.
74. Rodeghiero F, Castaman G, Tosetto A. How I treat von Willebrand disease. Blood. 2009;114(6):1158–65.
75. Abshire TC, Federici AB, Alvárez MT, et al. Prophylaxis in severe forms of von Willebrand's disease: results from the von Willebrand Disease Prophylaxis Network (VWD PN). Haemophilia. 2013;19(1):76–81.
76. Castaman G, Goodeve A, Eikenboom J. Principles of care for the diagnosis and treatment of von Willebrand disease. Haematologica. 2013;98(5):667–74.
77. Curnow J, Pasalic L, Favaloro EJ. Treatment of von Willebrand Disease. Semin Thromb Hemost. 2016;42(2):133–46.
78. Thompson AR, Gill JC, Ewenstein B, Mueller-Velten G, Schwartz B. Successful treatment for patients with von Willebrand disease undergoing urgent surgery using factor VIII/VWF concentrate (Humate-P®). Haemophilia. 2004;10(1):42–51.
79. deWee EM, Leebeek FWG, EikenboomJCJ. Diagnosis and management of von Willebrand disease in The Netherlands. Semin Thromb Hemost. 2011;37(5):480–7.
80. Mannucci PM, Franchini M. The use of plasma-derived concentrates. In: Federici AB, Lee CA, Berntorp EE, Lillicrap D, Montgomery RR, editors. Von Willebrand disease. 2nd ed. West Sussex, UK: Blackwell Publishing Ltd; 2010.
81. Nichols WL, Hultin MB, James AH, et al. von Willebrand disease (VWD): evidence-based diagnosis and management guidelines, the National Heart, Lung, and Blood Institute (NHLBI) Expert Panel report (USA). Haemophilia. 2008;14(2):171–232.
82. Dunkley S, Baker RI, Pidcock M, et al. Clinical efficacy and safety of the factor VIII/von Willebrand factor concentrate BIOSTATE in patients with von Willebrand's disease: a prospective multicentre study. Haemophilia. 2010;16(4):615–24.
83. Gill JC, Castaman G, Windyga J, Kouides P, Ragni M, Leebeek FW, et al. Hemostatic efficacy, safety, and pharmacokinetics of a recombinant von Willebrand factor in severe von Willebrand disease. Blood. 2015;126(17):2038–46.
84. Hancock JM, Escobar MA. An evaluation of von Willebrand factor (recombinant) therapy for adult patients living with severe type 3 von Willebrand disease. Expert Rev Hematol. 2023;16(3):157–61.
85. Gill JC, Castaman G, Windyga J, et al. Hemostatic efficacy, safety, and pharmacokinetics of a recombinant von Willebrand factor in severe von Willebrand disease. Blood. 2015;126(17):2038–46.
86. Leebeek F, Chapman M, Ploder B, Sytkowski A, Novack A, Ewenstein B. Treatment of gastrointestinal bleeding episodes with recombinant von Willebrand factor (rVWF) in patients with severe von Willebrand disease (VWD): sub-analysis from pivotal phase III on-demand study [abstract]. Res Pract Thromb Haemost. 2017;1(Suppl. 1):880.

87. Peyvandi F, Mamaev A, Wang JD, et al. Phase 3 study of recombinant von Willebrand factor in patients with severe von Willebrand disease who are undergoing elective surgery. J Thromb Haemost. 2019;17(1):52–62.
88. James A, Konkle B, Kouides P, Ragni M, Thames B, Gupta S, et al. Postpartum von Willebrand factor levels in women with and without von Willebrand disease and implications for prophylaxis. Haemophilia. 2015;21(1):81–7.
89. Simionescu AA, Buinoiu NF, Berbec N. Von Willebrand disease type 2 in pregnancy—a critical clinical association. Transfus Apher Sci. 2017;56(3):269–71.

Hemophilia A: Diagnosis and Management

4

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4.1 Introduction

The word "**Hemophilia**" (loosely translated to love of bleeding) was first used by Hopff in 1828 [1]. Hemophilia (either A or B) is a bleeding disorder that is inherited in an X-linked recessive manner. The most common type of hemophilia is hemophilia A, caused by a defect or deficiency in coagulation factor (F) VIII [2]. Hemophilia A or classical hemophilia is the most common severe bleeding disorder comprising ~80% of all types of hemophilia [2]. The main feature of hemophilia A is prolonged and abnormal bleeding, especially into joints and soft tissues.

The precise incidence of the disorder is unknown, but the estimated incidence ranges between 5 and 20 per 100,000 males, with the majority of patients remaining undiagnosed [3]. Approximately 40–60% of patients with hemophilia A suffer the severe form of the disease, and they are at risk of bleeding after surgery, such as dental extraction and circumcision, or traumatic visceral hemorrhages. They also exhibit spontaneous bleeding without trauma or injury or other interventional causes. Intra-articular joint hemorrhage is the most common spontaneous bleeding,

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_4

and this can lead to chronic physical disability due to arthropathy. In fact, currently, the majority of severe hemophilia develops chronic disability, typically in the form of hemophilic arthropathy, because of repeated joint bleeds [4].

Hemarthrosis is the main cause of functional disability and even psychological depression of patients with hemophilia [4]. Hemorrhage into the central nervous system (CNS) is the leading cause of death in hemophilia, accounting for ~20% of non-infective deaths, as reported in developed countries [5]. Primary prophylaxis is the treatment of choice in patients with severe hemophilia A. The goal is to maintain FVIII levels at a concentration that avoids most bleeding episodes, especially microscopic joint bleeds. Several therapeutic choices are available including fresh frozen plasma (FFP) and cryoprecipitate as traditional options and plasma-derived FVIII concentrate and recombinant FVIII (rFVIII) and non-factor concentrates as more advanced choices, but the most promising therapeutic strategy is gene therapy [6].

Based on FVIII activity, hemophilia A is classified into mild (5–40%), moderate (1–5%), and severe (<1%) deficiency. FVIII coagulant activity (FVIII:C) can be measured by either one-stage or chromogenic assays, but chromogenic assay is recommended for FVIII:C assay in emicizumab use [7, 8].

4.2 Factor VIII Structure and Function

The mature FVIII protein consists of three homologous A domains, two homologous C domains, and a unique B domain, which are arranged in the order of A1–A2–B–A3–C1–C2 from the N-terminus to the C-terminal end of protein (Fig. 4.1) [9, 10].

The A1 and A2 domains have about 30% homology with each other. A1–A2–B domains compose the heavy chain of FVIII, while the A3–C1–C2 domains comprise the light chain of the FVIII protein. The two chains are bound non-covalently and require a metal ion-dependent linkage, with the responsible residues located within the A1 and A3 domains. Each domain contains a copper atom [11, 12]. Two main antibody epitopes have been detected in the A2 domain. One epitope is located at amino acids 484–508, while the other is placed between amino acids 558 and 565. Immunization against these epitopes affects FVIII's interaction with activated FIX (FIXa) [13, 14]. There are three acidic peptides named a1, a2, and a3, which are clusters of Aspartic and Glutamic residues and surround the A domains.

The C domains of FVIII and FV are structurally related discoid domains and belong to the phospholipid-binding domain family [15]. The B domain is unique, without significant homology with any other known protein [16]. This domain does

Fig. 4.1 Structure of factor VIII protein; Human factor VIII is a single chain protein with a molecular weight of 300 kDa, and consists of six domains including A1–A2–B–A3–C1–C2. The mature factor VIII protein consists of three homologous A domains, two homologous C domains, and a B domain

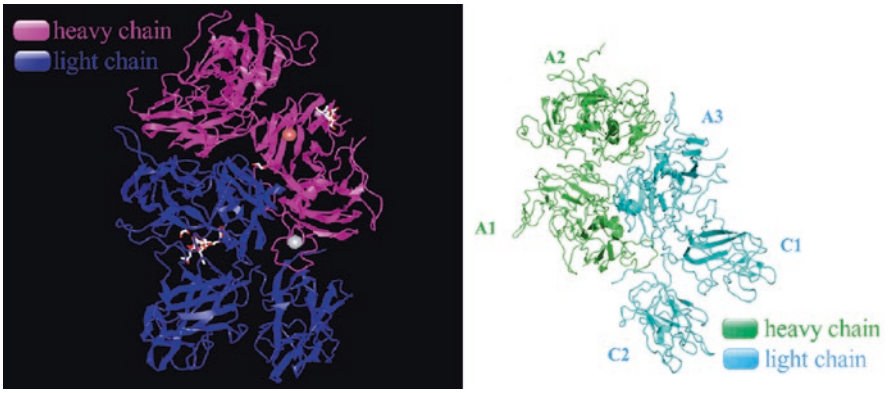

Fig. 4.2 Three-dimensional crystalloid structure of B domain deleted factor VIII protein. There are three A domains: A1, A2, and A3, two C domains: C1 and C2 domains. PDB of factor VIII crystal structure was downloaded from Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank (www.pdb.org)

not affect FVIII activity in the process of blood coagulation. When the B domain is cleaved and released, FVIII is converted to the active form (FVIIIa). The protein in active form consists of a heavy chain and a light chain. This process of activation requires two cleavages by thrombin (Fig. 4.2).

The different domains of the FVIII protein have different functions. An acidic peptide, which spans from amino acid 337–374 and called a1, separates the A1 from the A2 domain. Second acidic peptide, a2, links the A2 to the B domain. The next short acidic peptide termed a3 is connected with the A3 domain. A pair of homologous C domains (C1 and C2) resides at the C-terminal of FVIII protein. A region of the C2 domain contains a membrane binding site and the site for interaction with von Willebrand factor (VWF) [17]. The 2303–2332 sequence of the C2 domain has been suggested to form an interactive site for both VWF and phospholipids. The organization of the FVIII domains and linker peptides can thus be ordinated as A1.a1.A2.a2.B.a3.A3.C1.C2. Notably, there are seven disulfide bonds within the FVIII molecule: two in each A domain and one within the C1 domain [18]. In the A1 domain, Cys310, Met320, His267, and His315 create a copper-binding site and Cys2000, His1954, and His2005 constitute another copper-binding site in the A3 domain [12, 19].

The first cleavage of FVIII by thrombin at Arg1689 (at the B-a3) position generates a heavy chain with variable-size (90–210 kDa) and consists of A1 and A2 domains and heterogeneous fragments of the partially proteolyzed B domain; during this process, a 40-amino-acid acidic peptide (a3) is released from the C-terminal product to form a 73 kDa product that includes A3–C1–C2 domains, which is called the light chain. Further cleavage by thrombin cleaves the protein between the A1 and A2 domains and removes most of the B domain: cleavage at Arg372 (between the A1 and A2 domains) and at Arg740 (between the A2 and B domains) generates a 54 kDa A1 and a 44 kDa A2 domain (Fig. 4.3) [20, 21].

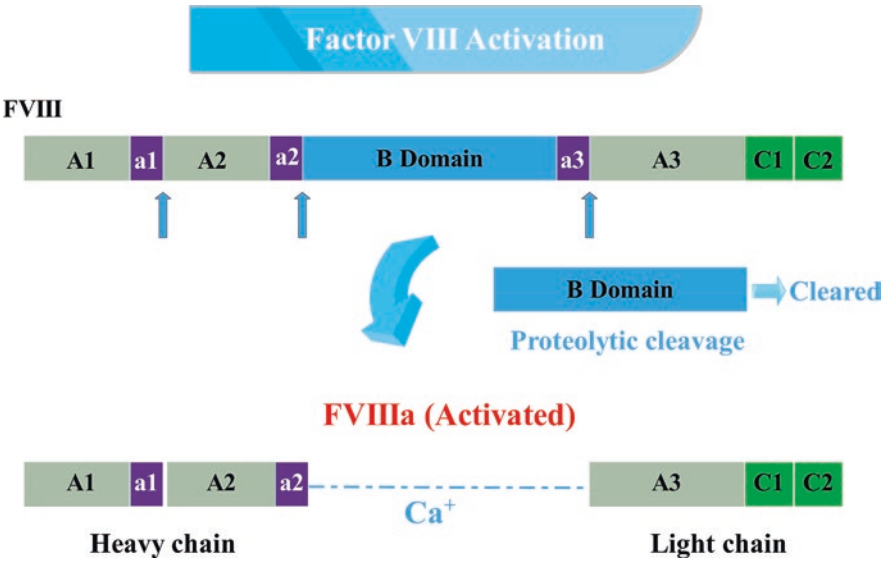

Fig. 4.3 Activation of factor VIII. Cleavage by thrombin removes the B domain and divides factor VIII to two chains: the heavy and light chain. Then the calcium cations link them together. Factor VIII in its nonactivated form circulates in plasma in a complex with von Willebrand factor (vWf). Upon activation by thrombin or factor Xa-mediated site-specific proteolysis, activated FVIII (FVIIIa) serves as a cofactor for factor IXa. Both chains form a non-covalently linked complex in a calcium-dependent manner. This complex is the pro-coagulant activated FVIII (FVIIIa) [22]. Next step in coagulation cascade is activation of FX by tenase complex, which consists of FVIIIa, and FIXa, platelet surface phospholipids and Calcium cation (Table 4.1) [23]

4.3 Hemophilia A

The word hemophilia comes from two Greek words: the "**haima**," which means blood, and the "**philia**" meaning affection. Hemophilia A or classic hemophilia is an X-linked recessive disorder characterized by dysfunctional blood clotting, due to a mutation in the gene for the clotting component, FVIII. This condition was described in the second century AD, when the Talmud, a collection of Jewish Rabbinical writings stated that male newborns should not be circumcised if their two brothers had already died from this procedure. During the twentieth century, hemophilia is estimated to affect one in 5032 live male births in the United States. Approximately 70% of mothers who have a child with hemophilia have a family history. While there is a history of hemophilia, 30% have a negative family history, and this is the first time a hemophilic child is present. These mothers are considered sporadic cases. [24, 25].

Clinical presentations of patients with hemophilia A vary from a mild condition to a severe bleeding tendency. Approximately 50% of people with hemophilia A have the severe form of the disease. Patients with hemophilia A are at higher risk of post-traumatic bleeding such as post-dental hemorrhage and post-surgical bleeding. Spontaneous bleeding is relatively common in patients

Table 4.1 Characteristics of factor VIII domains

Domains	Numbers of amino acids	Function	Molecular manifestation of the defected domain
A	(FVIII A domains consist of: A1(336 aa), A2 (337 aa) and A3 (329 aa))	– Coagulation FVIII A domains are homologous with the ones of coagulation FV and ceruloplasmin. – 40% amino acid identity with each other and to the A domains of FV	–
A1	336	– Creates the heavy chain (A1–A2) – Contains single copper atom	– Reduced stability of FVIIIa – Impaired thrombin activation
A2	337	– Creates the heavy chain (A1–A2) – The main antibody epitope is present in the A2 domain in amino acid 484–508 sites	– Alter FVIII intracellular trafficking and/or secretion – Alter interaction with FIXa – Impaired thrombin activation
A3	329	– Creates the light chain (A3–C1–C2) linked by metal ions – Contain single copper atom	– Alter interaction with FIXa – Reduce stability of the FVIIIa
B[a]	907	– Creates the heavy chain (A1–A2) – Intracellular synthesis quality control: enables interaction of FVIII with chaperone proteins that distinguish properly folded tertiary structure of proteins; stabilizes folded domains, prevents aggregation – Secretion: interacts with cargo-specific sorting receptor complex that enables endoplasmic reticulum to Golgi transport; increases secretion efficiency – Plasma activation: possibly shields thrombin activation site from premature proteolysis – Platelet binding: decreases affinity of inactivated FVIII for activated platelets, thus preserving circulating FVIII – Inactivation: reduces proteolysis by activated protein C and FXa – Clearance: may play a role in FVIII quality control through interaction with asialoglycoprotein receptor	–

continued

Table 4.1 Continued

Domains	Numbers of amino acids	Function	Molecular manifestation of the defected domain
C1	153	– C1 domain an impact on VWF and C2 domain linkage strengthening – Creates the light chain (80 kDa) (A3–C1–C2) linked by metal ions	– Reduction of FVIII binding to VWF – FVIII intracellular trafficking and/or secretion – Misfolded protein – Defect at phospholipid binding surface
C2	160	– C2 domain surface responsible for phospholipid linkage to coagulation FVIII – Creates the light chain (80 kDa) (A3–C1–C2) linked by metal ions	– Reduce the FVIII secretion rate – Misfolded protein – reduction of FVIII binding to VWF – Defect at phospholipid binding surface
a1	37	–	Impaired thrombin activation
a2	31	–	Impaired thrombin activation
a3	42	–	– Impaired thrombin activation – Reduction of FVIII binding to VWF

aa Amino acids, *FVIIIa* activated factor VIII, *FIXa* activated factor IX, *FXa* activated factor X, *VWF* von Willebrand factor
[a]Comprising 40% of FVIII mass

Table 4.2 Classification of hemophilia A and severity of clinical presentations

Severity of disease	Factor level % activity (IU/mL)	Sign and symptoms	Relative incidence	Age at diagnosis
Severe	<1% (<0.01)	Spontaneous bleeding, predominantly in joints, muscles, and internal organs	50%	First year of life
Moderate	1–5% (0.01–0.05)	Occasional spontaneous bleeding, severe bleeding after trauma or surgery	30%	Up to age 5–6 years
Mild	5 – 40% (0.05–0.40)	Severe bleeding with major trauma or surgery	20%	Often later in life

with severe hemophilia A. Recurrent intraarticular joint hemorrhage can lead to chronic disability as a result of arthropathy causing loss of a range of motion and severe pain. In fact, the majority of people with severe hemophilia A develop chronic disability, typically in the form of hemophilic arthropathy, because of repeated joint bleeds [4]. Furthermore, people with severe hemophilia have less than 1% of the normal level of FVIII in their blood. They can experience multiple spontaneous bleeding episodes per month. People with moderate hemophilia usually bleed less than severely affected patients, and post-minor traumatic hemorrhages can occur. Finally, people with mild hemophilia A have even fewer hemorrhages. Sometimes they are not aware of their bleeding problem although an invasive procedure such as surgery or a tooth extraction can bring the issue to light (Table 4.2) [26].

Diagnosis of this disorder can be made based on clinical presentations, appropriate laboratory assessment, and familial history. Because hemophilia A is an X-linked disease, females can be carriers. Carriers have one normal and one abnormal gene. They have a 50% chance of giving the hemophilia gene to each child. Boys who receive the abnormal gene will have hemophilia; girls who inherit it will become carriers. Therefore, if the father of a family has hemophilia and the mother has a hemophilic gene, their daughter can have hemophilia. With regards to phenotype, carriers are usually unaffected or only mildly affected with some bleeding symptoms. Carriers who have >50% of clotting FVIII levels generally do not demonstrate a bleeding tendency [27]. But half of them have less than 50% FVIII and therefore manifest a bleeding tendency proportional to how low the level is in their blood.

Carriers are divided into two groups: (1) mandatory carriers, who necessarily carry the affected X chromosome, and (2) possible carriers [27].

Mandatory Carriers Are

- All female offspring of a man with hemophilia
- Mothers who have a boy with hemophilia and at least one other family member with hemophilia (brother, grandparent, uncle, nephew, or cousin)
- Mothers who have a boy with hemophilia and a family member carrying the hemophilia gene (mother, sister, mother's grandmother, aunt, or uncle)
- Mothers of two or more boys with hemophilia

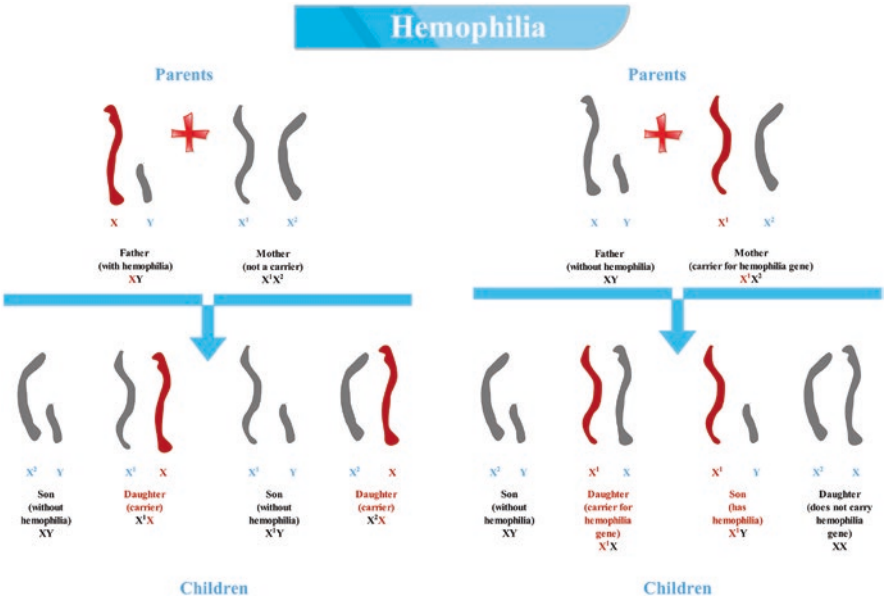

Fig. 4.4 Hereditary chance of giving the hemophilia gene in each child: left, when the father is affected, right, when the mother is a carrier

Possible Carriers Are

- All girls born of a carrier
- Mothers of one boy with hemophilia who have no other family members who have hemophilia or are carriers of hemophilia
- Sisters, mothers, maternal grandmothers, aunts, nieces, and female cousins of carriers (Fig. 4.4)

4.4 Clinical Manifestations

Hemophilia A is the most common severe congenital bleeding disorder, and patients suffer variable clinical presentations. There is a direct correlation between FVIII coagulant activity (FVIII:C) and severity of clinical presentations. Based on residual FVIII:C level, the disorder is classified into severe (<0.01 IU/dL (<1% of normal)), moderate (0.01–0.05 IU/dL (1–5% of normal)), and mild (0.05–0.40% IU/dL (5–40% of normal) deficiency [28].

Mild, moderate, and severe forms comprise 20%, 30%, and 50% of all cases with hemophilia, respectively [29]. Individuals with FVIII:C higher than 30% are usually asymptomatic, but a mild bleeding tendency presents in hemophilia A carrier females with low to low-normal FVIII:C level [30].

Severely affected individuals present with severe bruising and joint hemorrhage during their first 2 years of life, often bleeding with circumcision [31].

Phenotypically, hemarthrosis (particularly in the knee, ankle, and elbow joints), soft-tissue hematoma, easy bruising, retroperitoneal bleeding, intracranial hemorrhage (ICH), and post-surgical bleeding are the most concerning problems in hemophilia A that may present at any age [32]. ICH is the main concern of newborns that can occur immediately after birth or during birth. Severity of the hemophilia and type of delivery can affect the rate of ICH in neonates. Neonates with forceps delivery and Cesarean section delivery are at higher risk of ICH, while non-traumatic normal vaginal delivery imposes less risk. Overlaying the incidence of ICH in hemophilia is variable, but 3.5–4% is estimated to be typical (Table 4.3).

In early childhood, large hematomas might also be observed following intramuscular injections (e.g., immunizations) in boys and should persuade the physician to rule out a bleeding disorder [2, 33].

Bleeding symptoms also may appear later when the teeth erupt. Prolonged severe oral bleeding can lead to anemia [34].

As the child's activity increases with age, the presentation of bleeding episodes is changed. During the toddler age, bumps and falls can cause prolonged oral bleeding from the frenula, superficial hematoma and intramuscular hemorrhages. Physicians must thoroughly examine the patients who have such symptoms to differentiate hemophilia from child abuse. The family history of bleeding disorders can be helpful [2, 35].

Spontaneous hematuria may occur in children and adults, but usually disappears in a few days [36, 37]. Large intramuscular hematoma is also a major bleeding problem in childhood and adulthood periods and should immediately be treated by clotting factor concentrate. Bleeding into a muscle causes it to be swollen hard and tender. Hemorrhages in large muscles may cause anemia and severe pain [2, 38]. Bleeding into iliopsoas may present as an acute abdominal pain and could be mistaken as appendicitis or other intraabdominal surgical emergencies [39]. On the other hand, some mouth problems can occur during infancy, when teeth's roots of infant are mobile. Bleeding also occurs when a tooth falls out or is extracted by a dentist. The teenage years sometimes need for orthodontic braces. The orthodontist must be aware of the child's disorder and should consider the necessary considerations. Another problem is Gum diseases. Chronic periodontitis (pyorrhoea), which is typically seen in adults, and gingivitis are both painless, but may lead to gum bleeding, particularly during brushing [33]. Furthermore, one study suggests that temporomandibular joint open/close movements may result in silent bleeding into the chin joints in patients with inherited coagulation disorder. This study demonstrated that two patients with severe hemophilia developed temporomandibular disorders [41].

Moreover, gastrocnemius, quadriceps and forearm bleedings may increase pressure on neurovascular bundles, causing a situation that is known as compartment syndrome. It can lead to ischemia, fibrosis, neuropathy, atrophy and even may require amputation [42].

Table 4.3 Clinical manifestations of congenital factor VIII deficiency

Prevalence	Bleeding episodes	Iran% (*N*: 885)[a]	North-Eastern Iran% (*N*: 287)	Guidelines for the management of hemophilia (%)	India%)*N*: 56)[b]	Pakistan%)*N*: 229(	Iran% (*n*: 100)	Iran%	
								Severe/ moderate (*n*: 50)	Mild (*n*: 50)
~20–100%	Ecchymosis	13.2	71	–	–	–	–	–	–
	Hemarthrosis	8.7	72.6	70–80	73.21	–	86	86	6
	Oral cavity bleeding	–	–	–	–	–	64	64	30
	Post-partum bleeding	–	–	–	–	–	36	–	–
	Post-operative bleeding	–	–	–	–	18.4	–	76	30
	Post-dental extraction bleeding	6.8	89	–	–	0.9	–	–	–
	Epistaxis	–	55.9	–	26.78		20	20	12
~5–20%	Post-circumcision bleeding	14.5	–	–	–	62	–	–	–
	Gastrointestinal bleeding	0.4	21	–	3.57	–	10	10	0
	Hematuria	–	32.3	–	1.78	–	12	12	0
	Hematoma	1.2	4.2	–	–	0.9	93	82	8
	Skin bleeding	–	–	–	80.35	–	–	–	–
	Muscle bleeding			10–20	46.42	–	–	–	–
~<5%	Central nervous system bleeding	–	2.4	<5	–	–	4	4	0
	Umbilical cord bleeding	0.5	1.8	–	–	5.2	–	–4	–
	Menorrhagia	0.3	–	–	–	–	–	–	–
	Lliopsoas muscle	–	–	–	1.78	–	–	–	–

[a]First clinical presentations
[b]51 cases had hemophilia A, while five cases had hemophilia B

The major bleeding problem throughout the patient's life is hemarthrosis that requires immediate treatment. Bleeding into the joint leads to synovitis, causing increased proliferation and vascularization. Repeated bleeding episodes, causing a hot swollen joint, which resulted from the synovial hypertrophy and, with further bleeding, muscle weakness and loss of joint stability. This cycle of bleeding leads to erosion of joint cartilage, resulting in arthritis and the paralyzed, disfigured limb of patients with hemophilia [2, 43, 34].

4.4.1 Molecular Basis

FVIII gene that encodes FVIII protein is one of the largest genes located on the long arm of chromosome X (Xq28 position). *FVIII* gene contains high GC content and within the 9.1 kb coding region, includes 25 introns and 26 exons organized in several domains, A1, A2,A3, B and C1–C2 [45]. Exons are ranged from 69 bp (exon 5) to 3.1 Kb (exon 14) in size. The FVIII precursor protein of 2351 amino acids is encoded by FVIII mRNA which is 9.1 Kb in size [46].

4.4.2 Factor VIII Gene Mutations

The sequence of the *FVIII* gene was published in 1984, and since that time, a large number of mutations that cause hemophilia A have been identified [35].

FVIII deficiency is caused by a wide spectrum of mutations, which occur along the entire length of the *FVIII* gene. The mutations can cause quantitative or qualitative defects. In quantitative, there are defects in the level of transcription or translation and in qualitative defects, changes of individual amino acids in FVIII protein occur. Severe hemophilia is typically caused by inversions, deletions, insertions, missense mutations, and nonsense mutations. Milder forms of hemophilia are usually caused by missense mutations, while single nucleotide deletions or splicing errors also may occur [21]. There are about 70 CpG dinucleotides within *FVIII* gene. This results in to hyper-mutation status, and approximately 30% of variants are usually novel [34]. Human gene mutation database (HGMD) has reported that there are 2320 mutations within the *FVIII* gene [36]. The most frequent gene defect is the intron 22 inversion, which leads to hemophilia A with a frequency rate of 52% among individuals with severe hemophilia A [29]. The second most frequent mutation is the intron 1 inversion with a prevalence of about 1–5% among hemophilia A patients (Table 4.4) [37, 38].

The remaining cases are caused by numerous mutations spread all over the gene. As outlined in the FVIII HAMSTeRS (homology, mutation, structure, testing, and site resources) mutation database, there are over 1209 mutations in *FVIII* coding and untranslated regions. Most of these mutations are point mutations or small rearrangement. Most of the missense mutations, which are detected in mild to

Table 4.4 Frequency of factor VIII gene mutation [34, 38]

Type of mutation	Frequency percentage	Most reported relevant type of disease
Intron 1 and 22 inversions	29%	Severe
Large deletions	7%	Severe
Small deletions	6%	Severe
Non sense mutations	8%	Severe
Insertions	2%	Severe
Duplications	3%	Severe
Missense mutations	45%	Severe

moderately severe hemophilia A, are located within the exons coding for the three A domains or the two C domains [39, 40].

4.4.3 Intron 22 Inversion

About 40–50% of patient with severe hemophilia A show intron 22 inversion of FVIII, the most frequent inversion mutation in severe hemophilia A [41]. The intron 22 of the *FVIII* gene contains a 9.5 kb region, which is present outside of the gene, near the telomere of the X chromosome in two additional copies; these are termed *int22h2* and *int22h3*. *int22h1* is the sequence in intron 22 that includes the *FVIII* gene [42, 43]. The sequence identity of the three regions is 99.9%. An inversion of the *FVIII* gene can be caused by an intrachromosomal homologous recombination between *int22h1* and one of its two telomeric copies. This inversion occurs almost particularly in the male germ cells because the proposed intrachromosomal recombination would be probably inhibited by pairing Xq with its homologous in female meiosis (Fig. 4.5) [44].

4.4.4 Insertions and Deletions in the Factor VIII Gene

Insertions and deletions that cause severe hemophilia can be large (more than 50 bp) or small, usually one or more nucleotides. Insertions and deletions of a single nucleotide lead to a shift of the reading frame and thus, cause severe form of the disease. Several types of repetitive sequences, including SINES and LINES, which are present in the *FVIII* gene, may be mutated. Hemophilia in such patients is somewhere on the border between the severe and moderate forms. In severe hemophilia A, gross gene alterations (including large deletions or insertions, frame shift and splice junction changes, and nonsense and missense mutations) of FVIII account for approximately 50% of mutations detected. The most recent update of the HAMSTeRS database listed more than 200 small deletions (50 nucleotides or less than) and more than 80 insertions in hemophilia A [45–47].

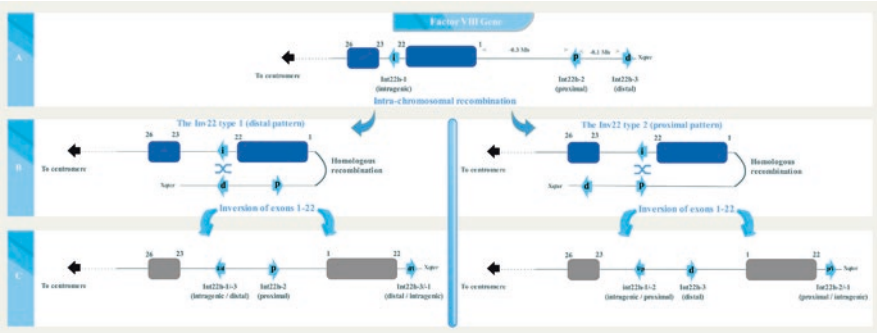

Fig. 4.5 The intron 22 inversion of the FVIII gene. Intrachromosomal homologous recombination between inverted repeats on the X chromosome account for about half of severe hemophilia A cases. Identification of a third rearrangement at Xq28 that causes severe hemophilia A as a result of homologous recombination between inverted repeats. (**a**) Show normal alleles, (**b**) Show the intra chromosomal homologous recombination, (**c**) Show the Inv22 type 1 (distal pattern) and type 2 (proximal pattern). Functional F8 is indicated by blue arrows, while non-functional F8 sequences are indicated by Gray arrows. (*Xqter* The end of the long arm of the X chromosome, *Int* intron, *FVIII* Coagulation Factor VIII, *i* Int22h-1 (intragenic), *P* Int22h-2 (proximal), *d* Int22h-3 (distal))

Genetic defects can affect the interaction sites of FVIII with other clotting factors such as FIX or VWF and cause hemophilia A [9, 10].

4.4.5 Diagnosis

Diagnosis and management of hemophilia A requires precise laboratory assessment of patients, especially precise determination of FVIII:C level. FVIII:C assay can be used for precise classification of disease, determination of the dose of replacement therapy and pre-operative management, and therapeutic response in patients with hemophilia A. Since FVIII is a cofactor and not an enzyme, FVIII assays use an indirect method performed by clot or chromogenic-based methodologies. Different methods are available for FVIII one-stage and chromogenic assays. Each of these methods has its own advantages and disadvantages. Large variation in FVIII concentrations measured in different laboratories is observed. This variation can be more profound with the use of new recombinant modified FVIII products.

4.4.6 Chromogenic Assay

The chromogenic assay uses a principal similar to the two-stage clot-based assay. In the first stage, activated FX (FXa) is generated and subsequently during the second stage, the amount of FXa is determined. In the first stage, patient's plasma is incubated with FIXa, excess FX, thrombin, calcium, and phospholipids. In this step,

activated FVIII (FVIIIa) with FIXa generates the tenase complex (FIXa–FVIIIa) and converts FX to FXa. In the second step, FXa cleaves the chromogenic substrate and the intensity of generated color is directly proportional to the amount of FXa and therefore the amount of FVIII in patient's plasma (Fig. 4.6).

The reagent consists of purified activated FIXa, purified FX, phospholipids (PL), calcium and chromogenic substrate. Added to the reagent is highly diluted patient's plasma or reference plasma as a source of FVIIIa. FX is converted to FXa and this activated coagulation factor hydrolyzes a FX specific chromogenic substrate, leading to release of chromophoric group para-Nitro Aniline (pNA). The extinction can be read on 405 nm (Fig. 4.7).

This method was recommended by the FVIII and FIX Subcommittee of International Society of Thrombosis and Hemostasis (ISTH) as a reference method

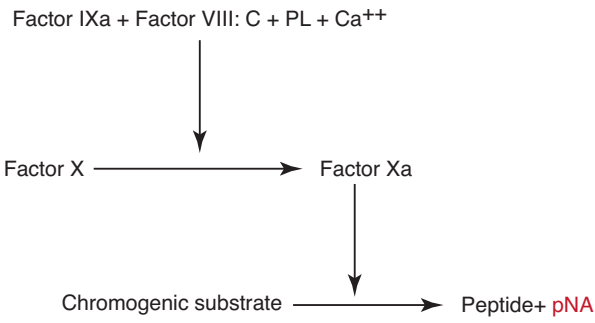

Fig. 4.6 Principle of chromogenic assay

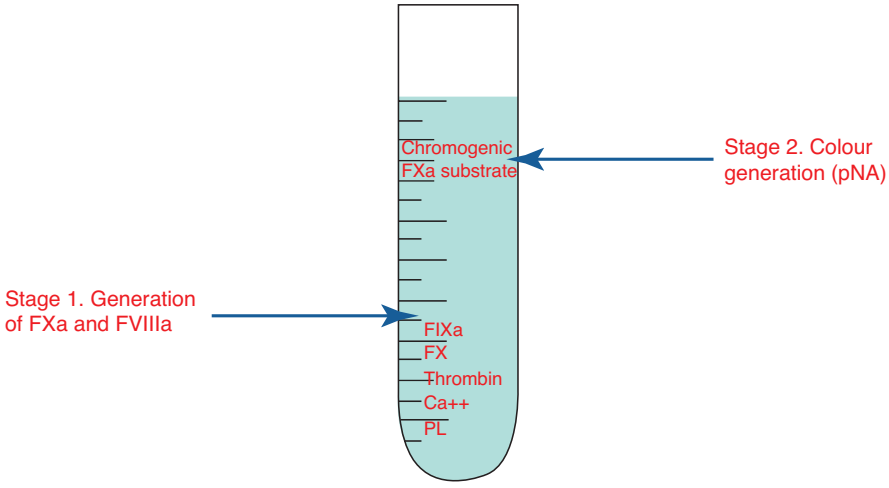

Fig. 4.7 Color generation of FXa and FVIIIa*FVIII* Factor VIII, *FIXa* Factor IX activated, *FX* factor X, *FXa* activated factor X, *Ca* calcium, *PL* phospholipid, *pNA* para-Nitro Aniline

for FVIII:C assay in plasma and/or concentrate. Chromogenic assays demonstrate less variation between laboratories than one-stage assay (Table 4.5).

The advantages of the chromogenic assay over clot-based one-stage assays include improved precision and sensitivity to low amounts of factor, but the complexity of procedure and operator burden may increase the risk of inaccuracy and errors.

Advantages:

1. FVIIIa cannot affect the chromogenic assay as it may the two-stage assay**.**
2. Direct thrombin inhibitors (DTI) or heparin can impact the chromogenic assay less than the one-stage assay.
3. In comparison with the one-stage assay, variability of the chromogenic assay is significantly lower.
4. The chromogenic assay is not dependent on FVIII deficient plasma.

Disadvantages:

1. Falsely reduced FVIII activity may be measured when a direct FXa inhibitor is present.
2. Generally, a chromogenic FVIII assay is more expensive than a clot-based FVIII activity assay such as the one-stage assay [6, 48, 49].

Table 4.5 Characteristics of chromogenic factor VIII assay kits

Kit	Vendor	Intended use	FXa substrate	Thrombin inhibitor	Detection limit	Total CV	Advantage	Measuring range
Coatest® SP4[a]	Chromogenix, Milan, Italy	Plasma sample and concentrate	S-2765: N-a-Z-D-Arg-Gly-Arg-pNA	I-2581	<1%	Range 1–20%: 5.6 (n = 80) Range 20-150%: 5.3 (n = 80)	Rapid Applications for a wide range of automated instruments	1. 1–20% 2. 20–150%
Coatest® SP[a]	Chromogenix, Milan, Italy	Plasma and concentrate	S-2765: N-a-Z-D-Arg-Gly-Arg-pNA	I-2581	<1%	Range 1-20%: 5.6 (n = 80) Range 20–150%: 5.3 (n = 80)	1.No drug interference reported 2. Heparin concentrations 0.2 IU/mL do not interfere 3. 0.5 IU/mL gives 5% inhibition 4. Due to the high dilutions used, there is no Underestimation of FVIII activity in samples containing lupus anticoagulant	1. 1–20% 2. 20–150%
Coamatic®	Chromogenix, Milan, Italy	Plasma and concentrate	S-2765: N-a-Z-D-Arg-Gly-Arg-pNA	I-2581	<1%	Range 1–20%: 5.6 (n = 80) Range 20–150%:	Applications for a wide range of automated instruments	20–150% (0.2–1.5 IU/ml) • 1–20% (0.01–0.2 IU/ml).

Technochrom® FVIII:C	Technoclone, Vienna, Austria	Plasma	FXa-1 (pNA)	α-NAPAP	<1%	–	Stability on board 24 h Reagents may be capped and refroze 2 weeks at −20 ° C after opening. Excellent accuracy and precision Economical use For plasma samples and Concentrates Adaptation sheets for common Autoanalyzers are available	0.6–1.5 IU F VIII (60–150% of normal)
DG-Chrom FVIII	Grifols, Barcelona, Spain	Plasma	FXa-1 (pNA)	α-NAPAP		4.06%	Rapid	1–130%
FVIII chromogenic assay	(Siemens, Marburg, Germany)	Plasma	CH3OCO-D-CHG-Gly-Arg-pNA. AcOH;	α-NAPAP		–	Extended onboard reagent stability	–
Biophen	Hyphen biomed, Neuville-Sur-Oise, France	Plasma and concentrate	SXa-11 (pNA)	Thombin inhibitor	-(high range) ~10% -(low range) ~2%	FVIII deficient plasma is not required Insensitive to lupus anticoagulant	More expensive than one stage More sophisticated than one stage Difficult for automation	(Low: 0 to 25% and High: 0 to 200%).

[a]The formulation of the two kits is the same, they are optimized for different test volumes

4.5 One-Stage Assay

The most common method to measure FVIII activity is the one-stage assay, which is based on partial thromboplastin time (PTT) or activated partial thromboplastin time (APTT).

In this assay, a plasma standard and FVIII-deficient plasma are required. The plasma standard, used for assay calibration, can be made in laboratory or a commercial standard can be used. In any case, this standard should be calibrated against an international standard for FVIII. It is not acceptable to assume that pooled plasma has 100 U/dL FVIII:C. FVIII-deficient plasma can be commercially available or can be obtained from patients with FVIII level <1 U/dL as long as there is no inhibitor and liver function tests are normal.

In this assay, FVIII-deficient plasma is added to patient's plasma diluted in buffer and is mixed with equal amount of deficient plasma along with the APTT reagent and incubated for appropriate time (most often 3–5 min) at 37 °C. Then calcium is added to this mixture and the clotting time is recorded. To determine the FVIII activity in patient's plasma, the clotting time of patient's sample is compared with standard curve. The standard curve is constructed by plotting the clotting time of serial dilution of standard plasma versus FVIII activity on logarithmic/linear scale graph paper. An important issue with this assay is parallelism between standard dilutions and patient's plasma dilutions. Therefore, FVIII activity assay should be performed at least on three different dilutions to check parallelism. Non-parallelism of FVIII activity will lead to inaccurate FVIII activity determination. It is generally accepted that the main reasons for non-parallelism are presence of a specific FVIII inhibitor, lupus anticoagulants, or other non-specific inhibitors.

4.5.1 Benefits of the One-Stage Methodology

1. Simplicity and readily automatable
2. The results are derived from an activity curve designed and built using clotting times of dilutions of normal reference plasma and specific factor VIII-deficient plasma.

4.5.2 Limitations of the One-Stage Assay

1. Lipemia will likely affect the one-stage assay than the chromogenic assay.
2. This method can also be influenced by anticoagulant drugs, specifically heparin, direct thrombin inhibitors, or direct FXa inhibitors.
3. If FVIII is converted to the FVIIIa during sample collection, the one-stage assay will demonstrate a false increase in FVIII activity.
4. The one-stage assay may be non-optimal for detecting an increased level of FVIII as shown by CV.
5. Finally, due to the presence of different APTT reagents, various analyzers, calibration standards, and factor deficient plasmas, there is a significant difference between one stage FVIII tests performed in different laboratories [47].

4.5.3 Discrepancy Between Chromogenic and One-Stage Assays

One-stage and chromogenic assays generally give comparable results, but discrepancies can occur in a number of circumstances including some hemophilia A phenotypes related to the presence of specific mutations and with some recombinant FVIII therapeutic agents. In 30% of patients with mild hemophilia A, discrepancy between the one-stage and chromogenic assays can be observed. In approximately 5–10% of patients with mild hemophilia A, the FVIII clot-based activity assay often falls in the normal range, while chromogenic assay yields a mildly abnormally low result. In these patients, if only the clot-based assay is used, the diagnosis may be missed; thus, simultaneous use of one-stage and chromogenic assays is recommended for diagnosis of mild hemophilia A.

When missense mutations occur in the A1–A2 and A3 domain interfaces, the result of FVIII activity by the chromogenic assay is lower than the one-stage assay. This difference can be as much as two-fold or more. The basis of this discrepancy is that such mutations can decrease FVIII heterodimer and FVIIIa heterotrimer stability. These changes have minimum effect on the one-stage assay, while in the chromogenic assay, this effect increases A2 dissociation impairing FVIII stability and leading to decreased activity.

FVIII mutations around the thrombin cleavage site, FXa binding site, and VWF binding site cause lower FVIII activity in the one-stage assay in comparison with the chromogenic assay. These mutations cause impaired FVIII activation by thrombin and impair its binding to VWF and FXa. In the one-stage assay, physiological concentration of thrombin is affected by mutations around the thrombin cleavage site, while in the chromogenic assay, high concentrations of thrombin and the longer time of incubation allow appropriate activation of FVIII and therefore this assay is less sensitive to these mutations.

4.5.4 Determination of Factor VIII Concentrates Potency

FVIII activity assay is important for monitoring patient's response to replacement therapy and for potency labeling of different FVIII products. Discrepancies were observed between FVIII activity assay by one-stage assay and chromogenic assays in the laboratory assessment of some long-acting recombinant FVIII products. One-stage assays are more sensitive to PEGylation than chromogenic assays, for example, N8-GP (Novo Nordisk A/S, Bagsværd, Denmark) a PEGylated, recombinant FVIII (rFVIII), causes the clot-based assay to be falsely elevated. The PEG moiety of this product, interferes with some particular APTT reagents, especially silica-based. Ellagic acid and polyphenol APTT reagents are not sensitive to PEGylated products and can be used for potency measurement of PEGylated rFVIII. The chromogenic FVIII assay is certified for potency testing of this product. Although one-stage assay can accurately determine FVIII activity in most plasma-derived FVIII products, they result in 20–50% lower FVIII activity than the chromogenic assay in B-domain deletion (BDD) rFVIII products. For full-length rFVIII products, the chromogenic assay yields 8–20% higher FVIII activity than the one-stage assay, which can be clinically significant [50–57] (Table 4.6).

Table 4.6 Compare the potency of FVIII replacement products

Product	Nature of product	Company	The certified potency of	Half life	Reference
N8-GP	A PEGylated, B-domain truncated derivative of recombinant FVIII (rFVIII), turoctocog alfa pegol	Novo Nordisk A/S, Bagsværd, Denmark	Chromogenic assay	19 h	[50]
NovoEight (turoctocog alfa)	rFVIII B-domain Truncation	Novoeight, Novo Nordisk A/S, Bagsværd, Denmark	One-stage clotting assay or the chromogenic substrate assay	10.8 h (in children is about 29% Shorter than in adults)	[50]
Advate®	Full-length rFVIII	rFVIII, Advate_, Baxter Bioscience, Deerfield, IL, USA	One-stage clotting assay	8.7–25.2 h	[51]
BAY 94-9027	K1804C directed PEGylation	Bayer, Leverkusen, Germany	One-stage FVIII assays, with ellagic acid APTT reagents	19 h	[52]
ReFacto	B-domain deletion (BDD) rFVIII	Pfizer, Sandwich, UK	One-stage clotting assay	14.8 ± 5.6 h (range 7.6–28.5 h)	[53, 54]
Efmoroctocog alfa	rFVIII-Fc fusion Protein	Eloctate®; Biogen Idec Inc., Cambridge, MA, USA	One-stage clotting or chromogenic assays	1.48 to 1.56-fold greater than that of RFVIII	[55]
BAX 855	PEGylated full-length rFVII	Baxter, Deerfield, IL, USA	Chromogenic FVIII assay.	1.4- to 1.5-fold increased half-life compared to Advate	[56]
CSL627	Single-chain variant of B-domain deleted FVIII	CSL Behring, King of Prussia, PA, USA	Chromogenic assay	13 h	[57]

For labeling of a FVIII product, both methods are acceptable when the results agree, but when a significant discrepancy is observed between one-stage and chromogenic assays, the more accurate method should be used. Although the one-stage assay is the most commonly used method, the advantages of the

chromogenic assay in accurately measuring some of the new modified products will increase its role in potency assignment and probably also in clinical monitoring in the future.

4.5.5 Treatment

In the 1960s and 1970s, the advance of factor concentrates, self-infusion method, and hemophilia treatment centers improved the quality of life in patients with hemophilia.

However, by the 1990's, almost 90% of severe hemophiliacs were infected with human immunodeficiency virus, acquired immunodeficiency syndrome (HIV/AIDS) [58]. Today, as a result of advances in the eradication of infectious agents in clotting factor concentrates, almost all hemophilia patients 30 years of age or younger are HIV-negative and Hepatitis C negative. Although hepatitis C infection continues as a complication of therapy, several strategies should be taken to help prevent infection. First preventive therapy, such as therapeutic abortion or vaccination against hepatitis in the prenatal and post-natal periods respectively; second, aggressive new treatment such as prophylaxis; and third, improved surgical techniques such as arthroscopic synovectomy, and finally better rehabilitation techniques [2].

4.5.6 Replacement Therapy

The traditional and common way to treat severe hemophilia A is regular supplementation with intravenous recombinant or plasma derived FVIII concentrate FVIII. However, some people with severe hemophilia and more often those with moderate and mild hemophilia are treated only as needed (also known as on demand). In children, to minimize frequent traumatic intravenous cannulations, an easily accessible intravenous port (Port-A-Cath) is often used [59]. These devices eliminate problems of finding a vein for infusion, which may be required, several times in a week. Therefore, such ports make prophylaxis programs easier. Studies have demonstrated a risk of clot formation at the tip of the indwelling catheters with high risk to become infected [60]. Individuals with mild hemophilia often are managed with desmopressin, a vasopressin analog that causes the release of stored FVIII from blood vessel walls [61].

4.5.7 Treatment of Hemarthrosis

Symptom relief, prevention of the progression of joint damage, and maintenance of function are the physician goals in hemophilic arthritis. These are similar to the goals in inflammatory arthropathy. In the subject with degenerative changes, function would be corrected by surgical/physical methods with adequate hemostatic

coverage [4]. These are similar to inflammatory arthropathy. A recent study concluded that if hemophilic patients have nonbleeding arthritis and extra-articular findings, physicians should be alert on the coexisting inflammatory disorders such as familial Mediterranean fever [62].

4.5.8 Management of Bleeding

The first choice for prevention of joint disease is prophylaxis with factor concentrates, as recommended by both the World Health Organization (WHO) and the World Federation of Hemophilia (WFH). The achieved level of the factor activity must be greater than 1% for the maximum profit. This can usually be achieved by giving 25–50 IU/kg of FVIII three times per week [63].

Extended half-life factor concentrates and subcutaneous non-factor replacement therapies have recently been used successfully to prevent bleeding in hemophilic patients [64].

4.5.9 Adjunctive Management

Analgesics such as paracetamol/acetaminophen can be prescribed to treat pain by joint bleeding, but milder opioid painkillers are more frequently used in Hemophilic joint pain.

4.5.10 Anti-Inflammatory Treatment

To treat chronic synovitis, some experts recommend intra-articular corticosteroid administrations.

4.5.11 Rest, Ice, Compression, and Elevation (RICE)

This strategy may be useful to relieve minor pain, but prolonged rest may cause motion limitation and muscle atrophy [65].

4.5.12 Physiotherapy

Physiotherapy is an important way to preserve or return movement and function to the joints. It also reduces swelling and pain, prevents injury, and maintains muscle strength [66].

4.5.13 Joint Aspiration

In hip hemarthrosis and other major and painful hemarthroses, aspiration can be prescribed and should be performed immediately after a bleeding episode (<12 h) [66].

4.5.14 Surgical Treatments

When conservative therapies fail, open surgical procedures are often the best or even the only way to cure patients with severe joint disease. The advantages of surgery must be more than the potential risks, particularly in patients with severe hemophilia and/or inhibitors.

4.5.15 Joint Debridement

Joint debridement is a surgical procedure that removes synovitis in young patients to prevent or delay articular arthroplasty [67].

4.5.16 Joint Arthroplasty

To reach the normal quality of life in patients with severe joint pain joint, replacement surgery, which also is known as arthroplasty, could be considered. Results from the most commonly replaced joints are generally good or excellent [68].

4.5.17 Fusion (Arthrodesis)

Fusion is also known as arthrodesis. It comprises joint removal and fusion of the bones. Ankle arthrodesis is carried out today and allows other joints in the foot to move normally so the patient can have a close-to-normal gait [68].

4.5.18 Treatment in Hemophilia Carriers

Most carriers are asymptomatic, but, if they are subject to trauma or surgical intervention, they may show prolonged bleeding. In these instances, treatment must be initiated using the same strategies as in patients with hemophilia. Pre-existing clotting factor level and the cause and severity of the bleed determine the dosage and duration of treatment [27].

4.5.19 Problems Related to Treatment of Hemophilia

Viral infections and immunization against the FVIII protein are the most reported problems related to treatment of patient with hemophilia. The main issue with factor replacement therapy is the development of inhibitor antibodies against FVIII. As the patient does not have its own copy, it recognizes the "normal form" of FVIII as foreign. Due to antibody development, FVIII infusions become ineffective. Recently, FVIIa has become available as a treatment for hemorrhage in patient with hemophilia with FVIII inhibitors [64, 69]. An inhibitor may be suspected when unexpected bleeding in the response to treatment is not stopped by factor substitution as quickly as it should. After suspicion, the inhibitor may be discovered during routine screening. As a follow-up, its titer must be determined. Inhibitor titer levels are important as a suitable treatment is based on the patient's inhibitor titer.

4.5.20 Viral Infections

Adults with hemophilia have one of the highest prevalence of hepatitis C virus (HCV) infections among all populations at risk of HCV infection. Prior to 1987, approximately all patients treated for hemophilia with plasma products were exposed to HCV, and more than 80% had chronic infections. In one study, of the 350 patients with hemophilia A and B, 232 individuals (about 66%) had been infected with HCV. There was no independent risk factor for infection based on multivariate logistic regression model [70]. The prevalence of HCV infection among HIV-infected patient with hemophilia was found to be 66%. Of the 31 patients with HCV/HIV, 21 persons (67.7%) had no history of transfusion. The authors noted that contaminated FVIII (clotting factor) could be responsible for disease acquisition [71].

4.5.21 Inhibitor

A FVIII inhibitor is a polyclonal high-affinity immunoglobulin G (IgG) that is directed against the FVIII protein [72]. IgG4 antibodies are the predominant antibodies that do not fix complement [72, 73]. The domains A2, A3, and C2 are the main domains that inhibitory antibodies are directed against [74].

The most dangerous complication of the hemophilia A management is the development of an inhibitor against FVIII. Based on data reported worldwide, the frequency of inhibitor development is 5–10% (totally) and 10–15% in severe patients [75].

Inhibitor development is defined as the number of new inhibitors diagnosed in a specific time period. Published articles report differences in the rate of inhibitor development likely due to disappearance of many transient low-titer inhibitors and successful tolerance of others [76, 77]. Based on different studies from around the

world, the prevalence of inhibitors in hemophilia A is greater compared with hemophilia B [78]. For example, in Saudi Arabia, patients with FVIII inhibitors were observed in 43 (29.3%) out of the 147 patients, and only 1 out of the 54 patients developed FIX inhibitor [79].

4.5.22 Inhibitor Associated Risk Factors

The following are the main risk factors for inhibitor development [74, 80].

1. Number of exposures to clotting factor concentrates
2. Increased dosage of clotting factor concentrations
3. Spanish ethnicity or black race
4. The positive history of inhibitors in family
5. High risk mutations such as multiple exon deletion or stop codon

4.6 Inhibitor Evaluation

The Bethesda assay is used to determine the presence of a factor inhibitor and to measure its titer. This assay uses dilutions of patient plasma with normal pooled plasma (NPP) and also control dilutions containing buffer and NPP. Following incubation, the remaining FVIII activity in the patient sample is compared to that of the control. The percent of residual FVIII activity in the patient mix is then converted to Bethesda units (BU) using an equation or read from a graph. One BU is defined as the amount of inhibitor producing a residual activity of 50% [81]. In the Nijmegen modified assay, the NPP that used in patient and control mixtures are buffered with Imidazole, and control tube contains FVIII-deficient plasma instead of buffer [81, 82]. This improves FVIII lability and helps prevent falsely elevated Bethesda results.

4.6.1 Treatment in Patient with Inhibitor

The healthcare costs associated with inhibitor development can be worrying because of the amount and type of treatment products required to stop bleeding in such patients. In addition, these patients are much likely to be hospitalized for a bleeding complication, and they are at increased risk of lethal bleeding complications [83–85].

4.6.2 The Treatment Lines

Following guidelines such as “treatment of bleeding in the presence of inhibitors” is usually recommended for patients with hemophilia with inhibitor [78].

4.6.3 Treatment of Bleeding in the Presence of Inhibitor

In persons with low-responding inhibitors and low Bethesda titers (less than 5 units), therapy with further FVIII replacement products is usually possible. In these cases, higher doses of the factor and/or more frequent (or continuous) factor infusions may be useful to overcome the effect of the antibody. However, when there is a large titer of antibody (at least 5 BU), higher doses of therapeutic FVIII or IX is usually not effective because the inhibitor can neutralize even large doses of factor. Plasmapheresis or immune absorption may be useful in eliminating inhibitors, especially when life-threatening bleeding is treated with an excess factor but a high level of inhibitor exists. However, as soon as the patient receives the factor, high concentrations of antibody develop within several days. Another approach is bypass therapy, which includes various therapeutic products such as prothrombin complex concentrate (PCC), activated PCC (aPCC), and recombinant activated FVII (rFVIIa). Therapeutic choice is based on circumstances such as inhibitor characteristics, nature, and severity of the bleed, age, and treatment response pattern of the individual (Table 4.7) [78].

Immune Tolerance Induction (ITI) Therapy: The purpose of ITI therapy is to halt the inhibitor production by the immune system of individuals with hemophilia. This method teaches the body to accept factor as a normal part of blood. In this way, patients receive more frequent, low dose factor [86]. People with low titer inhibitor (<5 BU) may be treated with higher amount or more frequent factor concentrate. Based on the inhibitor status of the patients, immune tolerance induction has been successfully used for years. High-dose (200 IU/kg/day) and low-dose (50 IU/kg × 3 times/week) ITI regimens are both effective at eliminating the inhibitor. However, bleeding manifestations are common during the administration of low-dose ITI. This bleeding problem has recently been overcome by using emicizumab prophylaxis in conjunction with low-dose ITI or reduced frequency of factor administration in a prospective, multicenter, phase IV study [87].

4.6.4 Bypassing Therapy Products

Bypassing agents, which can stimulate the coagulation cascade without the requirement for the desired factor, are used to treat bleeding complications in people with high-level inhibitors (>5 BU). Autoplex® was the first activated prothrombin complex concentrate (APCC) product. Despite the unknown mechanism of action for bypassing FVIII, Autoplex® appears to be a useful and necessary interim product that is safe and effective. To avoid thrombosis, use of fibrinolytic inhibitors at the same time must be avoided [88].

Today, the most well-known bypassing agents, such as activated prothrombin complex concentrate (APCC) (FEIBA®, anti-inhibitor coagulant complex, Shire, Dublin, Ireland) and recombinant factor VIIa (rFVIIa (NovoSeven®, eptacog alfa, NovoNordisk, Bagsvaerd, Denmark), are used to treat and prevent bleeding in hemophilic patients with inhibitors [89]. Factor eight-inhibitor bypass activity

Table 4.7 Treatment of bleeding in patients with hemophlia A and inhibitor

Inhibitor titer	Approach	Advantages	Disadvantages
<5 Bethesda units	Higher doses of the factor and/or more frequent, or continuous	May be useful to overcome the antibody	It may trigger stronger immune response and increases inhibit titer
≥5 Bethesda units with life-threatening Bleeding	Plasmapheresis or immune adsorption then factor replacement	Eliminate the risk of bleeding or lethal episodes	After the patient receives, the factor makes large amounts of new antibody within several days.
≥5 Bethesda units without is life-threatening Bleeding	Bypass therapy with (PCCs)	Bypasses the requirement for factor	As the same as APCC
	(APCC) with doses of 50–100 units/kg are every 8–24 h, depending on the severity of the bleed	Effective for 60–90% of musculoskeletal bleeds, major and minor surgery prophylaxis	1. Short-acting 2. Paradoxically causes either more bleeding or excess clotting. 3. Worsens problem if antifibrinolytic drugs are used along with it 4. Contains small amounts of FVIII and stimulates new antibody production
	Factor VII (rFVIIa). Multiple doses of 90 units/kg or more infused every 2–6 h may be required	1. Bypasses the requirement for factor 2. Effective in the prevention and treatment of joint hemorrhage, life-threatening bleeding and surgical bleeding 3. Contains no FVIII. So doesn't restimulate antibody Production	1. Short-acting 2. Excess blood clotting
	Immune tolerance induction (ITI) infusions of variable doses of FVIII or IX administered for a period of weeks to years in an effort to tolerize the immune system	Accept treatment with the missing clotting factor without producing further antibodies	Time-consuming and costly

Consequently, factor VII (rFVIIa) is frequently the bypass therapy of choice for patient with hemophilia

(FEIBA®) is also an anti-inhibitor coagulant complex, which is indicated in patients with hemophilia A and B inhibitors [90].

- Control and prevention of bleeding episodes.
- Perioperative management.
- As the prophylaxis to prevents or decreases the rate of acute bleeding.

A recent study investigated the efficacy and side effects of a new class of therapeutic drugs for hemophilia patients who have inhibitors that act by enhancing coagulation (emicizumab®) and inhibiting anticoagulant pathways (fitusiran® and concizumab®) [91]. Emicizumab is a licensed bispecific antibody that mimics factor VIII. A novel bispecific antibody that binds factor IX and factor X, partially mimicking the functional property of factor VIII in the tenase complex, has been shown to be highly effective in treating antibodies to factor VIII.

4.6.5 Salvage Treatment

Patients with hemophilia who have high inhibitor titers and persistent bleeding after major surgery may benefit from sequential bypassing agent therapy. In a previously reported case, this complication was controlled by sequential administration of recombinant factor VIIa and activated prothrombin complex concentrate [92].

4.6.6 Mouth Care in Hemophilia

For patients with severe hemophilia, factor replacement therapy is necessary before surgery or regional block injections. Some studies proposed a dose of 50 international units per kilogram of body weight (IU/kg) of FVIII before a tooth extraction in patients with hemophilia. The safe and cost-effective method to control bleeding of teeth extraction is the use of fibrin glue and anti-fibrinolytic agents such as tranexamic acid.

Lifestyle can be significantly responsible for gum diseases. Smoking and tobacco use may contribute to periodontal disease and oral cancer. Oral hygiene is the best way to prevent caries and gum diseases such as chronic periodontitis gingivitis [33, 93].

4.6.7 Bleeding Prevention Strategy

Reducing the frequency and amount of sugars in the diet, avoiding smoking, using fluoride, mouth protection and soft plastic mouth guards may be needed to prevent damage from trauma, tooth grinding (bruxism), and acid erosion in individuals with hemophilia [33, 94].

4.7 Gene Therapy

The most promising curative strategy in hemophilia is gene therapy. To be successful, the missing or defective clotting gene must be identified and replaced by a normal gene fragment, which is inserted in the genetic material of cells of the

hematopoietic system, liver, skin, muscle, or blood vessels. Because the *FVIII* gene is very large and difficult to insert compared with the *FIX* gene, hemophilia B was the first candidate to be treated by gene therapy. Several studies using test tubes and animal studies predicted that gene therapy would be effective. Recently, the FIX gene was successfully administered to the dogs with FIX deficiency [95]. A clinical trial that began in 2010 has reported long lasting conversion of severe hemophilia B to mild status by elevating factor IX levels above 5% [96]. Subsequently five other trials in hemophilia B have started using similar vectors based on Adeno Associated Virus.

The ultimate goal of treatment of gene-deficient diseases is the elimination of disease for the lifetime of the patient. Gene therapy is the best and only way to achieve this goal, which replaces the faulty gene sequence with the correct one. Continuous production of FVIII due to gene therapy is superior to non-continuous FVIII administration. This challenging task has now been accomplished. The first clinical trials attempting to treat hemophilia with gene therapy primarily demonstrated good safety, but without good efficacy. The next efforts, which re-engineered the vector plasmids and delivery systems, resulted in markedly improved outcomes in animal models of disease. Retroviral vectors that can permanently insert the *FVIII* gene into the DNA of the host cell appear the most suitable for this specific purpose (Fig. 4.8) [97, 98].

We divide the gene therapy strategies into two groups:

1. Gene replacement by an ex vivo or in vivo approach
2. Other proposed approaches

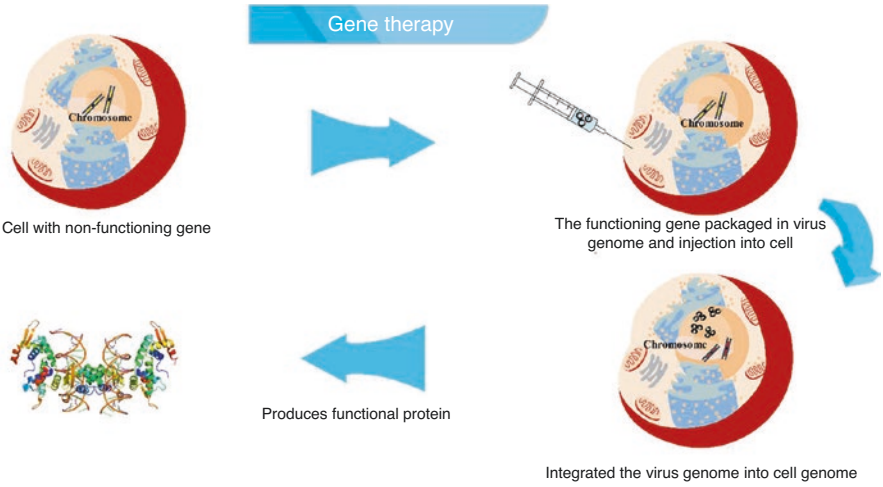

Fig. 4.8 The in vivo approach of gene therapy. The target gene packaged in virus genome, then delivered in a body organ such liver. The virus genome is integrated into cell genome and produces functional protein. (*ITR* Long terminal repeats, *PA* Palindromic repeat)

4.7.1 The Gene Replacement by an Ex Vivo or in Vivo Approach

Ex vivo and in vivo treatments for hemophilia gene therapy have been proposed. Ex vivo gene therapy involves isolating the cells from the patient, expanding and genetically modifying them in a culture medium with vectors which express FVIII, and then transferring the transfected cells to the patient's liver, skin etc. Different cell types such as skin fibroblasts, endothelial cells, epithelial cells, myoblasts, hematopoietic stem/progenitor cells, and bone marrow (BM) stromal cells have been considered as potential targets for hemophilia gene therapy [99–101].

Another particular creative target is megakaryocytes and platelets. An ex vivo hemophilic treatment gene that uses megakaryocytes and platelets has shown to be an effective homeostatic response in standard hemophiliacs [102, 103]. The initial cell capacity for transferring FVIII to the bloodstream is highly dependent on the site of implantation. The liver appears to be a suitable target for hemophilia gene therapy. FVIII produced in the transduced hepatocyte should have ready access to the circulation. In addition, the stability of FVIII increases when it is secreted in an environment with access to VWF either in the circulation or VWF produced in the sinusoidal endothelial. In contrast, coagulation factors produced in non-hepatic tissues might not be properly processed due to differences in post-translational modification. Ex vivo gene therapy is based on the use of viral vectors (especially retrovirus) and sometimes in non-viral transfer systems (Table 4.8) [104].

The in vivo gene therapy is more affordable than ex vivo. However, the host immune response towards the viral vector is the major drawback of in vivo gene therapy. The gene delivery system in the gene therapy should be efficient, safe, non-immunogenic and allow for long-term gene expression. Most importantly, the comparison between gene therapy for hemophilia A with existing protein replacement

Table 4.8 Viral-based-vectors used for gene therapy of factors VIII deficiency

Vector	Nucleic acid	Advantages	Disadvantages
Retroviral	RNA	Efficient transduction genomic integration persistent expression	Oncogene derivation random insertion cell division-dependent (except for lentiviral)
Adenoviral	DNA	Transduces non-dividing cells accommodates large cDNAs high level of don't have chronic toxicity	Immune responses to AV episomal (no integration) transient expression
Adeno-associated viral	DNA	Integration (partial) persistent expression different serotypes	Limited size of cDNA possible rearrangements pre-existing anti AAV antibody able to prevent successful transduction liver disease in patient especially HCV positives Immune response to the capsid

AV Adenoviral, *AAV* Adeno-associated viral, *HCV* Hepatitis C virus

therapies must be in favor of gene therapy [105]. Retroviral, lentiviral, adenoviral, and adeno-associated viral (AAV) vectors, each with their own benefits and limitations, are the most widely used vectors in hemophilia gene therapy. Moloney murine leukemia virus (MoMLV)-based retroviral vectors are the first and the most commonly used vectors for clinical trials [106]. Although non-viral vectors are less immunogenic than viral vectors, the viral vectors are the most chosen method since it is more manageable clinically (Table 4.7) [105]. If the immune system is stimulated, the treatment strategy includes immune-modulating drugs. For instance, inhibition of poly (ADP-ribose) polymerase-1 (PARP-1) and nuclear factor kappa B (NF-κB) signaling synergistically down regulates the immune response against recombinant AAV2 vectors. A combination of non-viral vector systems with some viral components can combine the benefits of both systems. The *FVIII* gene can be cloned, but this is too large to be packaged efficiently into any commonly used gene therapy vectors. However, there is a full-length recombinant Pegylated FVIII [107]. The B-domain deleted FVIII (FVIII-BDD) cDNA had been shown to express a higher level of FVIII protein as compared to the full-length FVIII cDNA and is suitable for the restricted packaging limits of vectors [105]. The packaging capacity of AAV is about 5 kb, but the FVIII expression cassette is about 7 kb. The use of a FVIII-BDD (4.37 kb in size) overcomes this challenge, but constrains the size of included regulatory elements that control FVIII expressions [108]. There are several FVIII-BDD products available: BAY94-9027, NovoEight, Eloctate, and others [107]. Studies in hemophilia A dogs that had developed inhibitors have demonstrated that inhibitors could be eradicated following AAV-mediated, liver-directed gene therapy [109]. Proprotein convertases are a family of proteins that activate other proteins by proteolytic activity. One member of this family is Furin [110]. When Furin processes FVIII-BDD, its procoagulant activity is degraded. This process also reduces its secretion. Therefore, inhibition of Furin leads to secretion of FVIII-BDD in mammalian cells [111]. Current challenges of gene therapy include ensuring long-term stable protein expression, vector immunogenicity, the potential risk of insertional mutagenesis, and hepatic toxicity (Fig. 4.8) [112].

4.7.2 Advantages of Lentivirus (LV) Transduction

1. Can result in a long-term therapeutic effect.
2. Some evidence suggests that a single treatment of LV-mediated gene therapy may be sufficient for life-long effect.
3. LV can transduce both non-dividing and dividing cells, and this is the most important advantage of LV in comparison with traditional retroviral vectors. This leads to a considerable increase in LV efficacy in the targeting of primitive stem cells.
4. Self-inactivating (SIN)-LVs provide improved safety by reducing transactivation capacity [113].

4.8 Adeno Associated Virus Gene Therapy for Hemophilia A

Recently, a trial of AAV gene therapy has reported that patients with severe hemophilia A treated by peripheral vein infusion of a single dose of vector achieved and have maintained normal level of factor VIII for more than a year. However, pre-existing AAV antibody is one of the most significant barriers to gene therapy in hemophilic patients. Pre-existing antibody seroprevalence, for example, was observed to be highest in Turkey [67% in Turkey, followed by patients from Dutch (27%) and Italian (14%) referral institutions]. This increased prevalence may reduce the number of potential candidates in hemophilic patients for upcoming gene treatments in some countries [114].

4.8.1 Other Proposed Approaches

1. Aminoglycoside antibiotics such as gentamycin can transiently suppress the nonsense mutations at the ribosomal level in a patient with hemophilia. This could potentially convert severe hemophilia to a moderately severe phenotype; however, this intervention has failed to show a clinically significant effect [115].
2. At the RNA level, trans-splicing could potentially be feasible and effective to correct hemophilia inducing inversions in mice [116].
3. Some prokaryotes like bacteria and even archaea use the Clustered Regularly Interspaced Short Palindromic Repeats or CRISPR and CRISPR-associated (Cas) system in their immune system. This provides the CRISPR/Cas9 genome editing technology that allows for permanent changes in the genes of organisms [117]. This process leads to the creation of a double strand break (DSB) at a distinctive region of the genome. Then The DSB(s) are typically repaired by an error-prone path, which is named non-homologous end joining (NHEJ), or error-free path, which is known as homology-directed repair (HDR). Thus, the HDR pathway could be used to correct the monogenetic diseases including hemophilia after CRISPR-induced-DSB. However, it has been reported that gene correction with NHEJ is also possible [118].

Acknowledgments We appreciate Professor Edward Tuddenham for his valuable comments that significantly improved the quality of this chapter.

References

1. Gitschier J, Wood WI, Goralka TM, Wion KL, Chen EY, Eaton DH, et al. Characterization of the human factor VIII gene. Nature. 1984;312:326–30.
2. Bell B, Canty D, Audet M. Hemophilia: an updated review. Pediatr Rev/Am Acade Pediatr. 1995;16(8):290–8.
3. Anwarul M, Yakub C. A review on hemophilia in children. Bangladesh J Child Health 2013;37(1):27–40.
4. Madhok R, York J, Sturrock RD. Haemophilic arthritis. Ann Rheum Dis. 1991;50(8):588.

5. Luck JV Jr, Silva M, Rodriguez-Merchan CE, Ghalambor N, Zahiri CA, Finn RS. Hemophilic arthropathy. J Am Acad Orthop Surg. 2004;12(4):234–45.
6. Srivastava A, Brewer A, Mauser-Bunschoten E, Key N, Kitchen S, Llinas A, et al. Guidelines for the management of hemophilia. Haemophilia. 2013;19(1):e1–e47. Moser KA. Chromogenic factor VIII activity assay. Am J Hematol 2014;89(7):781-4
7. Marlar RA, Strandberg K, Shima M, Adcock DM. Clinical utility and impact of the use of the chromogenic vs one-stage factor activity assays in haemophilia a and B. Eur J Haematol. 2020;104(1):3–14.
8. Potgieter JJ, Damgaard M, Hillarp A. One-stage vs. chromogenic assays in haemophilia a. Eur J Haematol. 2015;94(Suppl. 77):38–44.
9. Bowen D. Haemophilia a and haemophilia B: molecular insights. J Clin Pathol. 2002;55(2):127.
10. Keeney S, Mitchell M, Goodeve A. The molecular analysis of haemophilia a: a guideline from the UK haemophilia Centre doctors' organization haemophilia genetics laboratory network. Haemophilia. 2005;11(4):387–97.
11. Bihoreau N, Pin S, Kersabiec AM, Vidot F, Fontaine-Aupart MP. Copper-atom identification in the active and inactive forms of plasma-derived FVIII and recombinant FVIII-ΔII. Eur J Biochem. 1994;222(1):41–8.
12. Tagliavacca L, Moon N, Dunham WR, Kaufman RJ. Identification and functional requirement of cu (I) and its ligands within coagulation factor VIII. J Biol Chem. 1997;272(43):27428–34.
13. O'brien S, Mayewski P, Meeker L, Meese D, Twickler M, Whitlow S. Complexity of Holocene climate as reconstructed from a Greenland ice core. Science. 1995;270(5244):1962–4.
14. Bajaj MS, Birktoft JJ, Steer SA, Bajaj SP. Structure and biology of tissue factor pathway inhibitor. Thromb Haemost. 2001;86(4):959–72.
15. Macedo-Ribeiro S, Bode W, Huber R, Quinn-Allen MA, Kim SW, Ortel TL, et al. Crystal structures of the membrane-binding C2 domain of human coagulation factor V. Nature. 1999;402(6760):434–9.
16. Church WR, Jernigan RL, Toole J, Hewick RM, Knopf J, Knutson GJ, et al. Coagulation factors V and VIII and ceruloplasmin constitute a family of structurally related proteins. Proc Natl Acad Sci. 1984;81(22):6934–7.
17. Pratt KP, Shen BW, Takeshima K, Davie EW, Fujikawa K, Stoddard BL. Structure of the C2 domain of human factor VIII at 1.5 Å resolution. Nature. 1999;402(6760):439–42.
18. McMullen BA, Fujikawa K, Davie EW, Hedner U, Ezban M. Locations of disulfide bonds and free cysteines in the heavy and light chains of recombinant human factor VIII (antihemophilic factor a). Protein Sci. 1995;4(4):740–6.
19. Saenko EL, Shima M, Rajalakshmi K, Scandella D. A role for the C2 domain of factor VIII in binding to von Willebrand factor. J Biol Chem. 1994;269(15):11601–5.
20. Nogami K, Tanaka I, Shibata M, Yoshioka A, Shima M, Hosokawa K, Nagata M, Koide T, Saenko EL. Factor VIII C2 domain contains the thrombin-binding site responsible for thrombin-catalyzed cleavage at Arg1689. Journal of Biological Chemistry. 2000;275(33):25774–80.
21. Nogami K, Shima M, Hosokawa K, Suzuki T, Koide T, Saenko EL, Scandella D, Shibata M, Kamisue S, Tanaka I, Yoshioka A. Role of factor VIII C2 domain in factor VIII binding to factor Xa. Journal of Biological Chemistry. 1999;274(43):31000–7.
22. Thorelli E, Kaufman RJ, Dahlbäck B. The C-terminal region of the factor V B-domain is crucial for the anticoagulant activity of factor V. J Biol Chem. 1998;273(26):16140–5.
23. Swieringa F, Kuijpers MJ, Lamers MM, Van der Meijden PE, Heemskerk JW. Rate-limiting roles of the tenase complex of factors VIII and IX in platelet procoagulant activity and formation of platelet-fibrin thrombi under flow. Haematologica. 2015;100(6):748–56.
24. Mauser-Bunschoten EP. Symptomatic carriers of hemophilia. Treatment Hemophilia. 2008;46:1–9. World Federation of Hemophilia, 2008:No:46. https://www1.wfh.org/publication/files/pdf-1202.pdf
25. Soucie JM, Evatt B, Jackson D. Occurrence of hemophilia in the United States. Am J Hematol. 1998;59(4):288–94.

26. Manno CS. Difficult pediatric diagnoses: bruising and bleeding. Pediatr Clin N Am. 1991;38(3):637–55.
27. Mauser-Bunschoten EP. Symptomatic carriers of hemophilia. World Feder Hemophilia. 2008;46:2–3.
28. White G, Rosendaal F, Aledort L, Lusher J, Rothschild C, Ingerslev J. Recommendation of the scientific subcommittee on factor VIII and factor IX of the scientific and standardization committee of the international society on thrombosis and Haemostasis. Thromb Haemost. 2001;85:560.
29. Antonarakis SE, Rossiter J, Young M, Horst J, De Moerloose P, Sommer S, et al. Factor VIII gene inversions in severe hemophilia a: results of an international consortium study. Blood. 1995;86(6):2206–12.
30. Plug I, Mauser-Bunschoten EP, Bröcker-Vriends AH, van Amstel HKP, van der Bom JG, van Diemen-Homan JE, et al. Bleeding in carriers of hemophilia. Blood. 2006;108(1):52–6.
31. Khair K, Liesner R. Bruising and bleeding in infants and children—a practical approach. Br J Haematol. 2006;133(3):221–31.
32. Kasper CK. Hereditary plasma clotting factor disorders and their management. Haemophilia Oxford. 2000;6:13–27.
33. Jover Cerveró A, Poveda Roda R, Bagán JV, Jiménez SY. Dental treatment of patients with coagulation factor alterations: an update. Med Oral Patol Oral Cirugía Bucal. 2007;12(5):380–7.
34. Pezeshkpoor B, Oldenburg J, Pavlova A. Insights into the Molecular Genetic of Hemophilia A and Hemophilia B: The Relevance of Genetic Testing in Routine Clinical Practice. Hämostaseologie. 2022;42(6):390–9.
35. Margaglione M, Castaman G, Morfini M, Rocino A, Santagostino E, Tagariello G, et al. The Italian AICE-genetics hemophilia a database: results and correlation with clinical phenotype. Haematologica. 2008;93(5):722–8.
36. Stenson PD, Mort M, Ball EV, Howells K, Phillips AD, Thomas NS, et al. The human gene mutation database: 2008 update. Genome Med. 2009;1(1):13.
37. Salviato R, Belvini D, Radossi P, Tagariello G. Factor VIII gene intron 1 inversion: lower than expected prevalence in Italian haemophiliac severe patients. Haemophilia. 2004;10(2):194–6.
38. Schröder J, El-Maarri O, Schwaab R, Müller C, Oldenburg J. Factor VIII intron-1 inversion: frequency and inhibitor prevalence. J Thromb Haemost. 2006;4(5):1141–3.
39. Tantawy AA. Molecular genetics of hemophilia a: clinical perspectives. Egypt J Med Hum Genet. 2010;11(2):105–14.
40. Chen YC, Hu SH, Cheng SN, Chao TY. Genetic analysis of haemophilia a in Taiwan. Haemophilia. 2010;16(3):538–44.
41. Lakich D, Kazazian HH, Antonarakis SE, Gitschier J. Inversions disrupting the factor VIII gene are a common cause of severe haemophilia a. Nat Genet. 1993;5(3):236–41.
42. Levinson B, Kenwrick S, Lakich D, Hammonds G, Gitschier J. A transcribed gene in an intron of the human factor VIII gene. Genomics. 1990;7(1):1–11.
43. Naylor JA, Buck D, Green P, Williamson H, Bentley D, Gianneill F. Investigation of the factor VIII intron 22 repeated region (int22h) and the associated inversion junctions. Hum Mol Genet. 1995;4(7):1217–24.
44. Gitschier J. Molecular genetics of hemophilia a. Schweiz Med Wochenschr. 1989;119(39):1329–31.
45. Kemball-Cook G, Tuddenham EG. The factor VIII mutation database on the world wide web: the haemophilia a mutation, search, test and resource site HAMSTeRS update (version 3.0). Nucleic Acids Res. 1997;25(1):128–32.
46. Goodeve AC, Peake IR. The molecular basis of hemophilia a: genotype-phenotype relationships and inhibitor development. In: Seminars in thrombosis and hemostasis, vol. 29; 2003. p. 023–30. Copyright© 2003 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA.
47. El-Maarri O, Herbiniaux U, Graw J, Schröder J, Terzic A, Watzka M, et al. Analysis of mRNA in hemophilia a patients with undetectable mutations reveals normal splicing in the factor VIII gene. J Thromb Haemost. 2005;3(2):332–9.

48. Chandler WL, Ferrell C, Lee J, Tun T, Kha H. Comparison of three methods for measuring factor VIII levels in plasma. Am J Clin Pathol. 2003;120(1):34–9.
49. Kitchen S, McCraw A, Echenagucia M. Diagnosis of hemophilia and other bleeding disorders. A laboratory manual. World Feder Hemophilia; 2000.
50. Hillarp A, Bowyer A, Ezban M, Persson P, Kitchen S. Measuring FVIII activity of glycopegylated recombinant factor VIII, N8-GP, with commercially available one-stage clotting and chromogenic assay kits: a two-Centre study. Haemophilia. 2017;23(3):458–65.
51. Di Paola J, Smith M, Klamroth R, Mannucci P, Kollmer C, Feingold J, et al. ReFacto® and Advate®: a single-dose, randomized, two-period crossover pharmacokinetics study in subjects with haemophilia a. Haemophilia. 2007;13(2):124–30.
52. Mei B, Pan C, Jiang H, Tjandra H, Strauss J, Chen Y, et al. Rational design of a fully active, long-acting PEGylated factor VIII for hemophilia a treatment. Blood. 2010;116(2):270–9.
53. Peyvandi F, Oldenburg J, Friedman K. A critical appraisal of one-stage and chromogenic assays of factor VIII activity. J Thromb Haemost. 2016;14(2):248–61.
54. Reding M, Ng H, Poulsen LH, Eyster M, Pabinger I, Shin HJ, et al. Safety and efficacy of BAY 94-9027, a prolonged-half-life factor VIII. J Thromb Haemost. 2017;15(3):411–9.
55. Powell JS, Josephson NC, Quon D, Ragni MV, Cheng G, Li E, et al. Safety and prolonged activity of recombinant factor VIII fc fusion protein in hemophilia a patients. Blood. 2012;119(13):3031–7.
56. Turecek P, Bossard M, Graninger M, Gritsch H, Höllriegl W, Kaliwoda M, et al. BAX 855, a PEGylated rFVIII product with prolonged half-life. Hamostaseologie. 2012;32(1):S29–38.
57. Zhang Y, Limsakun T, Bensen-Kennedy DM, Veldman A, Yao Z. Population pharmacokinetic modeling and simulation of recombinant single-chain factor VIII (r VIII-singlechain) in patients with hemophilia a. Am Soc Hematol; 2014;124(21):2814
58. Evatt BL. The tragic history of AIDS in the hemophilia population, 1982–1984. J Thromb Haemost. 2006;4:2295–301.
59. Ma AD, Roberts HR, Escobar MA. Hemophilia and hemostasis: a case-based approach to management. John Wiley & Sons; 2012.
60. O'grady NP, Alexander M, Burns LA, Dellinger EP, Garland J, Heard SO, et al. Guidelines for the prevention of intravascular catheter-related infections. Clin Infect Dis. 2011;52(9):e162–e93.
61. Franchini M, Lippi G. The use of desmopressin in acquired haemophilia a: a systematic review. Blood Transfus. 2011;9(4):377.
62. Kirkiz S, Kaya Z, Gönen S, Yağcı M, Koçak U. Occurence of familial Mediterranean fever in haemophilia patients. Haemophilia. 2022; https://doi.org/10.1111/hae.14698.
63. Aledort L, Haschmeyer RH, Pettersson H. A longitudinal study of orthopaedic outcomes for severe factor-VIII-deficient haemophiliacs. J Intern Med. 1994;236(4):391–9.
64. Kavaklı K. New treatment modalities in hemophilia. Trends Pediatr. 2022;3(1):1–4.
65. Knobe K, Berntorp E. Haemophilia and joint disease: pathophysiology, evaluation and management. J Comorb. 2011;1(1):51–9.
66. Rodriguez-Merchan E. Articular bleeding (hemarthrosis) in hemophilia. In: An orthopedist's point of view. 2nd ed. Montréal: The World Federation of Hemophilia, Schulman S; 2008. p. 1–5.
67. Rodriguez-Merchan E. Ankle surgery in haemophilia with special emphasis on arthroscopic debridement. Haemophilia. 2008;14(5):913–9.
68. Rodriguez-Merchan E. Orthopaedic surgery in persons with haemophilia. Thromb Haemost. 2003;89(1):34–42.
69. Kavaklı K, Aktuğlu G, Kemahlı S, Başlar Z, Ertem M, Balkan C, Ar C. Inhibitor screening for patients with hemophilia in Turkey. Turk J Haematol. 2006;23(1):25–32.
70. Yazdani MR, Kassaian N, Ataei B, Nokhodian Z, Adibi P. Hepatitis C virus infection in patients with hemophilia in Isfahan. Iran Int J Prev Med. 2012;3(Suppl. 1):S89–93.
71. Al-Kubaisy W, Al-Naib K, Habib M. Prevalence of HCV/HIV co-infection among haemophilia patients in Baghdad. East Mediterr Health J. 2006;12(3/4):264.

72. Fulcher CA, de Graaf MS, Zimmerman TS. FVIII inhibitor IgG subclass and FVIII polypeptide specificity determined by immunoblotting. Blood. 1987;69(5):1475–80.
73. Lollar P. Pathogenic antibodies to coagulation factors. Part one: factor VIII and factor IX. J Thromb Haemost. 2004;2(7):1082–95.
74. Witmer C, Young G. Factor VIII inhibitors in hemophilia a: rationale and latest evidence. Therap Adv Hematol. 2013;4(1):59–72.
75. Wintrobe MM. Wintrobe's clinical hematology. Lippincott Williams & Wilkins; 2009.
76. Wight J, Paisley S. The epidemiology of inhibitors in haemophilia a: a systematic review. Haemophilia. 2003;9(4):418–35.
77. Astermark J, editor. Overview of inhibitors. Seminars in hematology. Elsevier; 2006.
78. DiMichele DM. Inhibitors in haemophilia: a primer. Haemophilia. 2000;6:38–40.
79. Owaidah T, Al Momen A, Alzahrani H, Almusa A, Alkasim F, Tarawah A, et al. The prevalence of factor VIII and IX inhibitors among Saudi patients with hemophilia: results from the Saudi national hemophilia screening program. Medicine. 2017;96(2):e5456.
80. Hoots W, Shapiro A. Factor VIII and factor IX inhibitors in patients with hemophilia. Waltham, MA: UpToDate; 2016.
81. Miller C, Platt S, Rice A, Kelly F, Soucie J. Validation of Nijmegen–Bethesda assay modifications to allow inhibitor measurement during replacement therapy and facilitate inhibitor surveillance. J Thromb Haemost. 2012;10(6):1055–61.
82. Duncan E, Collecutt M, Street A. Nijmegen-Bethesda assay to measure factor VIII inhibitors. Haemostasis: Methods Mol Biol. 2013;992:321–33.
83. Guh S, Grosse S, McAlister S, Kessler C, Soucie J. Healthcare expenditures for males with haemophilia and employer-sponsored insurance in the United States, 2008. Haemophilia. 2012;18(2):268–75.
84. Guh S, Grosse S, McAlister S, Kessler C, Soucie J. Health care expenditures for Medicaid-covered males with haemophilia in the United States, 2008. Haemophilia. 2012;18(2):276–83.
85. Walsh CE, Soucie JM, Miller CH. Impact of inhibitors on hemophilia a mortality in the United States. Am J Hematol. 2015;90(5):400–5.
86. Berntorp E, Shapiro A, Astermark J, Blanchette V, Collins P, Dimichele D, et al. Inhibitor treatment in haemophilias a and B: summary statement for the 2006 international consensus conference. Haemophilia. 2006;12(s6):1–7.
87. Matsushita T, Suzuki N, Nagao A, Nagae C, Yamaguchi-Suita H, Kyogoku Y, Ioka A, Nogami K. Akatsuki study: a prospective, multicentre, phase IV study evaluating the safety of emicizumab under and immediately after immune tolerance induction therapy in persons with congenital hemophilia a with factor VIII inhibitors. BMJ Open. 2022;12(3):e057018.
88. Abildgaard CF, Penner JA, Watson-Williams EJ. Anti-inhibitor coagulant complex (autoplex) for treatment of factor VIII inhibitors in hemophilia. Blood. 1980;56(6):978–84.
89. Shapiro AD, Mitchell IS, Nasr S. The future of bypassing agents for hemophilia with inhibitors in the era of novel agents. J Thromb Haemost. 2018;16(12):2362–74.
90. Tjonnfjord G, Holme PA. Factor eight inhibitor bypass activity (FEIBA) in the management of bleeds in hemophilia patients with high-titer inhibitors. Vasc Health Risk Manag. 2007;3(4):527.
91. Nogami K, Shima M. New therapies using non-factor products for patients with hemophilia and inhibitors. Blood. 2019;133(5):399–406.
92. Kaya Z, Orhan Ö, Turanlı S, Yenicesu İ, Koçak Ü, Gürsel T. Successful total hip replacement with sequential administration of bypassing agents in an adolescent boy with hemophilia a and high inhibitor titers. Blood Coagul Fibrinolysis. 2017;28(5):419–22.
93. Scully C, Dios PD, Kumar N. Special care in dentistry: handbook of oral healthcare. Elsevier Health Sciences; 2006.
94. Scully C, Dios PD, Giangrande P. Oral care for people with hemophilia or a hereditary bleeding tendency. In: Treatment of hemophilia monograph series. Montreal: The World Federation of Hemophilia; 2008. p. 10–1.
95. Wang L, Zoppè M, Hackeng TM, Griffin JH, Lee K-F, Verma IM. A factor IX-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci. 1997;94(21):11563–6.

96. Nathwani AC, Reiss UM, Tuddenham EG, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. N Engl J Med. 2014;371:1994–2004.
97. Murphy SL, High KA. Gene therapy for haemophilia. Br J Haematol. 2008;140(5):479–87.
98. Dwarki VJ, Belloni P, Nijjar T, Smith J, Couto L, Rabier M, et al. Gene therapy for hemophilia a: production of therapeutic levels of human factor VIII in vivo in mice. Proc Natl Acad Sci. 1995;92(4):1023–7.
99. Hoeben RC, Van der Jagt R, Schoute F, van Tilburg NH, Verbeet MP, Briet E, et al. Expression of functional factor VIII in primary human skin fibroblasts after retrovirus-mediated gene transfer. J Biol Chem. 1990;265(13):7318–23.
100. Fakharzadeh SS, Zhang Y, Sarkar R, Kazazian HH. Correction of the coagulation defect in hemophilia a mice through factor VIII expression in skin. Blood. 2000;95(9):2799–805.
101. Hao Q-L, Malik P, Salazar R, Tang H, Gordon EM, Kohn DB. Expression of biologically active human factor IX in human hematopoietic cells after retroviral vector-mediated gene transduction. Hum Gene Ther. 1995;6(7):873–80.
102. Swystun LL, Lillicrap D. Gene therapy for coagulation disorders. Circ Res. 2016;118(9):1443–52.
103. Shi Q, Fahs SA, Wilcox DA, Kuether EL, Morateck PA, Mareno N, et al. Syngeneic transplantation of hematopoietic stem cells that are genetically modified to express factor VIII in platelets restores hemostasis to hemophilia a mice with preexisting FVIII immunity. Blood. 2008;112(7):2713–21.
104. Rea CJ, Aledort L, Rangarajan S. Haemophilia: a race for cure. Hematol Transfus Int J. 2017;4(5):00094.
105. Ponder KP. Gene therapy for hemophilia. Curr Opin Hematol. 2006;13(5):301–7.
106. Chuah MK, Collen D, VandenDriessche T. Gene therapy for hemophilia: hopes and hurdles. Crit Rev Oncol Hematol. 1998;28(3):153–71.
107. Morfini M. A new era in the hemophilia treatment: lights and shadows! J Hematol Transfus. 2016;4(3):1051.
108. High KA, Anguela XM. Adeno-associated viral vectors for the treatment of hemophilia. Hum Mol Genet. 2016;25(R1):R36–41
109. Finn JD, Ozelo MC, Sabatino DE, Franck HW, Merricks EP, Crudele JM, et al. Eradication of neutralizing antibodies to factor VIII in canine hemophilia a after liver gene therapy. Blood. 2010;116(26):5842–8.
110. Artenstein AW, Opal SM. Proprotein convertases in health and disease. N Engl J Med. 2011;365(26):2507–18.
111. Siner JI, Samelson-Jones BJ, Crudele JM, French RA, Lee BJ, Zhou S, et al. Circumventing furin enhances factor VIII biological activity and ameliorates bleeding phenotypes in hemophilia models. JCI Insight. 2016;1(16):e8937. https://doi.org/10.1172/jci.insight.89371.
112. Powell JS, Ragni MV, White GC, Lusher JM, Hillman-Wiseman C, Moon TE, et al. Phase 1 trial of FVIII gene transfer for severe hemophilia a using a retroviral construct administered by peripheral intravenous infusion. Blood. 2003;102(6):2038–45.
113. Miao CH. Hemophilia a gene therapy via intraosseous delivery of factor VIII-lentiviral vectors. Thromb J. 2016;14(1):41.
114. Kavaklı K, Antmen B, Okan V, Şahin F, Aytaç S, Balkan C, Berber E, Kaya Z, Küpesiz A, Zülfikar B. Gene therapy in haemophilia: literature review and regional perspectives for Turkey. Ther Adv Hematol. 2022;13:20406207221104591.
115. James PD, Raut S, Rivard GE, Poon M-C, Warner M, McKenna S, et al. Aminoglycoside suppression of nonsense mutations in severe hemophilia. Blood. 2005;106(9):3043–8.
116. Chao H, Mansfield SG, Bartel RC, Hiriyanna S, Mitchell LG, Garcia-Blanco MA, et al. Phenotype correction of hemophilia a mice by spliceosome-mediated RNA trans-splicing. Nat Med. 2003;9(8):1015–9.
117. Zhang F, Wen Y, Guo X. CRISPR/Cas9 for genome editing: progress, implications and challenges. Hum Mol Genet. 2014;23(R1):R40–6.
118. Nguyen TH, Anegon I. Successful correction of hemophilia by CRISPR/Cas9 genome editing in vivo: delivery vector and immune responses are the key to success. EMBO Mol Med. 2016;8(5):439–41.

Hemophilia B: Diagnosis and Management

5

Giancarlo Castaman, Hoda Motlagh, and Behnaz Pezeshkpoor

5.1 Introduction

Blood coagulation factor IX (FIX) is a vitamin K-dependent glycoprotein that consists of 415 amino acids with a molecular weight of 57 kDa [1]. This coagulation factor that plays a vital role in coagulation cascade is converted into activated FIX (FIXa) by either tissue factor-FVII (TF-FVII) complex or coagulation FXIa, one of the contact pathway factors. FIXa, FVIIIa as its cofactor, phospholipids and calcium create intrinsic tenase complex, which transforms FX to FXa. This process plays a critical role in the propagation phase of the coagulation cascade (please refer to Chap. 1) [2].

Hemophilia B (HB) is a congenital bleeding disorder caused by mutations in the *F9* gene that is located in chromosome Xq27.1 [3]. Although HB is an inherited sex-*linked recessive* disorder, in ~30% of patients mutations are sporadic, with no family history of the disease. In addition to genetically determined defect, FIX deficiency can be an acquired defect due to vitamin-K deficiency or anti-vitamin-K treatments (e.g., warfarin), in which all vitamin K-dependent coagulation factors, FII, FVII, FIX, FX, and proteins C, S, and Z may be variably decreased [4].

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https://doi.org/10.1007/978-3-031-43156-2_5

Usually, there is a strong correlation between severity of clinical manifestations and residual FIX activity in plasma. Therefore, HB is classified according to the percentage of residual plasma FIX activity into severe (<1 U/dL), moderate (1–5 U/dL), and mild (6–40 U/dL) deficiency [5]. Patients with HB present large phenotypic heterogeneity, but hemarthrosis, muscle, and soft-tissue bleeding are usually observed in the severe deficiency and less frequently in moderate and mild cases. Life-threatening intracranial hemorrhage (ICH) and retroperitoneal bleeding are also more frequent in severe FIX deficiency [6]. Ecchymosis, epistaxis, and oral bleeding are also relatively present, especially in pediatric age. Surgery and trauma are also associated with an increased risk of bleeding according to the severity of FIX deficiency.

Patients with HB typically have a prolonged activated partial thromboplastin time (aPTT), with normal prothrombin time (PT), thrombin time (TT), and platelet count on initial laboratory examination. Factor assay must be performed to confirm the diagnosis. In addition, molecular studies can identify the causative variant, allowing also carrier detection and prenatal diagnosis (PND) [6].

Fresh frozen plasma (FFP) and prothrombin complex concentrate (PCC) were initially the therapeutic options available, followed by the production of specific plasma-derived FIX concentrates, but these products carried the risk of blood borne diseases transmission [2]. At present, prophylaxis with recombinant FIX (rFIX) and, recently, extended half-life (EHL) recombinant products is the current standard of treatment for patients with severe HB, and real world evidence studies are addressing their most appropriate time interval use according to patient clinical phenotype and life-style to improve quality of life [7, 8].

5.2 Factor IX Structure and Function

FIX is a vitamin K-dependent single chain glycoprotein (17% carbohydrates) structurally similar to FII, FVII, and FX, produced by hepatocytes in the liver. Its plasma half-life and average concentration are ~18 h and 2.5–5 μg/mL, respectively [9].

The complete sequence of *F9* gene was determined in 1985 [9]. The gene is located in the chromosome Xq27.1 and spans 33.5 kb. *F9* gene includes eight exons that transcribe to a 2.7 kb mRNA. Of this, 1.4 kb mRNA translates to a single chain precursor protein of 461 amino acids, from which the signal peptide (28 amino acid residues, responsible for protein secretion) and the propeptide (18 amino acid residues, responsible for γ-carboxylation) are removed by proteolytic cleavage. Signal peptide controls the co-translational translocation of FIX into endoplasmic reticulum lumen of hepatocytes. Propeptide contributes to initial connection of FIX to γ-glutamic carboxylase, which plays the main role in carboxylation of glutamate residues in Gla domain. However, both signal peptide and propeptide are removed to create a mature FIX zymogen glycoprotein of 415 amino acids with a 57 kDa molecular weight, which is released into the bloodstream (Fig. 5.1) [10, 11].

Circulating FIX zymogen has several domains including γ-carboxylation domain (Gla domain), epidermal growth factor-1 (EGF-1) like domain, EGF-2, activation

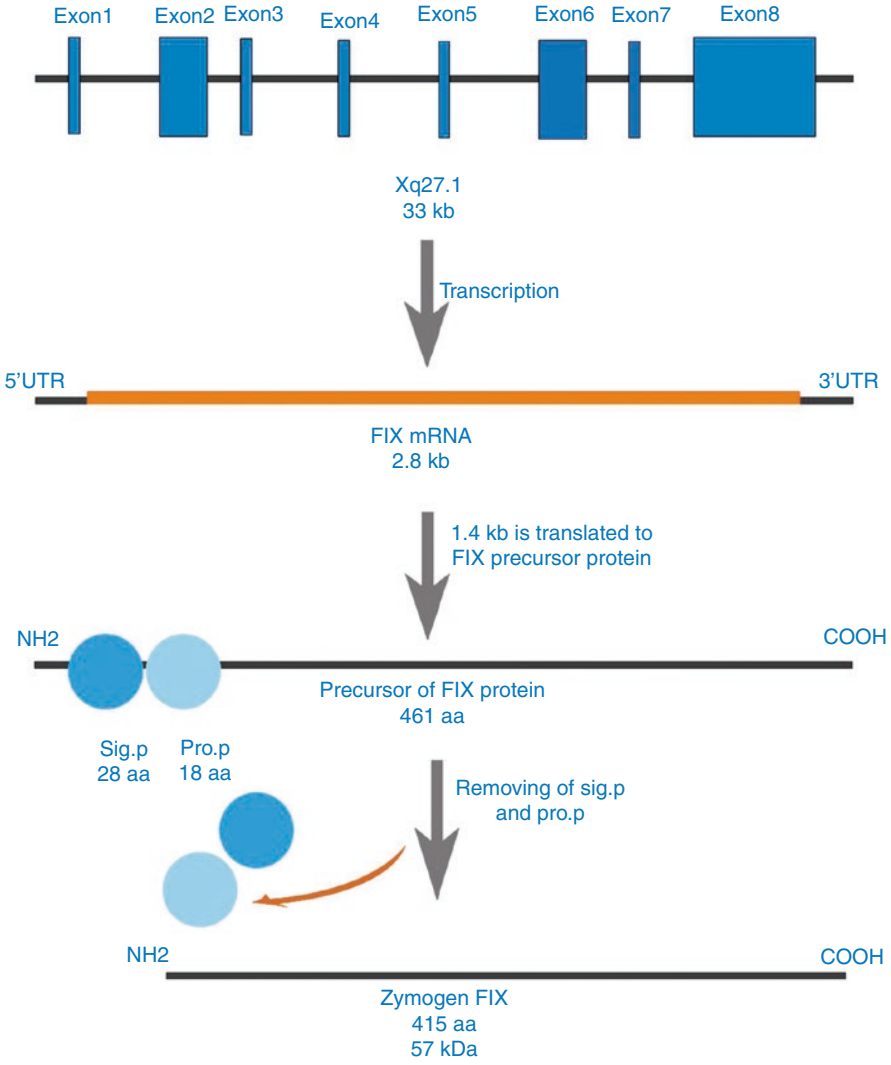

Fig. 5.1 *FIX* gene is mapped to Xq27.1. It has eight exons and spans 33.5 kilobase pairs (kb). *F9* gene is transcribed to an mRNA of 2.7 kb, from which 1.4 kb is translated to a precursor FIX with 461 amino acids. Then two domains of FIX, including signal peptide and propeptide, are removed by proteolytic cleavage. The remaining FIX zymogen has 415 amino acids with 57 kDa molecular weight. *UTR* untranslated terminal region, *aa* amino acid, *FIX* factor IX, *Sig.p* signal peptide, *Pro.p* propeptide

peptide (AP), and catalytic domain (Fig. 5.2) [12–14]. Gla domain consists of 12 glutamic acids that undergo post-translational modifications to convert to γ-carboxyglutamate through vitamin K-dependent gamma carboxylation. It is possible for the Gla domain to bind membrane phospholipids by interacting with calcium ions at these modified residues. Regions with high affinity for binding to

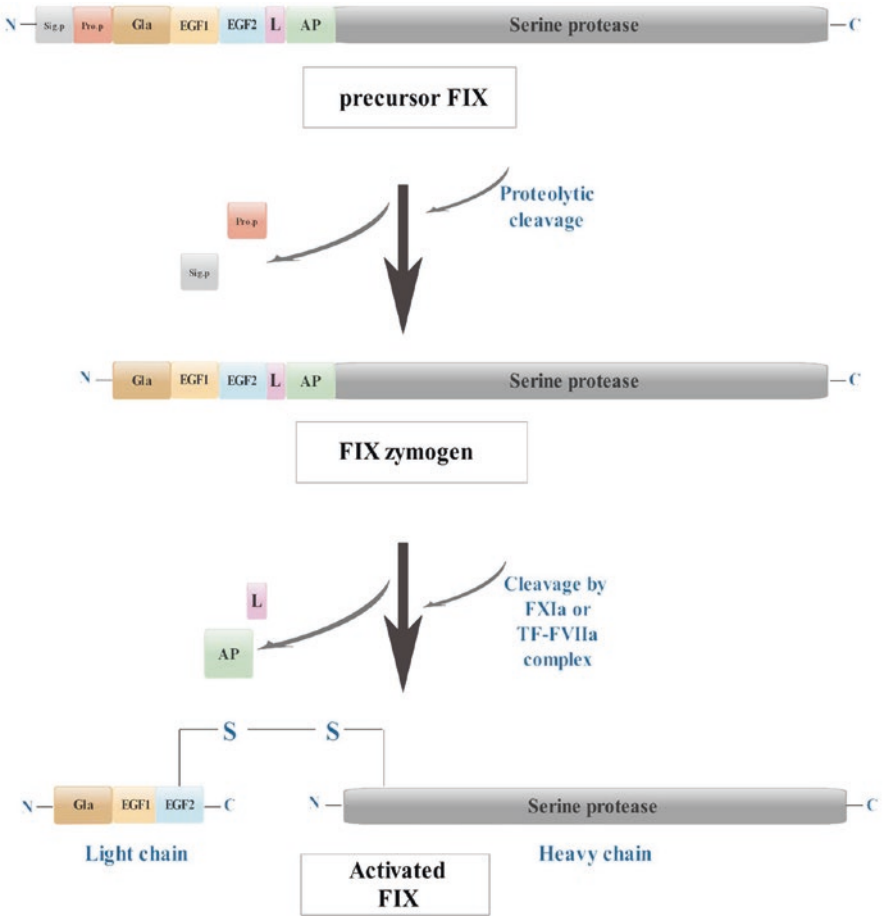

Fig. 5.2 Precursor factor IX (FIX) is synthesized in the liver, and it has several domains including Gla domain, EGF1 and EGF2 domains, and activation peptide and serine protease domains. Proteolytic cleavages remove signal peptide and propeptide containing domains. Thus initial FIX converts to FIX zymogen, which is released in circulation. Cleavage of activated peptide by activated factor XI (FXIa) or tissue factor-factor VII (TF-FVII) complex leads to conversion of FIX to activated FIX (FIXa). FIXa comprises a light chain (Gla, EGF-1, and EGF-2) and a heavy chain (serine protease domain), which are brought together by a single disulfide (*L* linker, *Gla* glutamic acid, *EGF* epidermal grow factor, *FIX* factor IX, *AP* activated peptide, *Sig.p* signal peptide, *Pro.p* propeptide, *TF-FVIIa* tissue factor-factor VII activated)

calcium were reported in EGF-1 and catalytic domains [10]. Both EGF domains, which are part of the EGF superfamily, contain six cysteine amino acids that form disulfide bridges. In order to convert FIX zymogen into FIXa, 35 amino acids of AP are removed. The catalytic domain has serine protease activity via catalytic triad of Ser411, Asp315, and His267 at the C terminus [1].

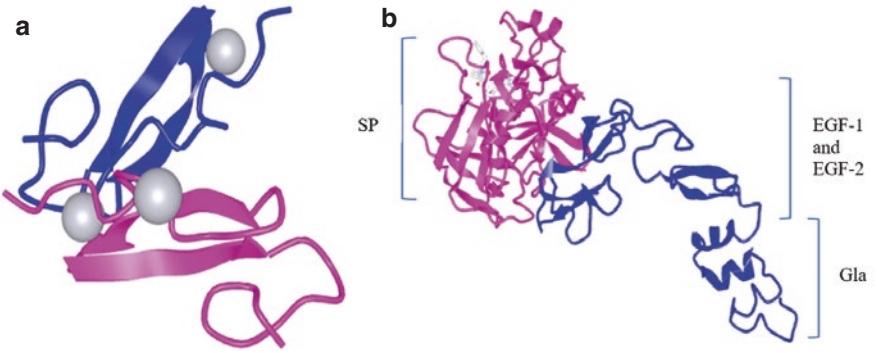

Fig. 5.3 (**a**) Epidermal Growth Factor-like (EGF) domain from human factor IX. B chain and C chain are shown in purple and blue, respectively. Calcium ions are illustrated in grey residues. (**b**) Porcine FIX domains. The blue color shows light chain, which includes Gla domain, EGF-1, and EGF-2 domains. The serine protease (SP) domain, which is the catalytic region of the coagulation factor, is shown in purple color

Although human FIXa crystal structure has not been identified, a structure model can be constructed by considering porcine FIXa (84% identity). It includes three partial structures of human FIXa including Gla, EGF-1, and EGF2-SP domains (Fig. 5.3). According to these findings, FIXa comprises a light chain with three domains (Gla, EGF-1, and EFG-2), and a heavy chain with one domain (catalytic domain), which are linked together by a single disulfide bond (Cys178-Cys335) [13].

The FIX light chain, which includes Gla, EGF-1, and EGF-2 domains, forms a stalk that connects Gla to phospholipid membranes. The catalytic domain sits above the structure, and FVIIIa interacts with the catalytic domain as well as key residues within the EGF-1 and EGF-2 domains in order to fulfill its cofactor function [15, 16].

FIX is activated either by FXI (intrinsic pathway) or TF-FVII complex (extrinsic pathway). Both pathways lead to the removal of AP by cleavages of peptide bonds after Ar145 and Arg180 residues resulting in the release of FIXa in plasma [17, 18]. FIXa function is dramatically enhanced by 50,000 folds when it binds to its cofactor, FVIIIa, and its bond with membrane phospholipids also enhances its activity. FIX, which is an integral component of intrinsic tenase complex containing FVIIIa, calcium, and phospholipids, cleaves an Arginine-Isoleucine bond to transform FX into FXa (Fig. 5.4). FXa then initiates the conversion of prothrombin to thrombin, which ultimately results in clot formation. Therefore, genetic mutations within the *F9* gene cause quantitative (type I) or qualitative FIX deficiency (type II) and lead to hemophilia B [13].

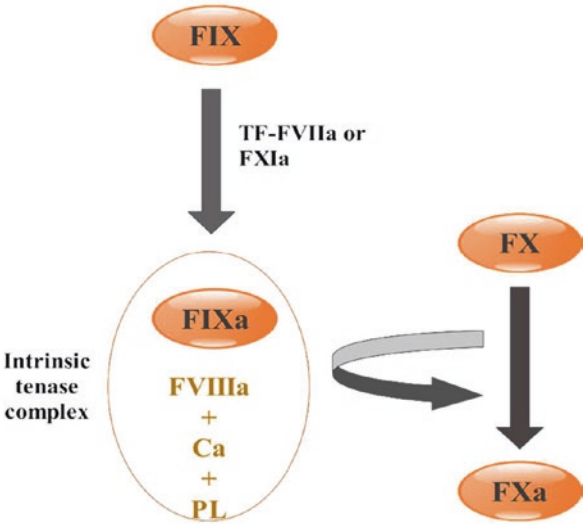

Fig. 5.4 Factor IX activated by tissue factor-factor VIIa complex or factor XIa, leads to formation of intrinsic tenase complex that converts factor X to activated FX. (*Ca* calcium, *PL* phospholipids, *TF* tissue factor, *FIX* factor IX, *FIXa* activated factor IX, *FVIIIa* activated factor VIII, *FX* factor X)

5.3 Hemophilia B

Hemophilia B or Christmas disease is a congenital X-chromosome linked bleeding disorder, which arises from different mutations in *F9* gene located in Xq27.1 [11, 19]. The overall incidence of HB is 1 per 30,000 live male births, whereas hemophilia A (HA) has a prevalence of one per 5000 [20]. Although most cases of HB have a clear family trait, 30% of cases are due to sporadic mutations and have no family history for the disease. Indeed, sporadic mutations may occur due to: (1) generation of a new allele in *F9* gene because of a mutation in egg or sperm cells; in such cases, a mother can be either heterozygous or non-heterozygous; (2) a mother with single affected allele in *F9* gene with unknown family history; and (3) maternal mosaicism [21].

As a result of the X-linked recessive inheritance pattern, a male with a single mutant allele and a female with two mutant alleles (although rare) both have hemophilia. Females with defect in one allele are carriers of this genetic abnormality. With a hemophilic father and a normal mother, all sons will be non-hemophilics, while daughters will be obligated hemophilic carriers. On the other hand, when a father has no mutant X chromosome and the mother is a carrier, 50% of their sons will have the mutant allele, and 50% of their daughters would be carriers (Fig. 5.5) [1].

Hemophilia B is characterized by the occurrence of spontaneous or secondary bleeding in response to trauma or surgery. Patients with severe HB present with recurring joint bleeding, soft-tissue hematoma, and, rarely, life-threatening manifestations such as ICH, retro-peritoneal, or gastrointestinal bleeding. Recurrent joint

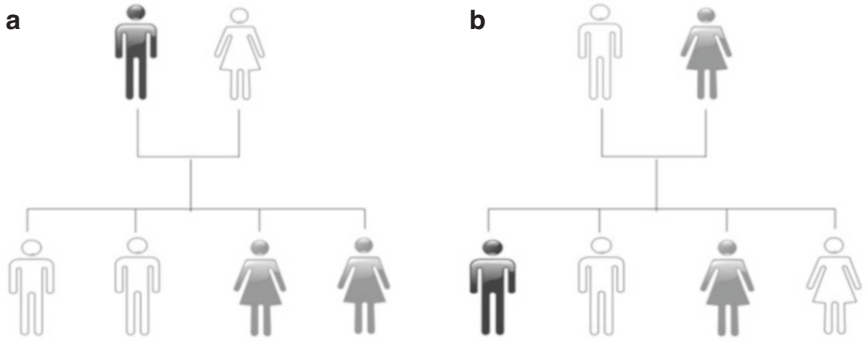

Fig. 5.5 Inheritance pattern in hemophilia B. (**a**) In a family with a hemophilic father and non-hemophilic mother, all daughters are genetic carriers and all sons are non-hemophilic. (**b**) In the case of a non-hemophilic father and a carrier mother with one mutant X-chromosome, 50% of sons are hemophilic and 50% of daughters are hemophilic carriers. Black: hemophilia patient, White: non-hemophilia, Grey: carrier

bleeds may lead to chronic disabling arthropathy. Ilio-psoas muscle bleeding may be potentially severe as to cause femoral nerve palsy or compartment syndrome. Diagnosis of disorder is based on clinical presentation, family history and appropriate laboratory approaches. aPTT is variably prolonged according to the severity of FIX activity reduction, which is usually tested by one-stage clotting assay. Molecular analysis can be used for diagnosis of the disorder in well equipped laboratories [11].

5.4 Molecular Basis

FIX gene is located on Xq27.1 and comprises eight exons and seven introns. Over 1000 distinct variants have been reported within *F9* gene based on FIX variant database [3]. The diagnosis of the causative mutations can potentially help to predict the development of inhibitors, and can help with genetic counseling and precise prenatal diagnosis. Mutations have been observed in exons, introns, or untranslated regions of the gene, with most of them occurring in the coding region of the FIX domains. According to CDC hemophilia mutation project (CHBMP), missense mutations account for the majority (58%), followed by frameshift mutations (16%), splice site changes (10%), nonsense mutations (8%), and others such as large and small structural changes and promoter changes (less than 5% each) (Fig. 5.6) [21]. Therefore, unlike HA, the most common abnormalities in HB are missense mutations, which often result in a normal or slightly reduced FIX antigen level (type II mutations or CRM^+) [20].

There are some hotspot regions within *F9* gene that are more prone to the occurrence of genetic alterations. For instance, the high rate of mutations in regions with high frequency of Cytosine and Guanine (CG-hotspots), typically near the initiation transcript region, revealed that they are mutational hotspots. *F9* gene mutations are

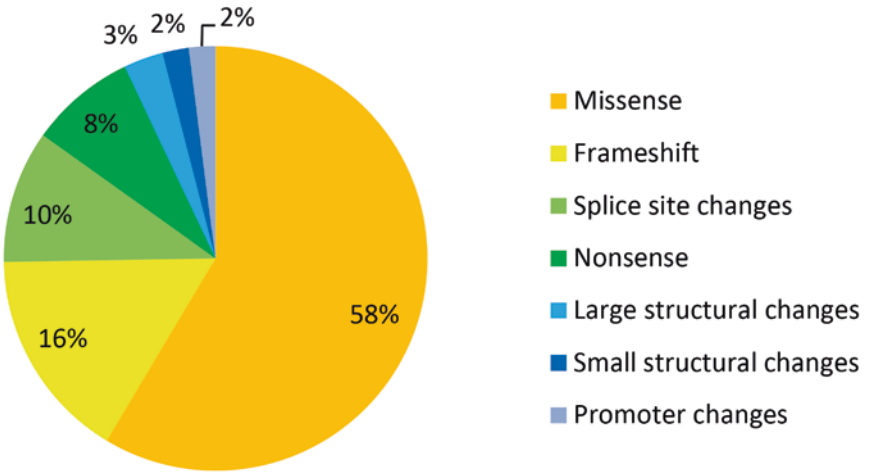

Fig. 5.6 The pie chart illustrates percentage of genetic abnormalities in patients with hemophilia B. Missense mutations are mostly prevalent (58%). Prevalence of other abnormalities reported within F9 gene are: Frameshift (16%), splice site (10%), Nonsense (8%), and other less than 5%

classified in two main types: type I and type II, which lead to quantitative deficiencies and qualitative defects, respectively. Type I mutations often result in reduced transcription and FIX secretion and can cause severe HB [1].

FIX mutations involving the signal peptide and the propeptide account for 6.5% of all mutations. A signal peptide (residues 1–28) directs intracellular trafficking of the protein and has no effect on the protein function, and therefore, its mutations lead to impaired FIX secretion (Type I). A mutation in the triad residues of the serine protease domain of the FIX gene results in a defective protein with decreased catalytic activity (Type II), which results in severe HB and increased susceptibility to inhibitor formation. Additionally, mutations in propeptide (residues 29–46), which mediates interaction with the vitamin K-dependent γ-carboxylase, lead to impaired phospholipid binding capacity that manifests as mild or moderate disease [13].

Generally, nonsense mutations are associated with severe type I defect, without any protein production. Nevertheless, a nonsense mutation occurs in C-terminal end results in producing a non-functional FIX. Although a splicing defect creates a variety range of disease severity, a single point mutation that impairs a splice donor site or occurs in the sequence of an intron, which creates an alternative splice acceptor site, and results in a nonsense mRNA and severe HB [1].

There are some mutations in the *F9* promoter that cause permanent low level of FIX throughout patient's life. However, in HB Leyden with more than 20 different identified mutations in proximal *F9* gene promoter, a severe FIX deficiency is present at birth. The FIX level starts to increase in the second decade of life at the onset of puberty, and finally reaches near normal levels in the third decade of life, remaining stable throughout life. In the past, it was thought that the change in FIX level

was associated with androgen receptors, but an increase in FIX level in some patients before puberty and in heterozygous females indicates that there is an independent X-linked mechanism [21, 22].

A small insertion or deletion (Indels) usually occurs within introns and is associated with dinucleotide repeats without affecting FIX protein. However, these indels can sometimes occur in regulatory regions or cause frameshift, resulting in severe HB.

Since inhibitor formation is influenced by the type of genetic abnormalities, a null allele that causes complete deletion of *F9* gene has the highest prevalence of inhibitor development [23]. Although the overall rate of inhibitor formation against therapeutic products in hemophilia B is up to 10%, it can be increased to more than 30% in cases with complete deletion of *F9* gene [13].

5.5 FIX Propeptide Mutation-Associated Hypersensitivity to Coumarin Therapy

There are two missense mutations in FIX propeptide, which influence the risk of significant bleeding after coumarin therapy. These mutations occur in Ala-10 locus, which is a highly protected site in vitamin K-dependent clotting factors (FII, FVII, FIX, and FX). These two point mutations, Ala-10(GCC) → Val(GTC) and Ala-10(GCC) → Thr(ACC) in exon 2 of *F9* gene, result in a propeptide for which carboxylase has low affinity and therefore exhibit extremely low FIX:C after coumarin therapy. It is considered that these patients have normal phenotype and coagulation screening test without any bleeding episodes in their lives. They only show a defect once oral anticoagulant therapy like Warfarin is given. After receiving anticoagulant, they would show extreme falls of FIX:C to levels as low as 0–3% and an abnormal aPTT prolongation, even when INR is in the therapeutic range. For other patients, FIX:C usually declines to 15–30% after coumarin therapy like other vitamin K-dependent factors [24–26].

Prothrombin complex factors (FIX, FII, FVII, and FX) require γ-carboxylation in NH2 terminal of mature protein for connection to membrane phospholipids. The propeptide is a carboxylase recognition site, and their connection is a key step of the reaction (Fig. 5.7). In cases with propeptide mutations, although the affinity of carboxylase enzyme is reduced, its activity is normal in the absence of coumarin therapy. Therefore, it could be the probable cause of normal FIX:C and lack of bleeding before treatment with anti-vitamin K oral anticoagulants as Warfarin. After coumarin therapy, in addition to low affinity, the activity of enzyme drops because of inhibition of vitamin K reductase and lack of KH2 as cofactor (Table 5.1). Therefore, FIX:C falls down to less than 1–3%, with an abnormal aPTT prolongation and severe bleeding manifestations after treatment with coumarin [24–26].

Propeptide mutations in other vitamin-K dependent clotting factors present less coumarin sensitivity due to their recessive autosomal inheritance (unlike FIX gene, which is located on X chromosome). Consequently, these variants will have a considerable impact in males. As a result, in cases with an unusual bleeding pattern

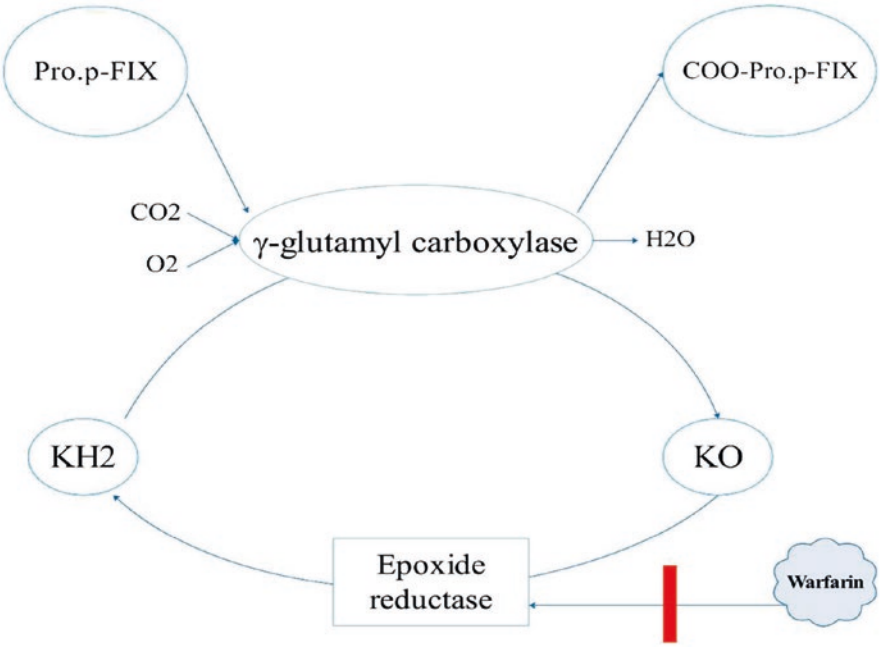

Fig. 5.7 The mechanism of vitamin K-dependent clotting factor carboxylation. The propeptide is the carboxylase recognition site, and after connection, KH2 is used as enzyme cofactor. Generated KO converts to KH2 through epoxide reductase function. It is the target enzyme for inhibition by warfarin. (*Pro.p* propeptide, *FIX* factor IX, *KH2* dihydro vitamin K, *KO* epoxide vitamin K)

Table 5.1 Activity and affinity of carboxylase enzyme in wild type FIX propeptide and mutant FIX propeptide in the presence and absence of coumarin

	Wild type FIX propeptide		Mutant FIX propeptide	
Enzyme characteristics[a]	Activity	Affinity	Activity	Affinity
Without warfarin	N	N	N	L
Warfarin therapy	L	N	L	L

In the absence of warfarin, although enzyme activity and affinity are normal in wild type FIX propeptide, affinity of enzyme is reduced in mutant propeptide. In wild type, enzyme activity is reduced during warfarin treatment. Low activity and low affinity during warfarin therapy in mutant FIX propeptide are the main causes of bleeding complications

[a]Enzyme is referred to as γ-glutamyl carboxylase; *N* normal, *L* low, *FIX* factor IX

after treatment with oral vitamin K antagonist anticoagulants, aPTT and FIX:C should be evaluated, even if INR is in the therapeutic range [25, 26].

5.6 Clinical Manifestations

According to FIX coagulant activity (FIX: C), HB is classified into three types: severe (FIX:C < 1 U/dL or 1%), moderate (FIX:C 1–5 U/dL or 1–5%), and mild (FIX:C 6–40 U/dL or 6–40%) deficiency. Usually, there is a strong correlation

Table 5.2 Classification and characteristics of hemophilia B according to residual plasma factor IX activity

Factor IX activity	Disease severity	Prevalence	Clinical symptoms	Bleeding frequency	Mean age at diagnosis
<1 U/dL	Severe	60%	Spontaneous joint or deep muscle bleeding	Up to 2–5 per month	<2 years
1–5 U/dL	Moderate	15%	Seldom spontaneous bleeding. Usually after minor trauma	Vary from 1 per month to 1 per year	2–5 years
6–40 U/dL	Mild	25%	No spontaneous bleeding. Usually bleeding after severe trauma, surgery, tooth extraction	Vary from 1 per year to 1 every 10 years	Often later in life

Table 5.3 reports genotype-phenotype correlation in HB [28]

Genetic abnormality	Severity of disease
Frame shifts	Severe (95%)[a]
Nonsense mutations	Severe (84%)[a]
Large deletions	Severe (100%)[a]
Splicing mutations	Severe (100%)[a]
Missense mutations	Severe, moderate, or mild deficiency, depending on the genetic abnormality location

[a]The percentage refers to phenotype caused by the mentioned genetic abnormality

between severity of clinical manifestations and residual FIX activity in plasma. Patients with HB present large phenotypic heterogeneity, but hemarthrosis, muscle, and soft-tissue bleeding are usually observed in severe deficiency and less frequently in moderate and mild cases. Life-threatening intracranial hemorrhage (ICH) and retroperitoneal or gastrointestinal bleeding are also more frequent in severe FIX deficiency [27]. Recurrent joint bleeds may lead to chronic disabling arthropathy. Ilio-psoas muscle bleeding may be potentially severe as to cause femoral nerve palsy or compartment syndrome. Muscle contractures and pseudo-tumors that result in disability and chronic pain are observed in elderly patients inadequately treated in the past [20].Ecchymosis, epistaxis and oral bleeding are also relatively present, especially in pediatric age. Surgery and trauma are also associated with an increased risk of bleeding according to the severity of FIX deficiency and often are the presenting symptoms in patients with mild deficiency (Table 5.2) [3, 13, 19] (Table 5.3).

Generally, HB is a less severe bleeding disorder with lower bleeding frequency and better long-term outcomes than HA [29, 30]. Almost one-third of heterozygous women are classified as symptomatic carriers because their FIX activity is between 40 and 60 U/dL, while those with levels similar to affected males are equally classified as women with hemophilia of different severity. Their clinical manifestations are similar to mild HB patients and usually mild, with heavy menstrual bleeding playing an important role in influencing the quality of life [1].

5.7 Diagnosis

The age of patients with HB at diagnosis is directly associated with the severity of factor deficiency. In the case of severe hemophilia, usually diagnosis occurs at age ≤2 years. Moderate hemophilia typically is diagnosed at age of 2–5 years, while mild deficiency is often diagnosed later in life, often for incidental reasons (bleeding after trauma or surgery, pre-surgery screening). Generally, the diagnosis of HB is based on the clinical manifestations, family history, and appropriate laboratory approaches. Since recurrent bleeding episodes are relatively similar in various coagulopathies, proper laboratory assessment is required for correct and timely diagnosis of the disorder [6]. Complete blood count (CBC) is normal in HB; however, a reduction of hemoglobin level may be observed in the presence of recurrent or significant bleeding. In screening coagulation tests, aPTT is variably prolonged according to the severity of FIX activity reduction, which is usually tested by one-stage clotting assay. Prothrombin time is normal. Diagnosis of HB should be differentiated from vitamin K deficiency, heparin use or contamination in sample tube, VWD, and hemophilia A, which all present an aPTT prolongation (Table 5.4) [31].

Factor assay must be used to assess FIX activity level in HB [32]. There are two methods for FIX activity assay including: one-stage clotting assay, which is based on aPTT, and chromogenic assay. The former is a traditionally commonly used method, whereas the later is rarely used for HB compared to HA. According to recent reports from seven countries, chromogenic assay is used in 68% laboratories for HA, while only 11% of laboratories used this method for HB [33].

The FIX clotting assay (FIX:C) requires blood collected in citrated tubes and immediately centrifuged at 2000 × *g* for 20 min. Then, plasma is separated and frozen at −70 °C [33]. After thawing, FIX:C can be measured by a one-stage or a chromogenic assay [34]. The one-stage assay is an aPTT-based method and the most routinely used one. FIX-deficient plasma and patient plasma are preincubated with aPTT reagent for 3–5 min. Once calcium has been added, the clotting time should be measured. The result of the patient's plasma clotting time is compared to a standard curve generated from plasma samples with determined FIX activity. At least three dilutions of each patient's plasma should be measured to analyze parallelism between standard dilutions and patient plasma dilutions. Unless an inhibitor is present, the two lines should be parallel [31].

Chromogenic assay includes two steps: initially, a reaction mixture consisting of FXIa, thrombin, phospholipid, and calcium chloride is added to the patient's plasma with unknown FIX activity. The amount of generated FXa is proportional to the residual plasma FIX. In the second step, a specific peptide of FXa, nitroanilin substrate, is measured through a peptide cleavage. Generated p-nitroanilin is analyzed photometrically at 405 nm absorbance length. According to a standard curve, the generated color is directly proportional to the amount of functional FIX in plasma (Fig. 5.8) [31, 33].

In one study in patients with HB, a discrepancy between the results with the two activity assay methods was not observed in severe HB patients. On the contrary, patients with nonsevere HB who had mutations at the N-terminus of the activation

Table 5.4 Differential diagnosis of hemophilia B

Bleeding disorders with a low FIX activity level				
Disease	PT	aPTT	Cause of disease	Differentiation
Combined vitamin K-dependent factors deficiency	↑	↑	Genetic abnormalities in *GGCX* and *VCORC1* genes	Prolonged PT, multiple coagulation factor deficiencies(factor II, VII, IX, X), autosomal recessive inheritance
Acquired vitamin K-dependent factor deficiency	↑↑	↑	Warfarin treatment, liver disease	Prolonged PT, multiple coagulation factor deficiencies (factor II, VII, IX, X)
Bleeding disorders with prolonged aPTT and a normal FIX activity level				
Hemophilia A	N	↑	Genetic abnormalities in *F8* gene	It is undistinguishable clinically from HB. A decreased FVIII activity level (<40%) with normal VWF level
Von Willebrand disease	N	↑or N	Genetic abnormalities in *VWF* gene	Specific von Willebrand factor assays
FXI deficiency	N	↑	Genetic abnormalities in *F11* gene	A specific FXI activity assay, which is normal in HB.
FXII, prekallikrein or HMWK deficiencies	N	↑	Genetic abnormalities in underlying gene	Do not cause any clinical bleeding, aPTT severely prolonged
Prothrombin (FII), FV or FX deficiencies	↑	↑	Genetic abnormalities in underlying gene	Specific coagulation factor assay
FVII deficiency	↑	N	Genetic abnormality in *F7* gene	Prolonged PT with normal aPTT
Inherited fibrinogen disorders	↑	↑	Genetic abnormality in *FGA*, *FGB* or *FGG* gene	Usually prolonged PT, aPTT, TT, RT
FXIII deficiency	N	N	Genetic abnormality in *F13A* or *F13B* genes	All routine coagulation tests are normal, specific FXIII assay must be used
Inherited platelet function disorders	N	N	Underlying gene defect	aPTT normal, platelet aggregation assays, flow cytometry, and platelet electron microscopy could be used for differential diagnosis

FIX factor IX, *PT* prothrombin time, *aPTT* activated partial thromboplastin time, *TT* Thrombin time; *RT* Reptilase time, *GGCX* Gamma-Glutamyl Carboxylase, *VKORC1* vitamin K epoxide reductase complex, subunit 1, *N* normal, *VWF* von willebrand factor, *VWD* von Willebrand disease, *HMWK* high molecular weight kininogen

peptide and propeptide domains of FIX had a two-fold greater levels by the chromogenic assay compared to one-stage assay [33].

Different aPTT reagents have no impact on discrepancies between these assays. There are actual advantages and disadvantages with each method (Table 5.5). The use of both assays contributes to an accurate diagnosis and classification of HB [33] (Table 5.6).

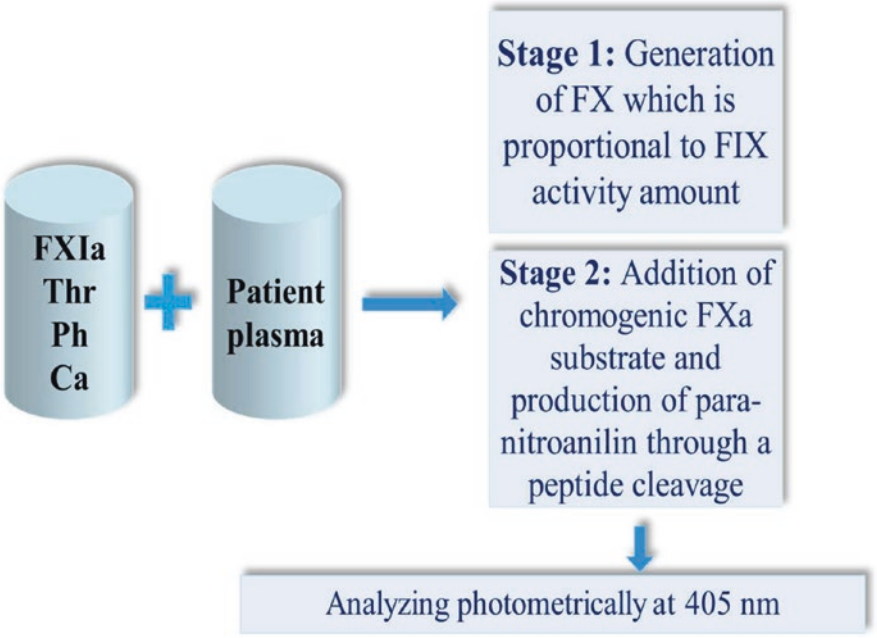

Fig. 5.8 Principle of factor IX (FIX) chromogenic assay. A reaction mixture (FXIa, Thr, Ph, and Ca) is added to patient's plasma with unknown FIX activity. In the first stage, generated FX is directly proportional to FIX activity in patient's plasma. In the second stage, a peptide cleavage of chromogenic FXa substrate, leading to generation of para-nitroanilin and therefore analyzing of this color in absorbance of 405 nm. *FXIa* activated factor XI, *Thr* thrombin, *Ph* phospholipid, *Ca* calcium, *FX* factor X, *FXa* activated factor X, *FIX* factor IX

Table 5.5 Advantages and disadvantages of one-stage assay and chromogenic assay

	One-stage assay	Chromogenic assay
Advantages	Simple, readily automated	Added thrombin allows unlimited FIX activation. High dilution of coagulation factors leading to reduction of interferences of heparin, anticoagulants, LAC
Disadvantages	Influenced by lipemia, heparin, direct thrombin inhibitors, direct FXa inhibitors. Differences between laboratories in results owing to using of different aPTT reagents, instruments and FIX deficiency plasma	More expensive, influenced by direct FXa inhibitors which cause false reduction

FIX factor IX, *LAC* lupus anticoagulant, *FXa* activated factor X, *aPTT* activates partial thromboplastin time

Table 5.6 Chromogenic assay reagents for FIX assay

Company	Kit	Country
Diagnostica Stago	STA-R FIX kit	France
Diagnostica Stago	Asserachrom IX:AG	France
Aniara	BIOPHEN chromogenic factor FIX:C kit	USA
Siemens healthcare	Unsp[a]	Germany
Rossix	Unsp[a]	Sweden

[a]*Unsp* unspecified

To assess inhibitor development in response to factor replacement therapy or when the presence of inhibitor is suspected, a mixing test is performed, in which normal pooled plasma as a source of FIX is mixed with patient's plasma.

After incubating this mixture at 37 °C for 1 h, aPTT is performed. Lack of aPTT correction of the mixture suggests that the presence of inhibitor and the Nijmegen assay to determine the inhibitor titer is required. One unit of inhibitor is the amount of inhibitor that will inactivate 50% or 0.5 unit of FIX activity over 10 min at 37°. This assessment helps to make a correct decision for management of patients with HB with inhibitor [6, 31].

If the mother is a known carrier of HB, a chorionic villus sampling (CVS) and amniocentesis can be performed to screen the fetus. Such cases should undergo coagulation tests after birth to determine clotting factor levels. While a low level of FVIII indicates HA, FIX results are not so conclusive. Because a normal level of FIX is reached approximately 6 months after birth, so the interpretation of FIX results at birth must take this into account. Thus, a mild decrease in FIX level at birth is not indicative of HB, while a marked reduction of FIX level (<1 U/dL) suggests the disorder [1].

Molecular analysis can confirm the presence of the disorder and provide valuable data about carrier detection, prenatal diagnosis (PND), and prediction of inhibitor formation, thus assisting with the management of the disorder [29]. Since there is no common reported mutation within *F9* gene, for molecular diagnosis, full *F9* gene sequencing is required. The DNA amplification by polymerase chain reaction (PCR) and then direct sequencing is a well-standardized procedure for molecular analysis; however, large deletions or other gross abnormalities due to the existence of other normal allele could not be detectable by this method. Multiplex ligation-dependent probe amplification (MLPA) and multiplex amplifiable probe hybridization (MAPH) could be used to detect *F9* gene mutations [35, 36].

5.8 Treatment

In the past, fresh frozen plasma (FFP) and prothrombin complex concentrate (PCC), which in addition to FIX contain other coagulation factors, were the only treatment available for patients with HB. PCC may cause a marked activation of coagulation cascade, with potential risk of thrombosis, while FFP may cause fluid overload and

risk of blood borne diseases, particularly before the era of virus screening in the donors and virucidal methods applied to plasma-derivatives [1, 2].

Next achievement for HB treatment was specific replacement therapy, either using plasma-derived or recombinant (rFIX) FIX concentrates. The purpose of replacement therapy is achievement of plasma FIX level to 60–80% and 20–40% for major and minor bleeding, respectively. There are several plasma-derived and recombinant FIX products for the treatment of HB (Table 5.8) [37]. Plasma-derived FIX allowed to start self-infusion and home-therapy, thus improving the life of patients. rFIX that is available since 1999 removed the risk of animal and human infectious agent transmission [38]. Notwithstanding the safety and effectiveness of unmodified FIX products, which require high injection frequency for prophylaxis, this approach became the standard of care for HB patients [39]. Standard dosing regimen for conventional rFIX prophylaxis is two injections per week that may be modified according to the pharmacokinetic profile and patient bleeding phenotype. The main obstacle regarding this procedure is frequent injections, which can be more challenging in children whose venous access is difficult as well as the fear, pain, and management issues related to needle-based injection of these products, which may cause poor adherence to prophylactic regimes [6].

The subsequent breakthrough of treatment in HB is replacement therapy using extended half-life (EHL) rFIX products. These novel products allow for prolonged dose intervals, which reduce the treatment burden [39]. Three different technologies have been used to increase the post-injection stability of rFIX including glycopegylation, Fc fusion, and albumin fusion [40]. In 2014, for the first time, Food and Drug Administration (FDA) approved a long acting rFIX Fc fusion protein (rFIXFc) as first EHL rFIX, which meets the current goal of producing a FIX with extended half-life in circulation, with dosing once weekly (50 IU/kg) or every 10–14 days, according to the patient's requirements [41]. rFIXc comprises a single chain FIX that is recombinantly connected to constant region (Fc) of IgG, and it has a half-life ~50 h compared to nearly 18 h of standard rFIX. Fc domain protects protein from catabolism via binding to Fc receptors (FcRn). These receptors protect IgG from degradation [37]. A novel product, BIVV002, is a new rFIXFc product designed to prolong FIX further, not available yet [42]. During a 4 year follow up of patients treated using rFIXFc, there was not any inhibitor formation resulting in confirmation of its safety and efficacy for previously treated patients with severe HB of all ages [43, 44].

GlycoPEGylated rFIX is another EHL rFIX product, which provides twofold recovery and five-fold longer half-life than conventional rFIX. PEG covalently connects to AP domain. PEGylation of this protein in this special site saves its biological activation and prolongs its circulation time with lower frequency of injection. Nanocog beta pegol (N9-GP) is an EHL FIX connected to poly ethylene glycol

Table 5.7 Recommended dosage of factor IX concentrate for treatment of hemophilia B

Factor IX dose (IU/kg)	Type of hemorrhage
20–40	Mild or moderate hemarthrosis or hematoma
40–60	Severe hemarthrosis or hematoma External bleeding with anemia Moderate posttraumatic bleeding
50–100	Cranial trauma Cerebral hemorrhage Surgery prophylaxis
30–40 twice weekly (recombinant or plasma-derived) 50 once weekly 50–60 once weekly or 10–14 days (recombinant EHL)	Primary or secondary prophylaxis
Factor IX dose (IU/kg)	**Site of hemorrhage**
30–40	Muscle
50–80	Joint
40–60	Gastrointestinal tract
30–40	Oral mucosa
30–50	Epistaxis
70–100	Hematuria
100	Retroperitoneal
100	CNS bleeding
100	Trauma or surgery

CNS central nervous system bleeding

molecule with 40 kDa molecular weight [45]. Despite 40 IU/kg injection weekly, fewer bleed and no evidence of inhibitor formation is reported. Moreover, it is safe and effective in the pre-operative setting [46–48].

A third type of EHL FIX fusion protein is albumin-rFIX (rFIX-FP), which has five-fold increased half-life compared with standard rFIX, allowing injection intervals up to 14 and, less frequently, 21 days, with an average dosing of 40–50 IU/kg [49–51].

Replacement therapy can be administered either on-demand or as prophylaxis [37]. The former occurs when bleeding is present, while the latter refers to a regular protein injection in the absence of bleeding as a protective approach. Currently, prophylaxis is the standard of care for HB that reduces bleeding episodes, prevents hemophilic arthropathy, and improves quality of life in patients compared to on-demand treatment [37, 39]. Recommended dosing of rFIX concentrate for HB treatment is shown in Table 5.7 [52]. Common prophylaxis with standard FIX products is twice weekly on average, but emergence of EHL rFIX products allow for more prolonged intervals, up to 14–21 days [53] (Table 5.8).

Table 5.8 Factor IX products for hemophilia B treatment

Product	Company	Country
	Plasma-derived factor IX	
IXED	Kedrion	Italy
AlphaNine SD	Grifols	Spain
Mononine/Berinin-P	CSL Behring	Australia
Betafact	LFB	Several countries
Factor IX Grifols	Grifols	Spain
Haemonine	Biotest	Germany
Hemo-B-RAAS	Shanghai RAAS	China
Immunine	Baxter BioScience	USA
Nanotiv	Octapharma	Switzerland
Nonafact	Sanquin	The Netherlands
Octanine F	Octapharma	Switzerland
Replenine	BPL	England
TBSF FIX	CSL biotherapies	Australia
	Recombinant FIX	
RIXUBIS	Baxter/Baxalta	USA
ALPROLIX (rFIXFc)	Biogen Idec	USA
IXINITY (trenonacog alfa)	Emergent BioSolutions (previously Cangene)	USA
IDELVION	CSL Behring	Australia
REFIXIA (N9-GP)	Novo Nordisk	Denmark
BeneFIX	Pfizer	USA

FDA food and drug administration, *FIX* factor IX

5.9 Inhibitor Formation: A Challenge of Replacement Therapy

Development of inhibitor against the infused FIX is the most important side-effect associated with the modern replacement therapy [54]. Inhibitor development in patients with severe HB usually occurs early, after a median of ten exposure days (ED). The FIX inhibitor is commonly of IgG4 subclass with affinity to Gla domain and catalytic domain in light and heavy chains, respectively (Fig. 5.9). It not only reduces the effectiveness of replacement therapy, worsening the health status and quality of life of the patient, but also increases the need of significant economic resources [55]. At variance with HA, inhibitor occurrence in HB is uncommon. It is hypothesized that the low frequency of inhibitor in HB may result from the similarity of FIX structure with other vitamin K-dependent factors or because the majority of patients have missense mutations allowing the production of dysfunctional proteins able to induce tolerization by the immune system [56]. Although, the prevalence of inhibitor in hemophilia B is less than 10%, in severely affected patients with major gene defects (large or complete gene deletion), this may increase to up to >40% [25, 38, 54, 56]. These FIX antibodies are associated with considerable morbidity due to the risk of life-threatening allergic reactions or anaphylaxis and

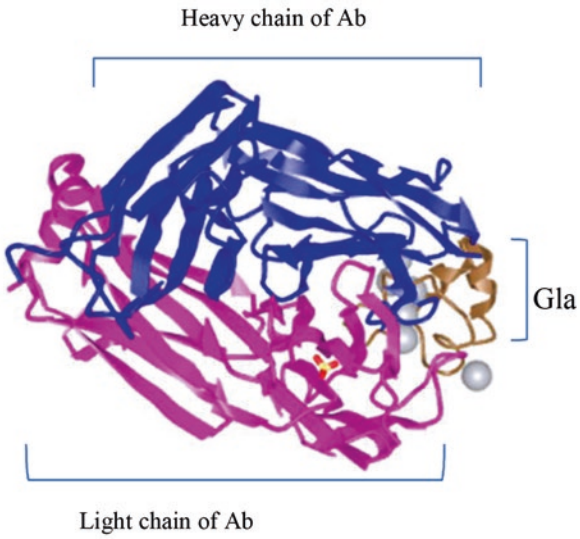

Fig. 5.9 Crystal Structure of Human Factor IX GLA Domain in Complex of an inhibitory antibody. Heavy chain and light chain of inhibitory antibody are shown in blue and purple, respectively. Gla domain is shown in brown color. The grey residues are calcium ions (*Ab* antibody)

nephrotic syndrome that may occur in 50% of patients with inhibitor when continuing treatment or during immunotolerance protocols [57].

Inhibitor development is a result of genetic and non-genetic reasons, and the type of mutation is the major risk factor. Null mutations, such as large deletions or nonsense mutations, prevent coagulation factor synthesis, leading to the development of inhibitors at 48% and 37%, respectively (Fig. 5.10) [58]. Conversely, for milder molecular defects such as small deletions/insertions, splice-site, frameshift, and missense mutations, which result in coagulation factor synthesis but loss of function, inhibitory development risk is significantly lower [54, 58]. The other risk factors, which are non-genetic, are less studied in HB due to the rarity of inhibitors' development in these individuals [25].

The treatment strategy for these patients depends on the inhibitor titer. When the inhibitor titer is low (<5 Bethesda unit (BU) in patients without allergic reactions, the treatment approach is injection of high concentrations of FIX to overcome the inhibitor titer. In contrast, high titer inhibitors (>5 BU) require more rigorous therapeutic strategies. In these cases, management of patients is divided into two parts: prevention of further bleeding episodes and their treatment and eradication of inhibitor. Bypassing agents, recombinant activated factor VII (rFVIIa) at a dose of 90 μg/kg every 3 h depending on the severity of bleeding, and activated prothrombin complex concentrate (APCC) at a dose of 50–100 U/kg body weight every 8–12 h, not exceeding 200 U/kg daily, are the main options for management of bleeding episodes. Unlike APCC, rFVIIa does not contain FIX and is preferred as therapeutic option for patients who developed the inhibitor and manifest anaphylactic reactions

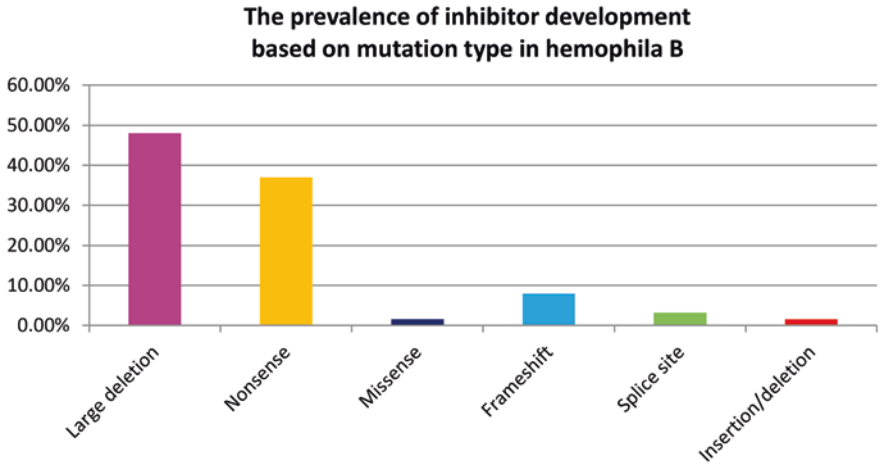

Fig. 5.10 The bar chart shows the relationship between inhibitor information and mutation type in patients with hemophilia B. Large deletion (48%) and nonsense (37%) mutations are responsible for the highest rate of inhibitor development. Missense (1.6%), frameshift (8%), splice sites (3.2%), and insertions/deletions (1.6%) are less likely to develop inhibitor

to FIX injections [59]. Immune tolerance induction (ITI), requiring high dose injection of FIX every day (100–200 U/kg/daily) or thrice weekly (25–50 U/kg) for months to years, is used for inhibitor eradication. In cases of ITI-resistant, immunosuppressive drugs such as rituximab are the alternative choice for inhibitor elimination (Fig. 5.11). However, patients with HB are less likely to benefit from ITI than those with hemophilia A, with 15–30% and 60–80% success rate, respectively [54, 60].

Recently, non-substitutive treatments have been developed to address the unmet needs of an effective prophylaxis in patients with HA and HB with inhibitors [61]. Concizumab is a monoclonal anti-tissue factor (TF) pathway inhibitor (TFPI) antibody under investigation for subcutaneous prophylaxis in all hemophilia subtypes [62]. Concizumab inhibits TFPI activity by high-affinity binding to the TFPI Kunitz-2 domain, blocking TFPI binding to and inhibition of active factor X (FXa), TF-FVIIa complex, maintaining FXa production by the TF-FVIIa complex. Thrombin generation therefore becomes sufficient to prevent bleeding episodes, achieving adequate hemostasis [62–64].

Results from the concizumab phase 2 explorer4 study (NCT03196284) established proof of concept in patients with HA and HB with inhibitors [65, 66]. The agent is administered as a daily subcutaneous injection (0.2 mg/kg/daily after a loading dose of 1 mg/kg) and is not yet approved for use in clinical practice, apart from Canada for HB with inhibitors. Recent data from a phase 3 study showed a median ABR of 0 in a group of patients with HA and HB with inhibitors treated with Concizumab for 24–32 weeks, thus representing a promising safe and useful prophylaxis tool for HB patients with inhibitors [67].

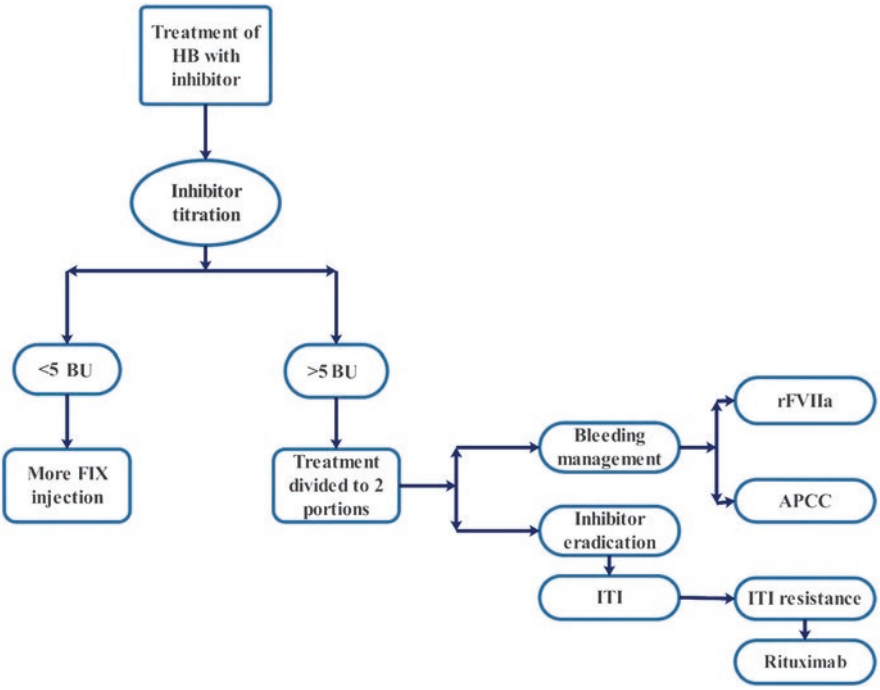

Fig. 5.11 The treatment approach for patients with hemophilia B and inhibitor antibody. (*HB* hemophilia B, *BU* bethesda units, *FIX* factor IX, *ITI* inhibitor tolerance induction, *rFVIIa* recombinant activated factor VII, *APCC* activated prothrombin complex concentrate

5.10 Gene Therapy

Despite the significant clinical benefits with prophylaxis, there remains several unmet needs for patients with hemophilia. Prophylactic treatment is often associated with a high treatment burden because of the requirement for life-long, repeated intravenous injections, placing a burden on family and caregivers and negatively impacting on appropriate education and employment. Current treatments with factor replacement products also result in fluctuating factor levels, and falling below minimum levels can increase the possibility of breakthrough bleeds; therefore, some patients with hemophilia who are treated with prophylaxis still experience breakthrough bleeds [68–72]. The development of inhibitors is also a major complication of factor replacement products and is associated with significant morbidity, and emotional strain [30, 73]. The prevalence of inhibitor development in HA is approximately 25–30%, compared to between 1.5 and 10% in HB [23, 30, 56]. Finally, patients with hemophilia deal with an array of challenges and emotions related to their condition.

Consequently, gene therapy is being studied as a possible treatment option for HB since its monogenic nature and the possibility of consistent endogenous expression of FIX [74]. Over the years, gene therapy using viral vectors has emerged as potential long-term therapeutic option. Adeno-associated virus (AAV)-mediated gene therapy is full of promise due to variable immune cellular response to virus capsid [75, 76]. Moreover, it can lead to durable FIX expression resulting in elimination of spontaneous bleeding and requirement for frequent injections [77, 78]. AAV is a class of nonpathogenic viruses with a single strand genome, which is 4.7 kb in length. Various serotypes of AAV are investigated such as 2, 3, 5 (AMT-060 drug), and 8 serotypes [76]. Nevertheless, the main complication of gene therapy is liver toxicity and aminotransferase elevation 5–10 weeks after gene transfer [75], although to a lesser extent compared to gene therapy in HA. This phenomenon is a result of T cell mediated immune responses to the transducted hepatocytes, usually managed by using corticosteroid treatment without loss of transgene expression. FIX increases at a dose-dependent rate. In high dose vector injection with AAV8, FIX level reached to 8–12% of normal levels that were stable for several years [75, 77, 78]. The viral vector contained wild FIX gene delivered intravenously. AMT-060 is a gene transfer product that consists of an adeno-associated virus 5 (AAV5) vector incorporating a small gene cassette containing codon-optimized wild-type (WT) human FIX under the control of a liver-specific promoter [78]. Etranacogene dezaparvovec (AMT-061) is the successor to AMT-060, and has an identical design except for a two-nucleotide substitution resulting in a single amino acid change (R338L) in the FIX coding sequence, which results in the highly active FIX-Padua variant [79]. The FIX-Padua protein demonstrates a six- to eight-fold increase in FIX activity compared to WT FIX [79, 80]. The HOPE-B study is the largest open-label, single-dose, multi-center trial in 54 adult males with severe (FIX <1 U/dL; $n = 44$) or moderately severe (FIX 1–2 U/dL; $n = 10$) hemophilia B (NCT03569891). Participants were treated with a single infusion of etranacogene dezaparvovec (2×10^{13} gc/kg) after a lead-in period (≥6 months) of usual FIX prophylaxis and followed-up for 18 months. In total, 53 participants completed the follow-up period. One participant discontinued treatment following a treatment emergent adverse event (TEAE) of hypersensitivity after receiving a partial dose (~10%). The participant did not respond to treatment but continued in the study. Etranacogene dezaparvovec significantly increased FIX activity from baseline, with a mean of 39 U/dL, 38.8 U/dL, and 36.9 U/dL at 6-, 12-, and 18-months post-treatment, respectively. Mean ABR decreased from 4.2 (95% CI: 3.22, 5.45) during the lead-in period to 1.5 (95% CI: 0.81, 2.82) during Months 7–18 post-treatment. The mean AsBR decreased from 1.52 (95% CI: 1.01, 2.30) during the lead-in period to 0.44 (95% CI: 0.17, 1.12) during Months 7–18. Overall, 63% of participants had zero bleeding episodes post-treatment, compared with 26% of participants during lead-in. FIX prophylaxis was discontinued in 96.3% (52/54) participants from Day 21 through to Month 18. Interestingly, unlike gene therapy trials in HA, patients who tested positive for antibodies against AAV5 were enrolled and their outcome was not significantly different compared to negative patients. Of the two participants who continued with FIX prophylaxis, one had received only 10% of the full dose and the other had a

pre-existing AAV5 NAb titer of 3212 just before dosing [81]. A similar, smaller study with a different vector, but containing the FIX Padua variant, yielded similar results in ten patients with HB [82]. Transaminase elevation occurred in less than 20% of patients in these studies, without loss of expression upon corticosteroid treatment. In November 2022, the Food and Drug Administration (FDA) approved the first gene therapy for previously treated adult patients with severe hemophilia B (etranacogene dezaparvovec; HEMGENIX®; CSL Behring®) [83].

In conclusion, gene therapy appears a promising potential cure for HB because it induces long-term endogenous production of FIX, especially taking advantage of its small size and the hyperactive FIX natural variant, which is able to produce FIX levels on average >30 U/dL. Therefore, despite increase in aminotransferase levels easily manageable by corticosteroid treatment, without loss of gene expression, gene therapy could convert severe HB to a mild form or within the normal range. However, at present, one should always take into consideration that a proportion of patients are not eligible to gene therapy, like those <18 years, advanced liver disease, past or present FIX inhibitors. Notwithstanding these limitations, the future of gene therapy for management and treatment of hemophilia patients looks bright.

Acknowledgments We appreciate Professor Edward Tuddenham for his valuable comments that significantly improved the quality of this chapter.

References

1. Motlagh H, Pezeshkpoor B, Dorgalaleh A. Henophilia B in congenital bleeding disorders: Diagnosis and treatment. Springer 2018;138-160.
2. DeLoughery TG. Basics of coagulation. In: Hemostasis and thrombosis. Springer; 2015. p. 1–7.
3. Wang Q-Y, Hu B, Liu H, Tang L, Zeng W, Wu Y-Y, et al. A genetic analysis of 23 Chinese patients with hemophilia B. Sci Rep. 2016;6(1):1–7.
4. Eby CS. Bleeding and vitamin K deficiency. In: Management of bleeding patients. Springer; 2016. p. 145–50.
5. Parrado Jara YA, Yunis Hazbun LK, Linares A, Yunis Londoño JJ. Molecular characterization of hemophilia B patients in Colombia. Mol Genet Genomic Med. 2020;8(5):e1210.
6. Dorgalaleh A, Dadashizadeh G, Bamedi T. Hemophilia in Iran. Hematology. 2016;21(5):300–10.
7. Marchesini E, Morfini M, Valentino L. Recent advances in the treatment of hemophilia: a review. Biol: Targets Ther. 2021;15:221.
8. Tagliaferri A, Molinari AC, Peyvandi F, Coppola A, Demartis F, Biasoli C, Borchiellini A, Cultrera D, De Cristofaro R, Daniele F, Giordano P, Marchesini E, Margaglione M, Marino R, Pollio B, Radossi P, Santoro C, Santoro RC, Siragusa S, Sottilotta G, Tosetto A, Piscitelli L, Villa MR, Zanon E, Finardi A, Schiavetti I, Vaccari D, Castaman G. IDEAL study: a real-world assessment of pattern of use and clinical outcomes with recombinant coagulation factor IX albumin fusion protein (rIX-FP) in patients with haemophilia B in Italy. Haemophilia. 2023;29(1):135–44.
9. Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K. Complete nucleotide sequences of the gene for human factor IX (antihemophilic factor B). Biochemistry. 1985;24(14):3736–50.
10. Lee CA, Berntorp EE, Hoots WK. Textbook of hemophilia. John Wiley & Sons; 2011.

11. Abla Z, Mouloud Y, El Mahmoudi H, Emna G, Meriem A, Yamina O, et al. Mutations causing hemophilia B in Algeria: identification of two novel mutations of the factor 9 gene. Biodivers J Biol Divers. 2018;19(1):52–8.
12. Schmidt AE, Bajaj SP. Structure–function relationships in factor IX and factor IXa. Trends Cardiovasc Med. 2003;13(1):39–45.
13. Rallapalli P, Kemball-Cook G, Tuddenham E, Gomez K, Perkins S. An interactive mutation database for human coagulation factor IX provides novel insights into the phenotypes and genetics of hemophilia B. J Thromb Haemost. 2013;11(7):1329–40.
14. Zacchi LF, Roche-Recinos D, Pegg CL, Phung TK, Napoli M, Aitken C, et al. Coagulation factor IX analysis in bioreactor cell culture supernatant predicts quality of the purified product. Commun Biol. 2021;4(1):1–19.
15. Ngo JCK, Huang M, Roth DA, Furie BC, Furie B. Crystal structure of human factor VIII: implications for the formation of the factor IXa-factor VIIIa complex. Structure. 2008;16(4):597–606.
16. Autin L, Miteva M, Lee W, Mertens K, Radtke KP, Villoutreix B. Molecular models of the procoagulant factor VIIIa–factor IXa complex. J Thromb Haemost. 2005;3(9):2044–56.
17. Li T, Miller CH, Payne AB, Craig HW. The CDC hemophilia B mutation project mutation list: a new online resource. Mol Genet Genom Med. 2013;1(4):238–45.
18. Freato N, van Alphen FP, Boon-Spijker M, van den Biggelaar M, Meijer AB, Mertens K, et al. Probing activation-driven changes in coagulation factor IX by mass spectrometry. J Thromb Haemost. 2021;19(6):1447–59.
19. Li T, Miller CH, Driggers J, Payne AB, Ellingsen D, Hooper WC. Mutation analysis of a cohort of US patients with hemophilia B. Am J Hematol. 2014;89(4):375–9.
20. Mannucci P, Franchini M. Is haemophilia B less severe than haemophilia A? Haemophilia. 2013;19(4):499–502.
21. Miller CH. The clinical genetics of hemophilia B (factor IX deficiency). Appl Clin Genet. 2021;14:445.
22. Crossley M, Ludwig M, Stowell KM, De Vos P, Olek K, Brownlee GG. Recovery from hemophilia B Leyden: an androgen-responsive element in the factor IX promoter. Science. 1992;257(5068):377–9.
23. DiMichele D. Inhibitor development in haemophilia B: an orphan disease in need of attention. Br J Haematol. 2007;138(3):305–15.
24. Ulrich S, Brand B, Speich R, Oldenburg J, Asmis L. Congenital hypersensitivity to vitamin K antagonists due to FIX propeptide mutation at locus-10: a (not so) rare cause of bleeding under oral anticoagulant therapy in Switzerland. Swiss Med Wkly. 2008;138(7–8):100–7.
25. Chu K, Wu S-M, Stanley T, Stafford DW, High KA. A mutation in the propeptide of factor IX leads to warfarin sensitivity by a novel mechanism. J Clin Invest. 1996;98(7):1619–25.
26. Oldenburg J, Quenzel EM, Harbrecht U, Fregin A, Kress W, Müller CR, et al. Missense mutations at ALA-10 in the factor IX propeptide: an insignificant variant in normal life but a decisive cause of bleeding during oral anticoagulant therapy. Br J Haematol. 1997;98(1):240–4.
27. Motlagh H, Pezeshkpoor B, Dorgalaleh A. Hemophilia B. In: Congenital bleeding disorders. Springer; 2018. p. 139–60.
28. Surin V, Demidova EY, Selivanova D, Luchinina YA, Salomashkina V, Pshenichnikova O, et al. Mutational analysis of hemophilia B in Russia: molecular-genetic study. Russ J Genet. 2016;52(4):409–15.
29. Franchini M, Mannucci PM. Haemophilia B is clinically less severe than haemophilia A: further evidence. Blood Transfus. 2018;16(2):121–2.
30. Castaman G, Matino D. Hemophilia a and B: molecular and clinical similarities and differences. Haematologica. 2019;104(9):1702–9.
31. Kitchen S, McCraw A, Echenagucia M. Diagnosis of haemophilia and other bleeding disorders: a laboratory manual. World Federation of Hemophilia Montreal; 2000.
32. Kizilocak H, Young G. Diagnosis and treatment of hemophilia. Clin Adv Hematol Oncol. 2019;17(6):344–51.

33. Kihlberg K, Strandberg K, Rosén S, Ljung R, Astermark J. Discrepancies between the one-stage clotting assay and the chromogenic assay in haemophilia B. Haemophilia. 2017;23(4):620–7.
34. Kitchen S, Signer-Romero K, Key N. Current laboratory practices in the diagnosis and management of haemophilia: a global assessment. Haemophilia. 2015;21(4):550–7.
35. Sellner LN, Taylor GR. MLPA and MAPH: new techniques for detection of gene deletions. Hum Mutat. 2004;23(5):413–9.
36. Kwon MJ, Yoo KY, Kim HJ, Kim SH. Identification of mutations in the F9 gene including exon deletion by multiplex ligation-dependent probe amplification in 33 unrelated Korean patients with haemophilia B. Haemophilia. 2008;14(5):1069–75.
37. Franchini M, Frattini F, Crestani S, Sissa C, Bonfanti C. Treatment of hemophilia B: focus on recombinant factor IX. Biol: Targets Ther. 2013;7:33.
38. Bolton-Maggs PH, Pasi KJ. Haemophilias A and B. Lancet. 2003;361(9371):1801–9.
39. Castaman G. The benefits of prophylaxis in patients with hemophilia B. Expert Rev Hematol. 2018 Aug;11(8):673–83.
40. Davis J, Yan S, Matsushita T, Alberio L, Bassett P, Santagostino E. Systematic review and analysis of efficacy of recombinant factor IX products for prophylactic treatment of hemophilia B in comparison with rIX-FP. J Med Econ. 2019;22(10):1014–21.
41. Nolan B, Klukowska A, Shapiro A, Rauch A, Recht M, Ragni M, et al. Final results of the PUPs B-LONG study: evaluating safety and efficacy of rFIXFc in previously untreated patients with hemophilia B. Blood Adv. 2021;5(13):2732–9.
42. Weyand AC, Pipe SW. New therapies for hemophilia. Blood, J Am Soc Hematol. 2019;133(5):389–98.
43. Ragni M, Kulkarni R, Pasi KJ, Fischer K, Mahlangu J, Shapiro A, et al. B-YOND final results confirm established safety, sustained efficacy, and extended dosing interval for up to 4 years of treatment with rFIXFc in previously treated subjects with severe hemophilia B. Blood. 2018;132:1214.
44. Shapiro A, Chaudhury A, Jain N, Tsao E, Barnowski C, Feng J, et al. Real-world data on the use of rFIXFc in subjects with hemophilia B for up to 3.7 years demonstrates improved bleed control and adherence with reduced treatment burden. Blood. 2018;132:2493.
45. Chan AK, Alamelu J, Barnes C, Chuansumrit A, Garly ML, Meldgaard RM, et al. Nonacog beta pegol (N9-GP) in hemophilia B: first report on safety and efficacy in previously untreated and minimally treated patients. Res Pract Thromb Haemost. 2020;4(7):1101–13.
46. Elm T, Ostergaard H, Tranholm M. Dose response and prolonged effect of 40K PEG-FIX on bleeding in hemophilia B mice. J Thromb Haemost. 2009;7(Suppl. 2)
47. Carcao M, Kearney S, Santagostino E, Oyesiku J, Young N, Meunier J, et al. Insight into health-related quality of life of young children with haemophilia B treated with long-acting nonacog beta pegol recombinant factor IX. Haemophilia. 2017;23(3):e222–e4.
48. Santagostino E, Mancuso ME. GlycoPEGylated recombinant factor IX for hemophilia B in context. Drug Des Devel Ther. 2018;12:2933.
49. Metzner HJ, Weimer T, Kronthaler U, Lang W, Schulte S. Genetic fusion to albumin improves the pharmacokinetic properties of factor IX. Thromb Haemost. 2009;102(10):634–44.
50. Alvarez Roman MT, Benítez O, Canaro MI, Lopez Fernandez MF, López Jaime FJ, Mateo Arranz J, et al. Expert opinion paper on the treatment of hemophilia B with albutrepenonacog alfa. Expert Opin Biol Ther. 2021;21(9):1165–71.
51. Oldenburg J, Yan S, Maro G, Krishnarajah G, Tiede A. Assessing bleeding rates, related clinical impact and factor utilization in German hemophilia B patients treated with extended half-life rIX-FP compared to prior drug therapy. Curr Med Res Opin. 2020;36(1):9–15.
52. Brown DL. Congenital bleeding disorders. Curr Probl Pediatr Adolesc Health Care. 2005;35(2):38–62.
53. Nummi V, Jouppila A, Lassila R. Monitoring once-weekly recombinant factor IX prophylaxis in hemophilia B with thrombin generation assay and factor IX activity. Int J Lab Hematol. 2017;39(4):359–68.
54. Dolan G, Benson G, Duffy A, Hermans C, Jiménez-Yuste V, Lambert T, et al. Haemophilia B: where are we now and what does the future hold? Blood Rev. 2018;32(1):52–60.

55. Key NS. Inhibitors in congenital coagulation disorders. Br J Haematol. 2004;127(4):379–91.
56. Male C, Andersson NG, Rafowicz A, Liesner R, Kurnik K, Fischer K, et al. Inhibitor incidence in an unselected cohort of previously untreated patients with severe hemophilia B: a PedNet study. Haematologica. 2021;106(1):123.
57. Barg AA, Livnat T, Kenet G. Inhibitors in hemophilia: treatment challenges and novel options. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2018.
58. Santoro C, Quintavalle G, Castaman G, Baldacci E, Ferretti A, Riccardi F, et al. Inhibitors in hemophilia B. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2018.
59. White G, Rosendaal F, Aledort L, Lusher J, Rothschild C, Ingerslev J. Recommendation of the scientific subcommittee on factor VIII and factor IX of the scientific and standardization committee of the international society on thrombosis and Haemostasis. Thromb Haemost. 2001;85(3):560.
60. Barg AA, Levy-Mendelovich S, Avishai E, Dardik R, Misgav M, Kenet G, et al. Alternative treatment options for pediatric hemophilia B patients with high-responding inhibitors: a thrombin generation-guided study. Pediatr Blood Cancer. 2018;65(12):e27381.
61. Nogami K, Shima M. New therapies using nonfactor products for patients with hemophilia and inhibitors. Blood. 2019;133:399–406.
62. Hilden I, Lauritzen B, Sorensen BB, et al. Hemostatic effect of a monoclonal antibody mAb 2021 blocking the interaction between FXa and TFPI in a rabbit hemophilia model. Blood. 2012;119:5871–8.
63. Mast AE, Ruf W. Regulation of coagulation by tissue factor pathway inhibitor: implications for hemophilia therapy. J Thromb Haemost. 2022;20:1290–300.
64. Eichler H, Angchaisuksiri P, Kavakli K, et al. A randomized trial of safety, pharmacokinetics and pharmacodynamics of concizumab in people with hemophilia A. J Thromb Haemost. 2018;16:2184–95.
65. Shapiro AD, Angchaisuksiri P, Astermark J, et al. Subcutaneous concizumab prophylaxis in hemophilia a and hemophilia a/B with inhibitors: phase 2 trial results. Blood. 2019;134:1973–82.
66. Shapiro AD, Angchaisuksiri P, Astermark J, et al. Long-term efficacy and safety of subcutaneous concizumab prophylaxis in hemophilia a and hemophilia A/B with inhibitors. Blood Adv. 2022;6:3422–32.
67. Matsushita et al. Phase 3 trial of concizumab in hemophilia with inhibitors. N Engl J Med. 2023;389(9):783–94.
68. Srivastava A, Santagostino E, Dougall A, et al. WFH Guidelines for the management of hemophilia, 3rd edition. Haemophilia. 2020;26(Suppl. 6):1–158.
69. Burke T, Shaikh A, Ali TM, et al. Association of factor expression levels with annual bleeding rate in people with haemophilia B. Haemophilia. 2023;29(1):115–22.
70. Shapiro A, Potts J, Li S, et al. Association of bleeding tendency with time under target FIX activity levels in severe hemophilia B patients treated with recombinant factor IX fc fusion protein. Blood. 2013;122(21):2349.
71. Thornburg CD, Duncan NA. Treatment adherence in hemophilia. Patient Prefer Adher. 2017;11:1677–86.
72. Schrijvers LH, Beijlevelt-van der Zande M, Peters M, et al. Adherence to prophylaxis and bleeding outcome in haemophilia: a multicentre study. Br J Haematol. 2016 Aug;174(3):454–60.
73. duTreil S. Physical and psychosocial challenges in adult hemophilia patients with inhibitors. J Blood Med. 2014;5:115–22.
74. Nathwani AC. Gene therapy for hemophilia. Hematology 2014. the American Society of Hematology Education Program Book. 2019;2019(1):1–8.
75. Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, Linch DC, et al. Adenovirus-associated virus vector–mediated gene transfer in hemophilia B. N Engl J Med. 2011;365(25):2357–65.
76. Nienhuis AW, Nathwani AC, Davidoff AM. Gene therapy for hemophilia. Mol Ther. 2017;25(5):1163–7.

77. Nathwani AC, Reiss UM, Tuddenham EG, Rosales C, Chowdary P, McIntosh J, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. N Engl J Med. 2014;371(21):1994–2004.
78. Miesbach W, Meijer K, Coppens M, Kampmann P, Klamroth R, Schutgens R, et al. Gene therapy with adeno-associated virus vector 5–human factor IX in adults with hemophilia B. Blood J Am Soc Hematol. 2018;131(9):1022–31.
79. Simioni P, Tormene D, Tognin G, et al. X-linked thrombophilia with a mutant factor IX (factor IX Padua). N Engl J Med. 2009;361(17):1671–5.
80. Spronck EA, Liu YP, Lubelski J, et al. Enhanced factor IX activity following administration of AAV5-R338L "Padua" factor IX versus AAV5 WT human factor IX in NHPs. Mol Ther Methods Clin Dev. 2019;13(15):221–31.
81. Pipe S et al. Gene therapy with etranacogene dezaparvovel for hemophilia B. N Engl J Med. 2023;388(8):706–18.
82. Geurge LA, Sullivan SK, Giermasz et al. Hemuphilia B gene therapy with a high-specific-activity factoer IX variant. N Engl J Med. 2017;377:2215–2227.
83. FaDA (FDA). Prescribing Information—HEMGENIX 2022.

Part III

Rare Bleeding Disorders

Congenital Fibrinogen Disorders, Diagnosis, and Management

6

Alessandro Casini

6.1 Introduction

Congenital fibrinogen disorders encompass a large spectrum of fibrinogen anomalies [1]. The prevalence of congenital fibrinogen disorders is not known. Based on international databases, it has been estimated that they represent about 8% of the rare bleeding disorders [2]. The prevalence of afibrinogenemia is estimated to be 1 for 1,000,000 of person, and more frequent in countries with consanguinity [3, 4]. A recent study from the United States Hemophilia Treatment Centers Network reported a prevalence of 1.13 for 1,000,000 of persons, compared to 3.74 in the United Kingdom registry data and 0.70 in the World Federation of Hemophilia Global Survey [5]. However, this prevalence is probably underestimated since asymptomatic patients are often not included in such registries. In addition, national registries usually include patients with severe coagulation factors deficiencies, while dysfibrinogenemia and hypofibrinogenemic patients can have only moderate decreased fibrinogen levels.

In our previous chapter published in 2018 in "Congenital Bleeding Disorders" (Springer, Ed. A. Dorgalaleh), we summarized how to diagnosis a congenital fibrinogen disorders and reviewed the main clinical features [1]. In this update, we revise the classification, the diagnosis, the genetic, the clinical features, and the management of fibrinogen disorder focusing mainly on the last five-year publications.

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_6

6.2 Classification

Congenital fibrinogen disorders have historically been classified as quantitative (type I) or qualitative (type II) fibrinogen disorders [6]. Quantitative fibrinogen disorders include afibrinogenemia characterized by the complete absence of fibrinogen and hypofibrinogenemia characterized by proportionally decreased levels of functional and antigen fibrinogen levels [7]. On the other side, qualitative fibrinogen disorders include dysfibrinogenemia defined by normal levels of a dysfunctional fibrinogen molecule and hypodysfibrinogenemia characterized by decreased levels of a dysfunctional fibrinogen molecule [7]. The Fibrinogen and Factor XIII subcommittee of the International Society of Thrombosis and Hemostasis (ISTH) has proposed a new classification considering both the biological (fibrinogen levels and genotype) and the patient's clinical features [8]. Thus, afibrinogenemia is separated into type 1A (afibrinogenemia with bleeding symptoms) and 1B (afibrinogenemia with thrombotic phenotype). Hypofibrinogenemia is classified according to the functional fibrinogen level (<0.5 g/L severe, type 2A; 0.5– < 1 g/L moderate, type 2B; 1 g/L to the lower range of laboratory, mild, type 2C). A fourth sub-type is the fibrinogen storage disease (see session on genetics). Dysfibrinogenemia is classified in type 3A (asymptomatic, bleeding, or thrombotic phenotype) or 3B (thrombotic-related fibrinogen variant, see session on genetics). Hypodysfibrinogenemia is classified according to antigenic fibrinogen level (<0.5 g/L severe, 4A; 0.5– < 1 g/L moderate, 4B; 1 g/L to the lower range of laboratory, mild, 4C).

6.3 Diagnosis

An accurate diagnosis is essential to characterize the type and sub-type of fibrinogen disorder and drive the management. Diagnosis of can be made based on clinical presentations and appropriate laboratory assessment but even in well-equipped coagulation laboratory, sometimes, diagnosis of these disorders can be a sophisticated process especially for dysfibrinogenemia and hypodysfibrinogenemia [9–12]. Usually, a fibrinogen disorder is suspected according to the personal or familial history, but an acquired fibrinogen disorder should be excluded before to perform a complete fibrinogen work-up. Common causes of acquired fibrinogen disorders are summarized in Table 6.1 [13]. In case of suspicion of a congenital fibrinogen disorder, the first step is the assessment of the functional fibrinogen by the Clauss method [14]. The Clauss method is a modification of the thrombin time, in which citrated plasma is diluted and then excess thrombin, usually 100 U/ml (range 35–200 U/ml) is added, and time of clotting is measured. High concentration of thrombin is used to ensure that clotting time is independent of thrombin concentration [15–18]. The clotting time is inversely proportional to the amount of fibrinogen in the sample. For determination of the plasma fibrinogen level, a calibration curve with a serial dilution of reference plasma with known concentration of fibrinogen is provided. If the Clauss fibrinogen level is below the lower range of the laboratory, the screening should be completed with the assessment of the antigenic fibrinogen, which is

Table 6.1 Causes of acquired fibrinogen deficiency

Cause	Potential physiopathologic mechanism
Liver disease	Increased sialylation of Bβ and γ chains [93]
Malignancy (renal carcinoma, hepatoma)	Paraneoplastic synthesis of abnormal fibrinogen [94]
Plasma cells disorders	Paraproteinemia binding fibrinogen [95]
Systemic lupus erythematosus and rheumatoid arthritis	Autoantibody against fibrinogen [96]
Disseminated intravascular coagulation	Consumption of clotting factors [97]
Hemophagocytic lymphohistiocytosis	Induced fibrinolysis or histiocytes binding fibrinogen [50]
Medications (isoniazid, asparaginase, thrombolytic drugs, valproic acid, tigecycline)	Impaired hepatic synthesis, fibrinolysis or unknown [98]
Plasma exchange	Apheresis without plasma as replacement fluid [99]

Causes mainly associated with qualitative fibrinogen disorder are indicated in italic type

mandatory to distinguish between hypofibrinogenemia, dysfibrinogenemia, and hypodysfibrinogenemia. Several immunological assays are available to evaluate the fibrinogen concentration but unfortunately, only a minority of routine laboratory are equipped with. A good alternative is the indirect fibrinogen evaluation from the prothrombin time (PT) curve. In this method, the PT is performed on plasma dilutions with known amount of fibrinogen, and a curve is drawn based on optical changes against different fibrinogen concentrations. By this graph, an optical change in plasma with known amount of fibrinogen is converted to fibrinogen level [15, 19]. The PT-derived fibrinogen overestimates the functional fibrinogen in dysfibrinogenemia [19] but provides an excellent correlation with the antigenic fibrinogen (r = 0.898) [20]. In a study including 66 patients with dysfibrinogemia and 54 patients with hypofibrinogenemia, a cut-off >1.7 for the ratio PT-derived fibrinogen/Clauss fibrinogen gave a 100% of specificity and sensitivity for the diagnosis of dysfibrinogenemia [21]. As indicated in Fig. 6.1, the type of fibrinogen disorder is defined by the functional and antigen fibrinogen level. In afibrinogenemia, all tests based on fibrin clot as endpoint are infinitely prolonged. Fibrinogen functional and antigen are undetectable. In hypofibrinogenemia, a proportional reduction of activity and antigen fibrinogen level is observed. The prolongation of standard coagulation assays and of thrombin and reptilase time depends on the fibrinogen levels. Distinguishing severe hypofibrinogenemia from afibrinogenemia can be difficult and depends on the limit detection of the fibrinogen assay. In such cases, additional methods, such as mass spectrometry, can be useful [22]. Dysfibrinogenemia is suspected in case of discrepancy between functional and antigenic fibrinogen levels. A ratio activity/antigen below 0.7 is historically used to diagnose dysfibrinogenemia, although this cut-off has never been validated [16, 23]. Hypodysfibrinogenemia is defined by low level of dysfunctional fibrinogen. It is suspected in case of discrepancy between decreased activity and decreased antigen fibrinogen levels. Since

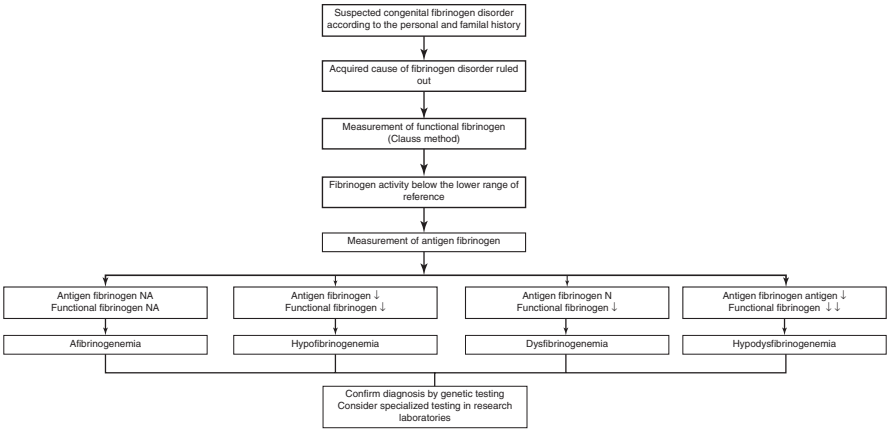

Fig. 6.1 Algorithm for diagnosis of fibrinogen disorders, adapted from [62]. *NA* not available as in afibrinogenemia both the antigen and the functional fibrinogen are not measurable. *N* in the normal range. ↓: below the lower range of reference. ↓↓: discordance between the antigen and functional fibrinogen

hypodysfibrinogenemia shares some features with both hypofibrinogenemia and dysfibrinogenemia, misdiagnosis is an important issue in this disorder. Distinguishing it from hypofibrinogenemia is difficult, especially in case with very low fibrinogen levels [24]. In systematic review of literature, the functional/antigenic fibrinogen ratio of <0.7 showed a poor sensitivity (86%) with a mean functional/antigenic fibrinogen ratio of 0.46 (0.07–1.25) observed in 32 cases [25].

When suspecting a fibrinogen disorder, it is important to underline two points. The first is that in some clinical situations as pregnancy, inflammatory disease, acute infection and active neoplasm, functional fibrinogen levels could be increased up to the normal levels. In such settings, if the suspicion of fibrinogen disorder is high, the assessment of the antigenic fibrinogen and eventually of the genotype is indicated even if the functional fibrinogen level is in the normal range. The second point is related to the diagnosis of dysfibrinogenemia. In dysfibrinogenemia, the sensitivity of the Clauss and PT-derived fibrinogen is dependent on the reagents, the assays, and the fibrinogen variant [17]. In an international study involving several European laboratories ($n = 86$), 39% of centers reported a normal or raised fibrinogen level for the Fibrinogen Longmont. The particularity of this fibrinogen variant is to form a translucent clot that can better be detected by a mechanical rather than a spectrophotometric clot assay [26]. Marked differences in Clauss fibrinogen results with different reagents were also noted for hotspot mutations (median 1.01 g/L vs 5.10 g/L for the two mostly widely used reagents) [27]. The thrombin origin (human or bovine) and the method for the detection of the fibrin clot (mechanical or optical endpoint) explain part of the variability observed in measurement of fibrinogen levels in dysfibrinogenemia [18, 28]. Recently, it has been reported that the clot waveform analysis (CWA) in the Clauss fibrinogen assay could be useful to detect functional fibrinogen abnormalities with no additional measurement of antigenic fibrinogen

[29, 30]. Of note, the thrombin time can be used when the fibrinogen Clauss is not available, even though the specificity and sensitivity of this assay are poor in fibrinogen disorders [31]. For the thrombin time measurement, a standard amount of thrombin is added to the patient's citrated plasma and then clotting time is measured. Thrombin cleaves both molecules of FpA and FpB to mediate fibrin formation. For the reptilase time, reptilase is used instead of thrombin to initiate the fibrin formation. In contrast to thrombin, reptilase induces fibrin formation only by cleavage of FpA. Thrombin time is prolonged in presence of unfractionated heparin or direct thrombin inhibitors in patient's plasma, while RT is not affected in this situation [14].

To better determine the patient's clinical phenotype, global hemostasis assays and an assessment of the fibrin clot properties are performed in research laboratories [11]. Point-of-care viscoelastic tests (i.e., rotational thromboelastometry or thromboelastography) can help to guide the hemostatic management in high bleeding risk surgeries [32, 33]. However, they have a limited sensitivity to certain changes in fibrinogen molecule structure and function, reducing their utility only to patients with hypofibrinogenemia [34, 35]. Similarly, in a small series of patients with dysfibrinogenemia, thrombin generation measurement seems not able to distinguish between specific clinical phenotypes [34].

6.4 Genetics

Since the identification of the first causative mutation of dysfibrinogenemia in 1968 [36] and of afibrinogenemia in 1999 [37], hundreds of mutations have been reported. Mutations are scattered throughout the three fibrinogen genes (i.e., *FGA*, *FGB*, *FGG*). Some hotspot mutations have been identified but number of new fibrinogen variants are still reported to date. Hotspot mutations are found worldwide, and some mutational clusters have been reported in some countries. List all the variants is out of the scope of this chapter, and we refer the reader to on-line database (https://site.geht.org/base-fibrinogene/, last accessed 20 June 2022) and to recent reviews that provide an exhaustive list of fibrinogen variants [33, 38–40]. The type and the localization of the mutation differ according to the fibrinogen disorder. Patients with afibrinogenemia are mostly either homozygous or compound heterozygous for null mutations (e.g., large deletions, frameshift mutations, early truncation, non-sense mutations, splice-site mutations). Overall, these molecular anomalies affect the fibrinogen assembly, stability, or secretion [37, 41–47]. Although traditionally afibrinogenemia and hypofibrinogenemia were considered as two completely separated clinical entities, in fact, they are the phenotypic expression of the heterozygote and homozygote allelic status for a given fibrinogen mutation. Two recurrent mutations have been identified in quantitative fibrinogen disorders; both localize in FGA. The IVS4 + 1G > T splice-site mutation results in an early α-chain truncation. The 11-kb deletion leads to the absence of the Aα chain.

Most dysfibrinogenemia cases are inherited in an autosomal dominant manner caused by heterozygote missense mutation in one of the three fibrinogen genes; although, rarely homozygotes or compound heterozygote have been reported. Dysfibrinogenemia is usually heterozygous for missense mutations, especially located in the thrombin cleavage site in the exon 2 of *FGA* (Arg35-Gly36), in knob A (Gly36, Pro37, and Arg38) and in residues Arg301 of exon 8 of *FGG* [38]. Two missense mutations (i.e., hotspot mutations) are frequent. The residue Arg35 of exon 2 of *FGA* (c.103C > T or c.104G > A) can be mutated in histidine or cysteine resulting in a defective thrombin binding and an abnormal release of FpA [8, 48–50]. The residue Arg301 of exon 8 of *FGG* (c.901C > T or c.902GA) can also be mutated in histidine or cysteine affecting the D:D interactions, causing a defect in the early stage of fibrin polymerisation [7]. Considering hotspot mutations and the surrounding residues in exon 2 of *FGA* and exon 8 of FGG, about 85% of dysfibrinogenemia can be identified.

Other mutations observed in both quantitative and qualitative fibrinogen disorders are clustered in the αC-region (composed by the αC-domain and connector, α221–α610) [51]. Interestingly, missense mutations in the C-terminal region of the γ chain lead to qualitative fibrinogen disorder demonstrating that this domain can tolerate structural change, at least for the assembly and secretion of fibrinogen [38]. On the contrary, mutations in the C-terminal domain of the β-chain result in quantitative fibrinogen disorders suggesting that this domain does not tolerate structural changes [52].

In hypodysfibrinogenemia, a total of 32 causative mutations, mainly missense, non-sense, and frameshift, have been identified [25]. The “hypo” phenotype of hypodysfibrinogenemia is due to impaired assembly of fibrinogen molecule, decreased secretion, or increased fibrinogen clearance, while the “dysf” phenotype is often due to defective fibrin polymerization or abnormal binding of calcium or tissue plasminogen activator. Several molecular mechanisms explain these phenotypes. On one hand, a single mutation can lead to production of an abnormal fibrinogen chain that is less effectively secreted. On the other hand, two distinct heterozygous mutations can cause the qualitative defect and the quantitative one, respectively [25, 53–56].

Next-generation sequencing technologies allow sequencing of the coding region of the genome at low cost and are of undeniable value for investigation of fibrinogen disorder [57, 58], especially in the setting of complex mutations [59]. Moreover, a whole exome sequencing can provide information on fibrinogen polymorphisms acting as genetic modifiers that could explain the phenotype variability in dysfibrinogenemia [60]. Genotype helps to determine the correct diagnosis of fibrinogen disorder, allows the familial and prenatal screening, and correlates to the clinical phenotype. Indeed, some fibrinogen variants are strongly associated with a thrombotic risk (type 3B in the ISTH classification) [61]. These variants are observed in multiple structural domains of the fibrinogen molecule [62]. Several mechanisms, often overlapping, lead to hypercoagulability such as defective binding of thrombin, hypofibrinolysis, defective clot retraction, and abnormal viscoelastic properties of the fibrin clot [63]. Other variants, clustered in the C-terminal part of the γ chain, are

associated with the fibrinogen storage disease [64]. The fibrinogen storage disease is a disorder characterized by protein aggregation in the endoplasmic reticulum, hypofibrinogenemia, and liver disease of variable severity [65].

6.5 Clinical Features

Bleeding is the main symptom of quantitative fibrinogen disorder and depends on the fibrinogen concentration [66]. Recently, a cross-sectional international multicentric study including 204 patients with afibrinogenemia has provided new insights on the bleeding phenotype of such patients [67]. The median ISTH bleeding assessment tool score was of 14 points. Most of patients reported at least one bleeding episodes per month and about half of patients suffered from muscle hematoma, hemarthrosis, and peri-operative bleeding. As previously observed [68], cerebral bleeding was frequent, occurring in 23% of patients in both adults and children group. Of note, the bleeding phenotype impacted the health-related quality of life, especially in women. Miscellaneous symptoms are typical complications of afibrinogenemia. In the aforementioned study, spontaneous spleen rupture and bone cysts were reported in 11 (5.4%) and 36 (17.6%) patients, respectively [67]. Thrombosis is a paradoxical complication of afibrinogenemia. In the cohort of 204 patients, a total of 37 (18.1%) patients experienced a thrombotic event in venous, arterial, or both territories. Venous thromboses occurred in young patients with a mean age at first event of 27 years. Arterial thromboses were also observed in young patients with a mean age at first event of 36 years. Thrombotic recurrence was reported in 15 (40.5%) patients [67]. Several mechanisms may explain the clinical conundrum of thrombosis in the absence of fibrin(ogen): the increased thrombin generation due to the lack of the antithrombin-like effect of fibrin and the tendency to embolism of platelets clots made in the absence of fibrinogen [69]. A potential link between fibrinogen infusion and thrombotic events has been often reported, even though no causality has been established, and pharmacovigilance data suggest that this risk, if any, is extremely low [70, 71]. In a recent study including 20 patients with afibrinogenemia, the thrombin generation analyzed by a calibrated automated thrombography after a single standard dose of fibrinogen concentrate increased but did not reach the levels measured in controls [71]. Pregnancy is a high-risk clinical situation in women with afibrinogenemia. Fibrinogen replacement should be started early in gestation to prevent miscarriage and kept throughout the pregnancy to reduce the risk of vaginal bleeding and fetal loss [72]. Patients with mild or moderate hypofibrinogenemia are more often asymptomatic. The bleeding risk is essentially related to trauma or surgeries [73].

Bleeding symptoms reported in dysfibrinogenemia are usually mild (Table 6.2). Recent and older series of patients indicated that most of patients suffer from cutaneous and mucosal bleeding. Heavy menstrual bleeding and post-surgical bleeding are more frequent. Of note, clinical data are often limited to the time of inclusion or diagnosis, but the clinical course of dysfibrinogenemia can be complicated by spontaneous or traumatic major bleeding [48, 74]. Post-partum hemorrhage is frequent

Table 6.2 Bleeding pattern from recent large series of patients with dysfibrinogenemia

Author, year	Number of patients, n	Bleeding, n (%)	Cutaneous and minor wound, n (%)	Heavy menstrual bleeding, n (%)	Epistaxis and oral cavity, n (%)	Post-surgery, n (%)	Major,[a] n (%)
Casini (2015) [48]	101	53 (52)	28 (27.7)	20 (29.4)	15 (14.9)	9 (8.9)	13 (12.9)
Zhou (2015) [100]	102	28 (27.5)	13 (12.7)	3 (6.1)	9 (8.8)	6 (5.9)	11 (10.8)
Smith (2018) [101]	13	7 (53.8)	1 (7.7)	ND	1 (7.7)	2 (15.3)	3 (23)
Castaman (2019) [102]	50	15[b] [30]	ND	2 (10)	NA	2 (4)	3 (6)
Wypasek (2019) [73]	14	6 (42.9)	2 (14)	3 (27.3)	2 (14)	2 (14)	ND
Simurda (2020) [103]	31	13 (42)	3 (9.6)	6 (31)	ND	1 (3.2)	1 (3.2)
Zhou (2020) [104]	15	5 (33.3)	2 (13.3)	1 (6.7)	1 (6.7)	ND	1 (6.7)
Mohsenian (2021) [105]	10	9 (90)	8 (80)	5 (71)	2 (20)	1 (10)	ND

[a]Bleeding requiring a blood transfusion, a surgical hemostasis or in a critical site (cerebral, hemarthrosis, muscle hematoma, retro-peritoneal bleeding)
[b]Indicated only patients requiring a treatment; *ND* no data

in dysfibrinogenemia. In a recent systematic literature review, 6/32 (18%) pregnancies were complicated by post-partum hemorrhage requiring a fibrinogen infusion [75]. Women with a bleeding phenotype seem particularly at risk of obstetrical complications [48, 76]. As previously mentioned, dysfibrinogenemia is also associated with a thrombotic risk and should be considered as a mild thrombophilia [62]. Patients with hypodysfibrinogenemia are more prone to bleeding symptoms due to lower levels of a dysfunctional fibrinogen [77].

6.6 Management

General recommendations according to specific clinical settings are reported in Table 6.3. Fibrinogen replacement is the main step in prevention and treatment of bleeding in fibrinogen disorders. Fresh-frozen plasma, cryoprecipitates, and fibrinogen concentrate can be administered to increase fibrinogen levels [70]. The latter has the advantages to be purified, to be virus-inactivated, to contain a defined fibrinogen concentration, and to be rapidly infused with relatively small volumes [78].

Table 6.3 Recommendation for management of congenital fibrinogen disorders in selected clinical settings adapted from [62, 108]

General
In quantitative fibrinogen disorders, all new symptoms should be considered as bleeding and treated accordingly until additional investigations are performed
In afibrinogenemia and severe bleeding phenotype, all invasive procedure (including venipuncture) should be avoided and performed only upon approval of hematologist
Anti-inflammatory drugs should be avoided in patients with a bleeding phenotype
Hormonal contraception should be suggested in women with heavy menstrual bleeding
Annual visit to a hemophilia center should be organized for all patients with fibrinogen disorders
Surgical procedure and pregnancy management should be performed under the guidance of specialized centers dealing with bleeding disorders
Thrombosis
Screening for dysfibrinogenemia should be considered as second-line investigation in the absence of more common causes of thrombophilia
The same recommendations as for the general population should be proposed, favoring a limited duration of anticoagulation (except for type 3B dysfibrinogenemia)
Anticoagulation with a direct anticoagulant is the first choice
A mechanical or pharmacological thromboprophylaxis should always be considered taking into account the bleeding risk
Pregnancy
A preconception counseling is mandatory with a multidisciplinary team
In afibrinogenemia and severe hypofibrinogenemia, fibrinogen replacement should be started as early in gestation as possible targeting at least >1 g/L
A quarterly assessment of fibrinogen level and a systematic monitoring of fetal growth should be proposed
Fibrinogen replacement to allow neuroaxial anesthesia targeting >1.5 g/L
Avoid invasive fetal procedures
Early fibrinogen replacement and tranexamic acid in case of post-partum hemorrhage
Consider thromboprophylaxis

Three fibrinogen concentrates are marketed and several other are in the process of authorization. However, they are still inaccessible in many areas of the world. In the aforementioned large cohort on afibrinogenemia, some patients from Asia (7.5%) and Africa (18%) had no access to fibrinogen supplementation [67].

Pharmacokinetics properties are similar among the fibrinogen concentrates even though slight differences in the half-lives and the recovery times have been observed (Table 6.4). In view of interindividual variations, an individual pharmacokinetics study should be proposed to each patient under fibrinogen prophylaxis [79–81]. Currently, there are no evidence-based data to drive the management of patients with fibrinogen disorders [82]. Conventionally, patients with quantitative fibrinogen disorders are treated "on-demand", although secondary prophylaxis regimens should be proposed after life-threatening bleeding. Long-term prophylaxis should be proposed as primary prophylaxis in patients with afibrinogenemia to decrease the risk of cerebral bleeding [69]. In case of primary or secondary prophylaxis, the target trough fibrinogen level should be more than 0.5 g/L. The long half-life of fibrinogen usually allows an administration every 5-7 to 14 days [70, 83, 84]. To determine

Table 6.4 Pharmacokinetic parameters for median (minimal–maximum) fibrinogen activity of three fibrinogen concentrates

		Manco-Johnson et al. [106]	Djambas-Khayat et al. [81]	Ross et al. [107]
Primary PK parameters	Clearance (ml/h/kg)	0.55 [0.45–0.86]	0.57 [0.38–0.77]	0.63 [0.40–1.17]
	Volume of distribution (ml/kg)	52.7 [36.22–67.67]	53.5 [36.3–60.4]	61.04 [36.89–149.11]
Secondary PK parameters	C_{max} (g/L)	1.3 [1.00–2.10]	1.34 [1.06–2.19]	1.24 [0.75–1.96]
	AUC $_{0\text{-}\infty}$ (g h/L)	126.8 [81.7–156.4]	105 [78.2–167]	111.14 [59.7–175.5]
	Half-life $t_{1/2}$ (h)	77.1 [55.73–117.26]	67.9 [51.0–99.9]	72.85 [40.03–156.96]
In vivo recovery	Incremental recovery (mg/dl mg/kg)	1.7 [1.30–2.73]	2.22 [1.77–3.65]	1.77 [1.08–2.62]
	According to the plasmatic volume (%)	61.8 [52.45–97.43]	89.0 [69.5–133.0]	64.83 [40.89–88.13]

PK pharmacokinetics, C_{max} maximum plasma concentration, *AUC* area under the plasma concentration-time curve from the start of infusion (time 0)

the required dose of fibrinogen, the following formula can be used: Amount of fibrinogen to be administered (g/L) = [target fibrinogen level (g/L) – basal fibrinogen level (g/L)] × 1/incremental recovery time (mg/dl mg/kg) × weight (kg). The optimal target of fibrinogen activity in cases of acute bleeding or to prevent bleeding during surgery is unknown and is mainly extrapolated from non-randomized clinical trials [81, 85, 86]. Recent expert's guidelines suggest raising peak fibrinogen levels to 1.5 g/L for most major or clinically relevant nonmajor bleeding events or high-risk bleeding surgeries [62]. Subsequent doses should be based on the clinical evolution and the patient's trough fibrinogen activity level, aiming at least 0.5 g/L throughout wound healing [87]. Keeping a minimal level of fibrin(ogen) is essential to maintaining an optimal activation of factor XIII and thus helping in wound healing and angiogenesis [49]. Tranexamic acid can be added in cases with bleeding involving mucous membranes [88]. In case of thrombosis, the same recommendations as for the general population are usually adopted. Increasing data support the utilization of direct oral anticoagulant [60, 89–92]. The overall management of patients with qualitative fibrinogen disorders should always consider the personal and familial history of bleeding and thrombosis as well as the genotype [62]. In case of bleeding, the same recommendations for quantitative fibrinogen disorder are valuable. Fibrinogen replacement prophylaxis before surgery is usually necessary only in case of a bleeding phenotype or in case of major surgery [62]. Most often, the fibrinogen replacement is required only in case of complications. Patients should receive an accurate thromboprophylaxis in high thrombotic risk situation.

References

1. Dorgalaleh A, Casini A, Rahmani P. Congenital Fibrinogen Disorders. In: Dorgalaleh A, editor. Congenital bleeding disorders. Springer; 2018. p. 163–81.
2. Palla R, Peyvandi F, Shapiro AD. Rare bleeding disorders: diagnosis and treatment. Blood. 2015;125(13):2052–61.
3. Casini A, de Moerloose P, Neerman-Arbez M. Clinical features and management of congenital fibrinogen deficiencies. Semin Thromb Hemost. 2016;42(4):366–74.
4. Dorgalaleh A, Alavi SE, Tabibian S, et al. Diagnosis, clinical manifestations and management of rare bleeding disorders in Iran. Hematology. 2017;22(4):224–30.
5. Miller CH, Soucie JM, Byams VR, et al. Occurrence rates of inherited bleeding disorders other than haemophilia and von Willebrand disease among people receiving care in specialized treatment centres in the United States. Haemophilia. 2022;28(3):e75–8.
6. de Moerloose P, Casini A, Neerman-Arbez M. Congenital fibrinogen disorders: an update. Semin Thromb Hemost. 2013;39(6):585–95.
7. Neerman-Arbez M, de Moerloose P, Casini A. Laboratory and genetic investigation of mutations accounting for congenital fibrinogen disorders. Semin Thromb Hemost. 2016;42(4):356–65.
8. Casini A, Undas A, Palla R, et al. Diagnosis and classification of congenital fibrinogen disorders: communication from the SSC of the ISTH. J Thromb Haemost. 2018;16(9):1887–90.
9. Miesbach W, Schenk J, Alesci S, Lindhoff-Last E. Comparison of the fibrinogen Clauss assay and the fibrinogen PT derived method in patients with dysfibrinogenemia. Thromb Res. 2010;126(6):e428–33.
10. Jennings I, Peyvandi F, Kitchen S, et al. A failure to diagnosis dysfibrinogenaemia: data from multicentre studies amongst UK Nequas and proRBDD project laboratories. In: XXV congress of the international society on thrombosis and Haemostasis, abstract PO500. Int J Lab Hematol. 2017;39(6):653–62.
11. Casini A. From routine to research laboratory: strategies for the diagnosis of congenital fibrinogen disorders. Hamostaseologie. 2020;40(4):460–6.
12. Verhovsek M, Moffat KA, Hayward CP. Laboratory testing for fibrinogen abnormalities. Am J Hematol. 2008;83(12):928–31.
13. Besser MW, MacDonald SG. Acquired hypofibrinogenemia: current perspectives. J Blood Med. 2016;7:217–25.
14. Undas A. Determination of fibrinogen and thrombin time (TT). Methods Mol Biol. 2017;1646:105–10.
15. Mackie IJ, Kitchen S, Machin SJ, et al. Guidelines on fibrinogen assays. Br J Haematol. 2003;121(3):396–404.
16. Llamas P, Santos AB, Outeirino J, Soto C, Tomas JF. Diagnostic utility of comparing fibrinogen Clauss and prothrombin time derived method. Thromb Res. 2004;114(1):73–4.
17. Shapiro SE, Phillips E, Manning RA, et al. Clinical phenotype, laboratory features and genotype of 35 patients with heritable dysfibrinogenaemia. Br J Haematol. 2013;160(2):220–7.
18. Vasse M, Francois D, Van Dreden P, de Mazancourt P. Different sensitivity of von Clauss reagents for the diagnosis of dysfibrinogenemia. Eur J Haematol. 2020;104(1):70–1.
19. Skornova I, Simurda T, Stasko J, et al. Use of fibrinogen determination methods in differential diagnosis of hypofibrinogenemia and dysfibrinogenemia. Clin Lab. 2021;67(4) https://doi.org/10.7754/Clin.Lab.2020.200820.
20. Xiang L, Luo M, Yan J, et al. Combined use of Clauss and prothrombin time-derived methods for determining fibrinogen concentrations: screening for congenital dysfibrinogenemia. J Clin Lab Anal. 2018;32(4):e22322.
21. Luo M, Xiang L, Yan J, et al. Fibrinogen Clauss and prothrombin time derived method ratio can differentiate dysfibrinogenemia from hypofibrinogenemia and hyperfibrinogenemia. Thromb Res. 2020;194:197–9.

22. Brennan SO, Mangos H, Faed JM. Benign FGB (148Lys-->Asn, and 448Arg-->Lys), and novel causative gamma211Tyr-->His mutation distinguished by time of flight mass spectrometry in a family with hypofibrinogenaemia. Thromb Haemost. 2014;111(4):679–84.
23. Krammer B, Anders O, Nagel HR, Burstein C, Steiner M. Screening of dysfibrinogenaemia using the fibrinogen function versus antigen concentration ratio. Thromb Res. 1994;76(6):577–9.
24. Lebreton A, Casini A. Diagnosis of congenital fibrinogen disorders. Ann Biol Clin (Paris). 2016;74(4):405–12.
25. Casini A, Brungs T, Lavenu-Bombled C, et al. Genetics, diagnosis and clinical features of congenital hypodysfibrinogenemia: a systematic literature review and report of a novel mutation. J Thromb Haemost. 2017;15(5):876–88.
26. Leung B, Beggs J, Mason J. Fibrinogen Longmont: a clinically heterogeneous dysfibrinogenemia with discrepant fibrinogen results influenced by clot detection method and reagent. TH Open. 2022;6(1):e18–20.
27. Jennings I, Kitchen S, Menegatti M, et al. Potential misdiagnosis of dysfibrinogenaemia: data from multicentre studies amongst UK NEQAS and PRO-RBDD project laboratories. Int J Lab Hematol. 2017;39(6):653–62.
28. Marchi R, Neerman-Arbez M, Gay V, et al. Comparison of different activators of coagulation by turbidity analysis of hereditary dysfibrinogenemia and controls. Blood Coagul Fibrinolysis. 2021;32(2):108–14.
29. Suzuki A, Suzuki N, Kanematsu T, et al. Clot waveform analysis in Clauss fibrinogen assay contributes to classification of fibrinogen disorders. Thromb Res. 2019;174:98–103.
30. Suzuki A, Suzuki N, Kanematsu T, et al. Development and validation of a novel qualitative test for plasma fibrinogen utilizing clot waveform analysis. Sci Rep. 2022;12(1):434.
31. Rodeghiero F, Pabinger I, Ragni M, et al. Fundamentals for a systematic approach to mild and moderate inherited bleeding disorders: an EHA consensus report. Hema. 2019;3(5):e286.
32. Simurda T, Casini A, Stasko J, et al. Perioperative management of a severe congenital hypofibrinogenemia with thrombotic phenotype. Thromb Res. 2020;188:1–4.
33. Simurda T, Asselta R, Zolkova J, et al. Congenital afibrinogenemia and hypofibrinogenemia: laboratory and genetic testing in rare bleeding disorders with life-threatening clinical manifestations and challenging management. Diagnostics (Basel). 2021;11(11):2140.
34. Szanto T, Lassila R, Lemponen M, et al. Whole blood thromboelastometry by ROTEM and thrombin generation by Genesia according to the genotype and clinical phenotype in congenital fibrinogen disorders. Int J Mol Sci. 2021;22(5):2286.
35. Young GA, Carmona R, Cano GV. Thromboelastography and thrombin generation assay in inherited afibrinogenemia. Haemophilia. 2018;24(6):e410–6.
36. Blomback B, Blomback M, Henschen A, et al. N-terminal disulphide knot of human fibrinogen. Nature. 1968;218(5137):130–4.
37. Neerman-Arbez M, Honsberger A, Antonarakis SE, Morris MA. Deletion of the fibrinogen [correction of fibrogen] alpha-chain gene (FGA) causes congenital afibrogenemia. J Clin Invest. 1999;103(2):215–8.
38. Richard M, Celeny D, Neerman-Arbez M. Mutations accounting for congenital fibrinogen disorders: an update. Semin Thromb Hemost. 2022;48(08):889–903.
39. Sovova Z, Pecankova K, Majek P, Suttnar J. Extension of the human fibrinogen database with detailed clinical information-the alphaC-connector segment. Int J Mol Sci. 2021;23(1):132.
40. Soria J, Mirshahi S, Mirshahi SQ, et al. Fibrinogen alphaC domain: its importance in physiopathology. Res Pract Thromb Haemost. 2019;3(2):173–83.
41. Asselta R, Duga S, Tenchini ML. The molecular basis of quantitative fibrinogen disorders. J Thromb Haemost. 2006;4(10):2115–29.
42. Duga S, Asselta R, Santagostino E, et al. Missense mutations in the human beta fibrinogen gene cause congenital afibrinogenemia by impairing fibrinogen secretion. Blood. 2000;95(4):1336–41.
43. Neerman-Arbez M, de Moerloose P, Bridel C, et al. Mutations in the fibrinogen aalpha gene account for the majority of cases of congenital afibrinogenemia. Blood. 2000;96(1):149–52.

44. Neerman-Arbez M. The molecular basis of inherited afibrinogenaemia. Thromb Haemost. 2001;86(1):154–63.
45. Vu D, Bolton-Maggs PH, Parr JR, et al. Congenital afibrinogenemia: identification and expression of a missense mutation in FGB impairing fibrinogen secretion. Blood. 2003;102(13):4413–5.
46. Vu D, Di Sanza C, Caille D, et al. Quality control of fibrinogen secretion in the molecular pathogenesis of congenital afibrinogenemia. Hum Mol Genet. 2005;14(21):3271–80.
47. Vu D, Neerman-Arbez M. Molecular mechanisms accounting for fibrinogen deficiency: from large deletions to intracellular retention of misfolded proteins. J Thromb Haemost. 2007;5(Suppl. 1):125–31.
48. Casini A, Blondon M, Lebreton A, et al. Natural history of patients with congenital dysfibrinogenemia. Blood. 2015;125(3):553–61.
49. Bridey F, Negrier C, Duval C, et al. Impaired factor XIII activation in patients with congenital afibrinogenemia. Haematologica. 2019;104(3):e111–3.
50. Valade S, Mariotte E, Azoulay E. Coagulation disorders in hemophagocytic Lymphohistiocytosis/macrophage activation syndrome. Crit Care Clin. 2020;36(2): 415–26.
51. McPherson HR, Duval C, Baker SR, et al. Fibrinogen alphaC-subregions critically contribute blood clot fibre growth, mechanical stability, and resistance to fibrinolysis. elife. 2021;10:e68761.
52. Yan J, Wu Y, Liao L, et al. The beta-chain mutation p.Trp433Stop impairs fibrinogen secretion: a novel nonsense mutation associated with hypofibrinogenemia. Int J Lab Hematol. 2021;43(6):1549–56.
53. Ridgway HJ, Brennan SO, Faed JM, George PM. Fibrinogen Otago: a major alpha chain truncation associated with severe hypofibrinogenaemia and recurrent miscarriage. Br J Haematol. 1997;98(3):632–9.
54. Martinez J, Holburn RR, Shapiro SS, Erslev AJ. Fibrinogen Philadelphia. A hereditary hypodysfibrinogenemia characterized by fibrinogen hypercatabolism. J Clin Invest. 1974;53(2):600–11.
55. Mukai S, Nagata K, Ikeda M, et al. Genetic analyses of novel compound heterozygous hypodysfibrinogenemia, Tsukuba I: FGG c.1129+62_65 del AATA and FGG c.1299+4 del a. Thromb Res. 2016;148:111–7.
56. Duval C. Fibrinogen levels and thrombosis prevention. Blood. 2022;139(9):1269–71.
57. Moret A, Zuniga A, Ibanez M, et al. Clinical and molecular characterization by next generation sequencing of Spanish patients affected by congenital deficiencies of fibrinogen. Thromb Res. 2019;180:115–7.
58. Cao Z, Dong Y, Zeng J, et al. Whole-exome sequencing identified novel mutations in FGA and FGG genes in the patients with decreased fibrinogen. Thromb Res. 2019;177:79–82.
59. Guipponi M, Masclaux F, Sloan-Bena F, et al. A homozygous duplication of the <I>FGG</i> exon 8-intron 8 junction causes congenital afibrinogenemia. Lessons learned from the study of a large consanguineous Turkish family. Haematologica. 2022;107(5):1064–71.
60. Bor MV, Feddersen S, Pedersen IS, Sidelmann JJ, Kristensen SR. Dysfibrinogenemia-potential impact of genotype on thrombosis or bleeding. Semin Thromb Hemost. 2022;48(2):161–73.
61. Vilar R, Fish RJ, Casini A, Neerman-Arbez M. Fibrin(ogen) in human disease: both friend and foe. Haematologica. 2020;105(2):284–96.
62. Casini A, de Moerloose P. How I treat dysfibrinogenemia. Blood. 2021;138(21):2021–30.
63. Casini A, Neerman-Arbez M, Ariens RA, de Moerloose P. Dysfibrinogenemia: from molecular anomalies to clinical manifestations and management. J Thromb Haemost. 2015;13(6):909–19.
64. Gu L, Wang B, Liu L, et al. Hepatic fibrinogen storage disease and hypofibrinogenemia caused by fibrinogen Aguadilla mutation: a case report. J Int Med Res. 2020;48(1):300060519898033.
65. Asselta R, Paraboschi EM, Duga S. Hereditary hypofibrinogenemia with hepatic storage. Int J Mol Sci. 2020;21(21):7830.

66. Peyvandi F, Palla R, Menegatti M, et al. Coagulation factor activity and clinical bleeding severity in rare bleeding disorders: results from the European network of rare bleeding disorders. J Thromb Haemost. 2012;10(4):615–21.
67. Casini A, von Mackensen S, Santoro C, et al. Clinical phenotype, fibrinogen supplementation, and health-related quality of life in patients with afibrinogenemia. Blood. 2021;137(22):3127–36.
68. Dorgalaleh A, Farshi Y, Haeri K, Ghanbari OB, Ahmadi A. Risk and management of intracerebral hemorrhage in patients with bleeding disorders. Semin Thromb Hemost. 2022;48(3):344–55.
69. Casini A, Neerman-Arbez M, de Moerloose P. Heterogeneity of congenital afibrinogenemia, from epidemiology to clinical consequences and management. Blood Rev. 2021;48:100793.
70. Shapiro A. The use of prophylaxis in the treatment of rare bleeding disorders. Thromb Res. 2020;196:590–602.
71. Djambas Khayat C, Marchi R, Durual S, et al. Impact of fibrinogen infusion on thrombin generation and fibrin clot structure in patients with inherited afibrinogenemia. Thromb Haemost. 2022;122(09):1461–8.
72. Saes JL, Laros-van Gorkom BAP, Coppens M, Schols SEM. Pregnancy outcome in afibrinogenemia: are we giving enough fibrinogen concentrate? A case series. Res Pract Thromb Haemost. 2020;4(2):343–6.
73. Wypasek E, Klukowska A, Zdziarska J, et al. Genetic and clinical characterization of congenital fibrinogen disorders in polish patients: identification of three novel fibrinogen gamma chain mutations. Thromb Res. 2019;182:133–40.
74. Wang X, Li Y, Luo Z, et al. Fibrinogen BOE II: intracerebral hemorrhage associated with a novel compound mutation in a Chinese family with dysfibrinogenemia. Thromb Res. 2020;196:63–6.
75. Valiton V, Hugon-Rodin J, Fontana P, Neerman-Arbez M, Casini A. Obstetrical and postpartum complications in women with hereditary fibrinogen disorders: a systematic literature review. Haemophilia. 2019;25(5):747–54.
76. Peterson W, Liederman Z, Baker J, et al. Hemorrhagic, thrombotic and obstetric complications of congenital dysfibrinogenemia in a previously asymptomatic woman. Thromb Res. 2020;196:127–9.
77. Marchi R, Vilar R, Durual S, et al. Fibrin clot properties to assess the bleeding phenotype in unrelated patients with hypodysfibrinogenemia due to novel fibrinogen mutations. Thromb Res. 2021;197:56–64.
78. Casini A, de Moerloose P. Fibrinogen concentrates in hereditary fibrinogen disorders: past, present and future. Haemophilia. 2020;26(1):25–32.
79. Bellon A, Djambas Khayat C, El Khorassani M, et al. Use of a population pharmacokinetic model to determine pharmacokinetic parameters of a new fibrinogen concentrate in pediatric afibrinogenemic subjects ≤12-year old. Res Pract Thromb Haemost. 2017;1(Suppl. 1):851.
80. Bellon A, Fuseau E, Roumanie O, et al. Population pharmacokinetics of a triple-secured fibrinogen concentrate administered to afibrinogenaemic patients: observed age- and body weight-related differences and consequences for dose adjustment in children. Br J Clin Pharmacol. 2020;86(2):329–37.
81. Djambas Khayat C, El Khorassani M, Lambert T, et al. Clinical pharmacology, efficacy and safety study of a triple-secured fibrinogen concentrate in adults and adolescent patients with congenital fibrinogen deficiency. J Thromb Haemost. 2019;17(4):635–44.
82. Mumford AD, Ackroyd S, Alikhan R, et al. Guideline for the diagnosis and management of the rare coagulation disorders: a United Kingdom Haemophilia Centre Doctors' Organization guideline on behalf of the British Committee for Standards in Haematology. Br J Haematol. 2014;167(3):304–26.
83. Peyvandi F. Epidemiology and treatment of congenital fibrinogen deficiency. Thromb Res. 2012;130(Suppl. 2):S7–11.
84. Menegatti M, Peyvandi F. Treatment of rare factor deficiencies other than hemophilia. Blood. 2019;133(5):415–24.

85. Lissitchkov T, Madan B, Djambas Khayat C, et al. Fibrinogen concentrate for treatment of bleeding and surgical prophylaxis in congenital fibrinogen deficiency patients. J Thromb Haemost. 2020;18(4):815–24.
86. Ross CR, Subramanian S, Navarro-Puerto J, et al. Pharmacokinetics, surrogate efficacy and safety evaluations of a new human plasma-derived fibrinogen concentrate (FIB Grifols) in adult patients with congenital afibrinogenemia. Thromb Res. 2021;199:110–8.
87. Luyendyk JP, Schoenecker JG, Flick MJ. The multifaceted role of fibrinogen in tissue injury and inflammation. Blood. 2019;133(6):511–20.
88. Maas D, Saes JL, Blijlevens NMA, et al. Treatment of patients with rare bleeding disorders in The Netherlands: real-life data from the RBiN study. J Thromb Haemost. 2022;20(4):833–44.
89. Choi C, Maus T. Pulmonary thromboendarterectomy requiring cardiopulmonary bypass and deep hypothermic circulatory arrest in a patient with congenital afibrinogenemia. J Cardiothorac Vasc Anesth. 2021;35(2):593–6.
90. Trelinski J, Witkowski M, Chojnowski K, et al. Fibrinogen Lodz: a new cause of dysfibrinogenemia associated with recurrent thromboembolic arterial events. Pol Arch Intern Med. 2019;129(12):934–5.
91. Nathoo N, Rydz N, Poon MC, Metz LM. Ischemic strokes in a man with congenital afibrinogenemia. Can J Neurol Sci. 2018;45(5):590–2.
92. Lasky J, Teitel J, Wang M, et al. Fibrinogen concentrate for bleeding in patients with congenital fibrinogen deficiency: observational study of efficacy and safety for prophylaxis and treatment. Res Pract Thromb Haemost. 2020;4(8):1313–23.
93. Lisman T, Ariens RA. Alterations in fibrin structure in patients with liver diseases. Semin Thromb Hemost. 2016;42(4):389–96.
94. Dawson NA, Barr CF, Alving BM. Acquired dysfibrinogenemia. Paraneoplastic syndrome in renal cell carcinoma. Am J Med. 1985;78(4):682–6.
95. Arai S, Kamijo T, Takezawa Y, et al. Acquired dysfibrinogenemia: monoclonal lambda-type IgA binding to fibrinogen caused lower functional plasma fibrinogen level and abnormal clot formation. Int J Hematol. 2020;112(1):96–104.
96. Lazarou I, Petitpierre N, Auger I, et al. Felty's syndrome and hypofibrinogenemia: an unusual target for anti-cyclic citrullinated peptide antibodies? Mod Rheumatol. 2015;25(5):790–3.
97. Taylor FB Jr, Toh CH, Hoots WK, et al. Towards definition, clinical and laboratory criteria, and a scoring system for disseminated intravascular coagulation. Thromb Haemost. 2001;86(5):1327–30.
98. Zhang Q, Wang J, Liu H, et al. Risk factors for tigecycline-induced hypofibrinogenaemia. J Clin Pharm Ther. 2020;45(6):1434–41.
99. Zollner S, Pablik E, Druml W, et al. Fibrinogen reduction and bleeding complications in plasma exchange, immunoadsorption and a combination of the two. Blood Purif. 2014;38(2):160–6.
100. Zhou J, Ding Q, Chen Y, et al. Clinical features and molecular basis of 102 Chinese patients with congenital dysfibrinogenemia. Blood Cells Mol Dis. 2015;55(4):308–15.
101. Smith N, Bornikova L, Noetzli L, et al. Identification and characterization of novel mutations implicated in congenital fibrinogen disorders. Res Pract Thromb Haemost. 2018;2(4):800–11.
102. Castaman G, Giacomelli SH, Biasoli C, Contino L, Radossi P. Risk of bleeding and thrombosis in inherited qualitative fibrinogen disorders. Eur J Haematol. 2019;103(4):379–84.
103. Simurda T, Zolkova J, Kolkova Z, et al. Comparison of clinical phenotype with genetic and laboratory results in 31 patients with congenital dysfibrinogenemia in northern Slovakia. Int J Hematol. 2020;111(6):795–802.
104. Zhou P, Yu M, Peng Y, Ma P, Wan L. Identification and characterization of novel mutations in Chinese patients with congenital fibrinogen disorders. Blood Cells Mol Dis. 2020;86:102489.
105. Mohsenian S, Seidizadeh O, Mirakhorli M, Jazebi M, Azarkeivan A. Clinical and molecular characterization of Iranian patients with congenital fibrinogen disorders. Transfus Apher Sci. 2021;60(6):103203.
106. Manco-Johnson MJ, Dimichele D, Castaman G, et al. Pharmacokinetics and safety of fibrinogen concentrate. J Thromb Haemost. 2009;7(12):2064–9.

107. Ross C, Rangarajan S, Karimi M, et al. Pharmacokinetics, clot strength and safety of a new fibrinogen concentrate: randomized comparison with active control in congenital fibrinogen deficiency. J Thromb Haemost. 2018;16(2):253–61.
108. Undas A, Casini A. Congenital structural and functional fibrinogen disorders: a primer for internists. Pol Arch Intern Med. 2019;129(12):913–20.

Congenital Prothrombin Deficiency: Diagnosis and Management

7

Raimondo De Cristofaro

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)

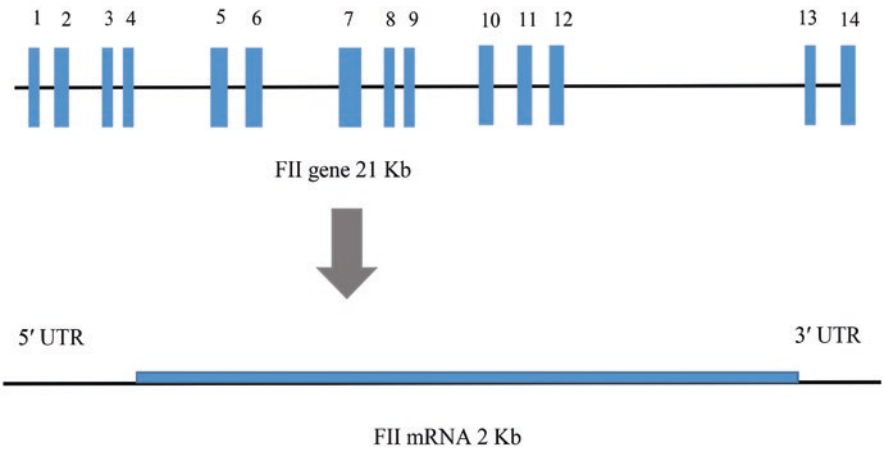

Fig. 7.1 factor (F) II structure. The schematic presentation of *F2* gene. The *F2* gene is composed of 14 exons and 13 introns covering a 21 kb region. This protein is mapped on short arm of chromosome 11 (11p11-q12)

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].

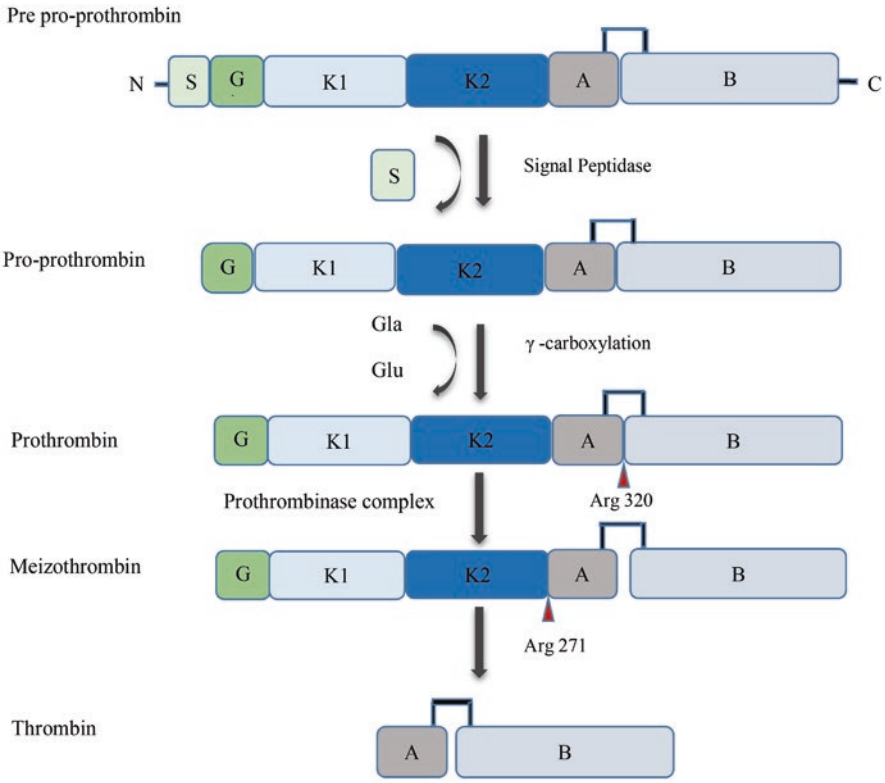

Fig. 7.2 Prothrombin synthesis and thrombin generation. Pre-pro-prothrombin is a precursor of prothrombin, which is synthesized in the liver. Following the removal of signal peptide by signal peptidase the pro-prothrombin which consists of Gla domain is generated. The vitamin K-dependent carboxylase 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.

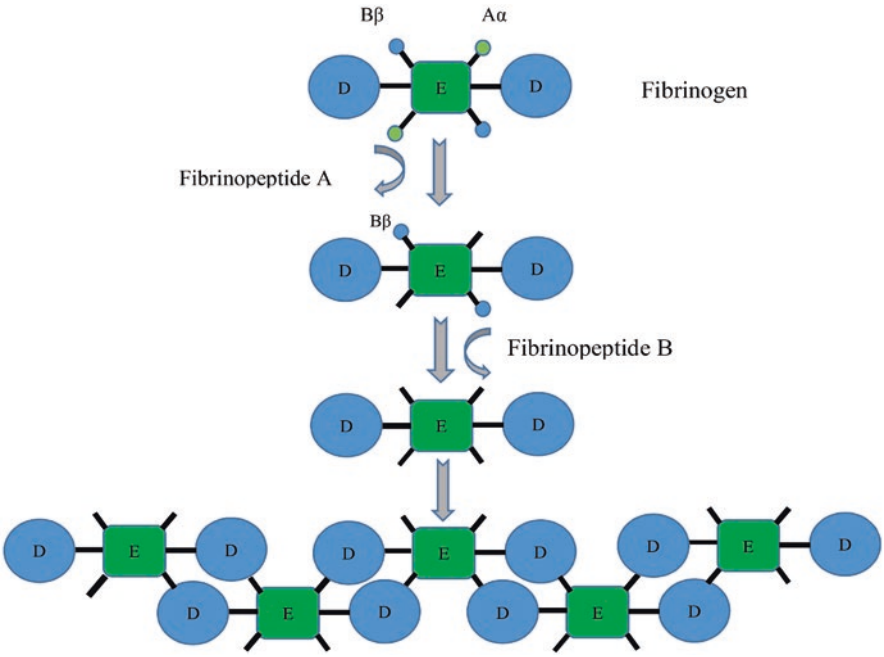

Fig. 7.3 Thrombin binds to the central E nodule of fibrinogen and cleaves this protein at four specific Arg-Gly bonds at the N-terminal of Aα and Bβ chains. This reaction leads to release of fibrinopeptide A (FPA) and B (FPB), respectively. Following the cleavage of FPA, a fibrin monomer which also termed 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].

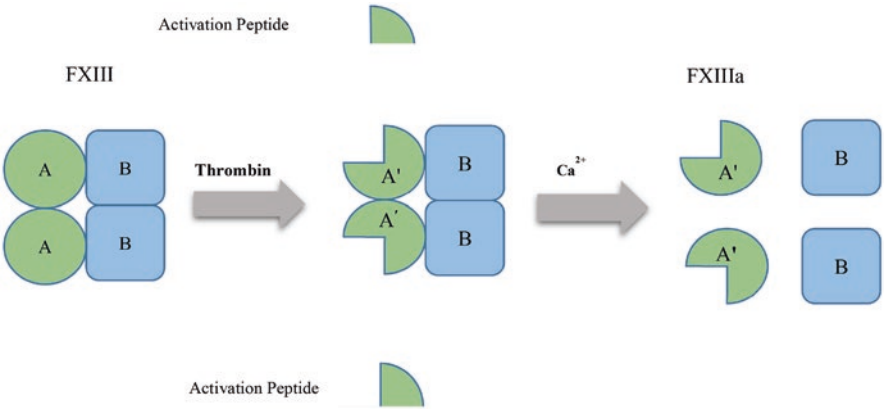

Fig. 7.4 Factor (F) XIII activation. Thrombin cleaves FXIII-A subunit (Arg37). Following the cleavage, the activation peptide is released and the active site is exposed. FXIII-A and FXIII-B subunits are separated from each other in the presence of $Ca^{++,}$ and therefore FXIII became activated

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].

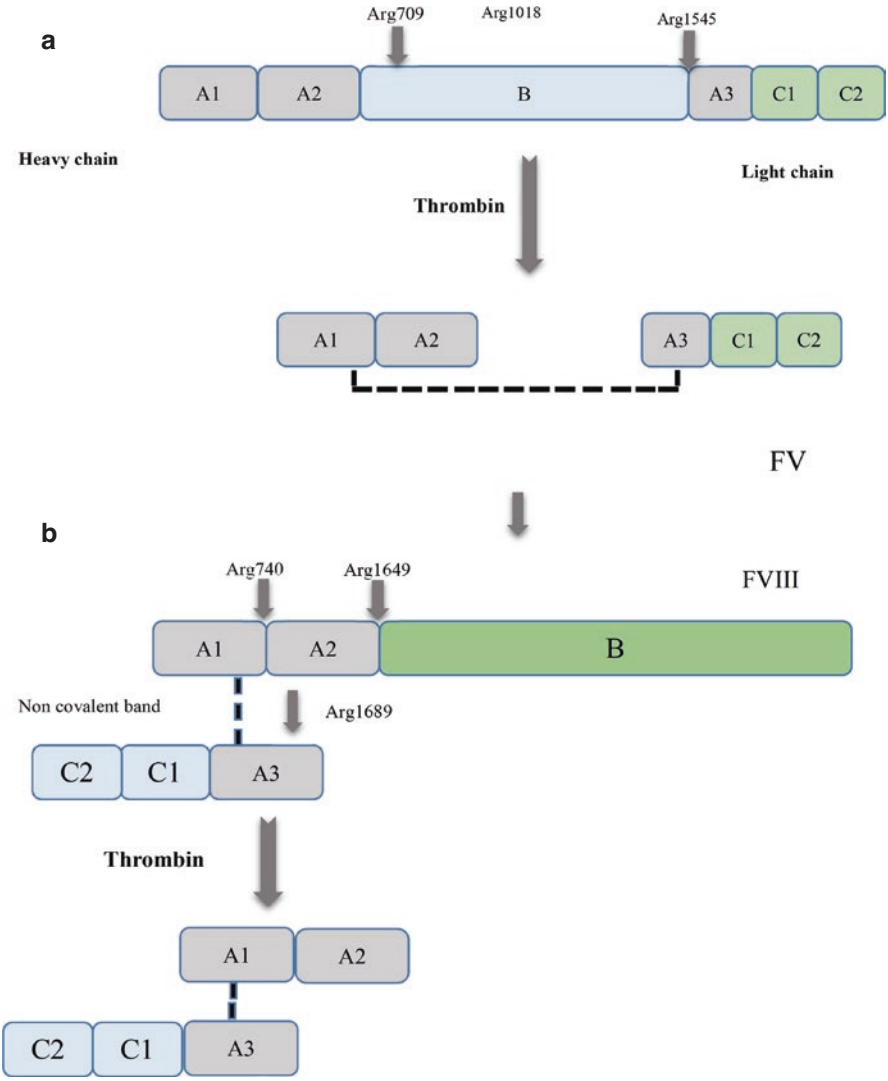

Fig. 7.5 Factor (F) V an FVIII activation. (**a**) FVIII consists of the heavy (A1, A2, and B domains) and light chains (C1, C2, and A3) that noncovalently linked to each other. Thrombin activates FVIII through cleavage between A1-A2, A2-B and at the A3 domains (are shown by arrows). Following the cleavages, 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].

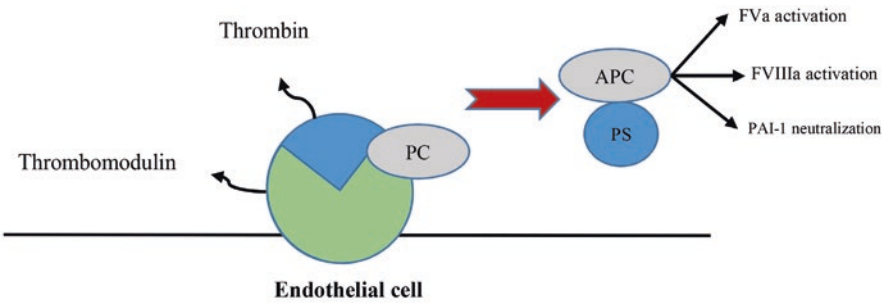

Fig. 7.6 Role of thrombin–thrombomodulin (TM) complex. Thrombin binds to the TM on the endothelial cells and shows its anticoagulant roles. In fact, thrombin–TM complex formation results in activation of protein C (PC). Activated PC (APC) with its cofactor, protein S (PS) inactivates the factor (F) V 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

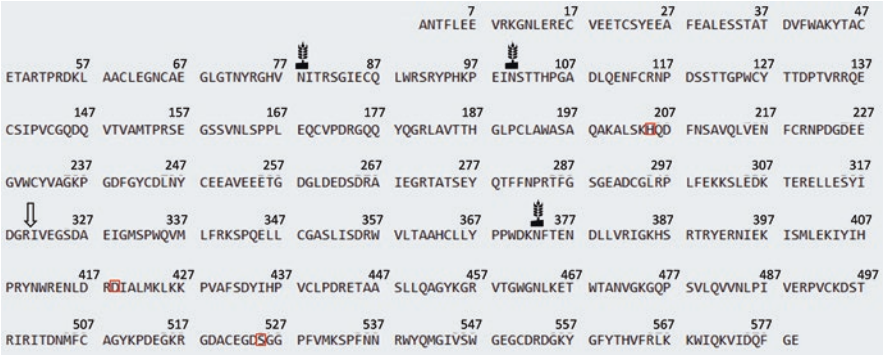

Fig. 7.7 Primary sequence of mature prothrombin. The residues forming the catalytic triad (H-S-D) are shown with a red rectangle at positions 205, 419, and 525, respectively. The putative N-glycosylation sites (N78, N100, and N373) are shown by symbols of black ears. The open arrow shows the factor Xa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

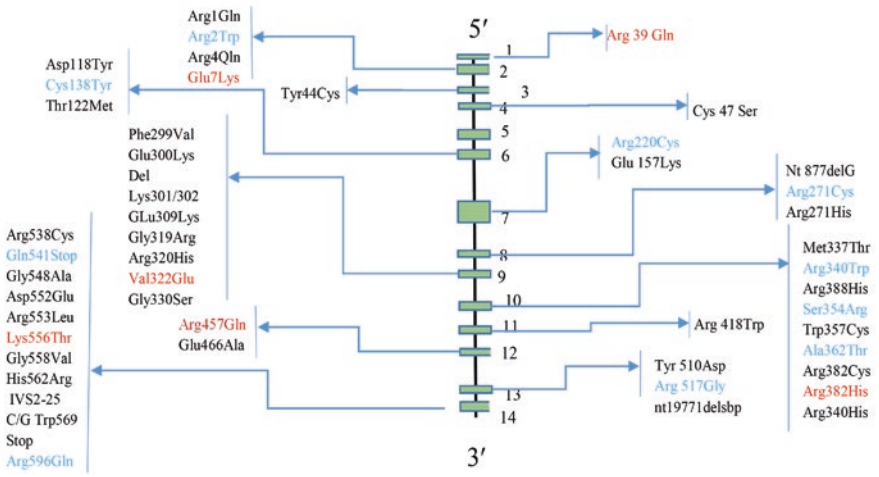

Fig. 7.8 The mutational spectrum of factor II deficiency. Almost all 60 variants (46 missenses, 3 splicing, 4 regulatory, and 7 frameshift) are represented. Exons and introns are shown by boxes and lines, respectively

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

References

1. Pozzi N, Chen Z, Gohara DW, Niu W, Heyduk T, Di Cera E. Crystal structure of prothrombin reveals conformational flexibility and mechanism of activation. J Biol Chem. 2013;288(31):22734–44.
2. Kamath P, Krishnaswamy S. Fate of membrane-bound reactants and products during the activation of human prothrombin by prothrombinase. J Biol Chem. 2008;283(44):30164–73.
3. Lancellotti S, Basso M, De Cristofaro R. Congenital prothrombin deficiency: an update. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; Stuttgart, Germany. 2013.
4. Hassanian SM, Dinarvand P, Rezaie AR. Adenosine regulates the proinflammatory signaling function of thrombin in endothelial cells. J Cell Physiol. 2014;229(9):1292–300.
5. Mannucci PM, Duga S, Peyvandi F. Recessively inherited coagulation disorders. Blood. 2004;104(5):1243–52.
6. Peyvandi F, Duga S, Akhavan S, Mannucci P. Rare coagulation deficiencies. Haemophilia. 2002;8(3):308–21.
7. Akhavan S, Luciani M, Lavoretano S, Mannucci PM. Phenotypic and genetic analysis of a compound heterozygote for dys-and hypoprothrombinaemia. Br J Haematol. 2003;120(1):142–4.
8. Rodriguez V, Warad D. Pediatric coagulation disorders. Pediatr Rev/Am Acad Pediatr. 2016;37(7):279.
9. Su K, Jin Y, Miao Z, Cheng X, Yang L, Wang M. Phenotypic and genetic analysis of dysprothrombinemia due to a novel homozygous mutation. Hematology. 2017;22(6):380–5.
10. Mumford AD, Ackroyd S, Alikhan R, Bowles L, Chowdary P, Grainger J, et al. Guideline for the diagnosis and management of the rare coagulation disorders. Br J Haematol. 2014;167(3):304–26.
11. Huntington JA. How na+ activates thrombin—a review of the functional and structural data. Biol Chem. 2008;389(8):1025–35.
12. Bode W. Editor the structure of thrombin: a Janus-headed proteinase. In: Seminars in thrombosis and hemostasis. New York, NY: Thieme Medical Publishers, Inc.; 2006.
13. Bode W, Turk D, Karshikov A. The refined 1.9-A X-ray crystal structure of D-Phe-pro-Arg chloromethylketone-inhibited human alpha-thrombin: structure analysis, overall structure, electrostatic properties, detailed active-site geometry, and structure-function relationships. Protein Sci. 1992;1(4):426–71.
14. Adams TE, Huntington JA. Thrombin-cofactor interactions: structural insights into regulatory mechanisms. Arterioscler Thromb Vasc Biol. 2006;26(8):1738–45.
15. Crawley J, Zanardelli S, Chion C, Lane D. The central role of thrombin in hemostasis. J Thromb Haemost. 2007;5(s1):95–101.
16. Carter IS, Vanden Hoek AL, Pryzdial EL, MacGillivray RT. Thrombin a-chain: activation remnant or allosteric effector? Thrombosis. 2010;2010:416167.
17. Hansson K, Stenflo J. Post-translational modifications in proteins involved in blood coagulation. J Thromb Haemost. 2005;3(12):2633–48.
18. Davie EW, Kulman JD. An overview of the structure and function of thrombin. In: Seminars in thrombosis and hemostasis. New York, NY: Thieme Medical Publishers, Inc.; 2006.
19. Mizuochi T, Fujii J, Kisiel W, Kobata A. Studies on the structures of the carbohydrate moiety of human prothrombin. J Biochem. 1981;90(4):1023–31.
20. Ruben EA, Summers B, Rau MJ, Fitzpatrick JAJ, Di Cera E. Cryo-EM structure of the prothrombin-prothrombinase complex. Blood. 2022;139(24):3463–73.
21. Krishnaswamy S. Prothrombinase complex assembly. Contributions of protein-protein and protein-membrane interactions toward complex formation. J Biol Chem. 1990;265(7):3708–18.
22. Krishnaswamy S. The transition of prothrombin to thrombin. J Thromb Haemost. 2013;11(s1):265–76.
23. Haynes LM, Bouchard BA, Tracy PB, Mann KG. Prothrombin activation by platelet-associated prothrombinase proceeds through the prethrombin-2 pathway via a concerted mechanism. J Biol Chem. 2012;287(46):38647–55.

24. Di Cera E, Dang Q, Ayala Y. Molecular mechanisms of thrombin function. Cell Mol Life Sci. 1997;53(9):701–30.
25. Boissel J, Le Bonniec B, Rabiet M, Labie D, Elion J. Covalent structures of beta and gamma autolytic derivatives of human alpha-thrombin. J Biol Chem. 1984;259(9):5691–7.
26. Mullin JL, Gorkun OV, Binnie CG, Lord ST. Recombinant fibrinogen studies reveal that thrombin specificity dictates order of fibrinopeptide release. J Biol Chem. 2000;275(33):25239–46.
27. Wolberg AS. Thrombin generation and fibrin clot structure. Blood Rev. 2007;21(3):131–42.
28. Standeven KF, Ariëns RA, Whitaker P, Ashcroft AE, Weisel JW, Grant PJ. The effect of dimethylbiguanide on thrombin activity, FXIII activation, fibrin polymerization, and fibrin clot formation. Diabetes. 2002;51(1):189–97.
29. Narayanan S. Multifunctional roles of thrombin. Ann Clin Lab Sci. 1999;29(4):275–80.
30. Camire R, Bos M. The molecular basis of factor V and VIII procofactor activation. J Thromb Haemost. 2009;7(12):1951–61.
31. Myles T, Yun TH, Leung LL. Structural requirements for the activation of human factor VIII by thrombin. Blood. 2002;100(8):2820–6.
32. Bouma BN, Mosnier LO. Thrombin activatable fibrinolysis inhibitor (TAFI)—how does thrombin regulate fibrinolysis? Ann Med. 2006;38(6):378–88.
33. Coughlin SR. Thrombin signalling and protease-activated receptors. Nature. 2000;407(6801):258–65.
34. Andersen H, Greenberg DL, Fujikawa K, Xu W, Chung DW, Davie EW. Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. Proc Natl Acad Sci. 1999;96(20):11189–93.
35. Yang L, Manithody C, Rezaie AR. Activation of protein C by the thrombin–thrombomodulin complex: cooperative roles of Arg-35 of thrombin and Arg-67 of protein C. Proc Natl Acad Sci U S A. 2006;103(4):879–84.
36. Girolami A, Santarossa L, Scarparo P, Candeo N, Girolami B. True congenital prothrombin deficiency due to a 'new'mutation in the pre-propeptide (ARG-39 GLN). Acta Haematol. 2008;120(2):82–6.
37. Acharya SS. Rare bleeding disorders in children: identification and primary care management. Pediatrics. 2013;132(5):882–92.
38. Bajaj S, Rapaport S, Barclay S, Herbst K. Acquired hypoprothrombinemia due to non-neutralizing antibodies to prothrombin: mechanism and management. Blood. 1985;65(6):1538–43.
39. Hankey GJ, Eikelboom JW. Dabigatran etexilate: a new oral thrombin inhibitor. Circulation. 2011;123(13):1436–50.
40. Ng JW, Mohd Tahir NA, Chin PKL, Makmor-Bakry M, Mohd SS. A systematic review and meta-analysis of dabigatran peak and trough concentration in adults. Br J Clin Pharmacol. 2022;88(10):4443–59.
41. O'Marcaigh AS, Nichols WL, Hassinger NL, Mullins JD, Mallouh AA, Gilchrist GS, et al. Genetic analysis and functional characterization of prothrombins Corpus Christi (Arg382-Cys), Dhahran (Arg271-his), and hypoprothrombinemia. Blood. 1996;88(7):2611–8.
42. Lancellotti S, De Cristofaro R. Congenital prothrombin deficiency. In: Seminars in thrombosis and hemostasis. © Thieme Medical Publishers; Stuttgart, Germany. 2009.
43. Miyawaki Y, Suzuki A, Fujita J, Maki A, Okuyama E, Murata M, et al. Thrombosis from a prothrombin mutation conveying antithrombin resistance. N Engl J Med. 2012;366(25):2390–6.
44. Sun WY, Smirnow D, Jenkins ML, Degen SJ. Prothrombin Scranton: substitution of an amino acid residue involved in the binding of na+ (LYS-556 to THR) leads to dysprothrombinemia. Thromb Haemost. 2001;85(4):651–4.
45. Flaujac C, Conard J, Horellou M, Le Flem L, Samama M. Atypical mutations of the prothrombin gene at positions 20 209 and 20 218, and a novel mutation at position 20 219. Report on 10 patients. J Thromb Haemost. 2007;5(5):1064–8.
46. Bezeaud A, Vidaud D, Guillin M-C. Les déficits constitutionnels en prothrombine et les informations qu'ils peuvent nous apporter sur la structure et les fonctions de la prothrombine. Hématologie. 2005;11(6):397–407.

47. Varga EA, Moll S. Prothrombin 20210 mutation (factor II mutation). Circulation. 2004;110(3):e15–e8.
48. Zivelin A, Rosenberg N, Faier S, Kornbrot N, Peretz H, Mannhalter C, et al. A single genetic origin for the common prothrombotic G20210A polymorphism in the prothrombin gene. Blood. 1998;92(4):1119–24.
49. Pihusch R, Hiller E, Buchholz T, Rogenhofer N, Hasbargen U, Thaler CJ, et al. Thrombophilic gene mutations and recurrent spontaneous abortion: prothrombin mutation increases the risk in the first trimester. Am J Reprod Immunol. 2001;46(2):124–31.
50. Carter AM, Sachchithananthan M, Stasinopoulos S, Maurer F, Medcalf RL. Prothrombin G20210A is a bifunctional gene polymorphism. Thrombosis Haemost-Stuttgart. 2002;87(5):846–53.
51. Warshawsky I, Makkar V, Rimmerman C, Kottke-Marchant K. Prothrombin 20209C> T: 16 new cases, association with the 19911A> G polymorphism, and literature review. J Thromb Haemost. 2009;7(9):1585–7.
52. Girolami A, Ferrari S, Cosi E, Girolami B, Lombardi AM. Congenital prothrombin defects: they are not only associated with bleeding but also with thrombosis: a new classification is needed. Hematology. 2018;23(2):105–10.
53. Peyvandi F, Menegatti M, Palla R. Rare bleeding disorders: worldwide efforts for classification, diagnosis, and management. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; Stuttgart, Germany. 2013.
54. Meeks S, Abshire T. Abnormalities of prothrombin: a review of the pathophysiology, diagnosis, and treatment. Haemophilia. 2008;14(6):1159–63.
55. Denson K, Borrett R, Biggs R. The specific assay of prothrombin using the taipan snake venom. Br J Haematol. 1971;21(2):219–26.
56. Girolami A, Scarano L, Saggiorato G, Girolami B, Bertomoro A, Marchiori A. Congenital deficiencies and abnormalities of prothrombin. Blood Coagul Fibrinolysis. 1998;9(7):557–70.
57. Acharya S, Coughlin A, Dimichele DM. Rare bleeding disorder registry: deficiencies of factors II, V, VII, X, XIII, fibrinogen and dysfibrinogenemias. J Thromb Haemost. 2004;2(2):248–56.
58. Kershaw G, Favaloro EJ. Laboratory identification of factor inhibitors: an update. Pathol J RCPA. 2012;44(4):293–302.
59. Peyvandi F, Di Michele D, Bolton-Maggs P, Lee C, Tripodi A, Srivastava A. Classification of rare bleeding disorders (RBDs) based on the association between coagulant factor activity and clinical bleeding severity. J Thromb Haemost. 2012;10(9):1938–43.
60. Deangelo J, Jarrell D, Cosgrove R, Camamo J, Edwards C, Patanwala AE. Comparison of 3-factor versus 4-factor prothrombin complex concentrate with regard to warfarin reversal, blood product use, and costs. Am J Ther. 2018;25(3):e326–32.
61. Bolton-Maggs P, Perry D, Chalmers E, Parapia L, Wilde J, Williams M, et al. The rare coagulation disorders–review with guidelines for management from the United Kingdom Haemophilia Centre Doctors' organisation. Haemophilia. 2004;10(5):593–628.
62. Kadir R, Chi C, Bolton-Maggs P. Pregnancy and rare bleeding disorders. Haemophilia. 2009;15(5):990–1005.

Congenital Factor V Deficiency, Diagnosis, and Management

8

Shadi Tabibian and Rodney M. Camire

8.1 Introduction

Factor V (FV), also known as labile factor or proaccelarin, is a coagulation factor that plays a critical role in coagulation through dual pro and anti-coagulant functions [1]. FV was discovered in 1943 by Paul Owren through study of a woman affected by a syndrome like hemophilia [2]. FV circulates in blood as an inactive procofactor protein and must be converted to active its active form, FVa. FVa is a cofactor that binds to the serine protease FXa on activated cells to form the prothrombinase complex which rapidly converts prothrombin to thrombin [1]. FV is synthetized by hepatocytes and circulates as a precursor molecule in plasma. However, approximately 20–25% of the total FV pool is stored in platelet α-granules which originates from endocytosis of plasma FV by megakaryocytes [3]. In addition to procoagulant activity, FV is thought to have anticoagulant activity via participation in the downregulation of FVIII [4]. FV deficiency is a rare bleeding disorder (RBD) with an estimated prevalence of 1 per one million in the general population. This disorder mostly presents in regions where parental consanguinity is commonly practiced [2, 5]. FV deficiency is classified into two types including type I deficiency (parallel reduction in antigen and activity level) and type II deficiency (normal or mildly reduced in antigen level with reduced activity) [6]. FV deficiency most often presents at birth or early childhood but several cases due to

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_8

mild clinical presentations remain undiagnosed until later in life. The most common clinical manifestations impacting patients are mucosal bleeds (epistaxis, gum bleeding, and menorrhagia in women) and post-surgical bleeding. Hematoma and hamorthrosis are presented in about one quarter of patients. Life-threatening episodes including central nervous (CNS) bleeding and gastrointestinal bleeding (GI) bleeding have rare presentations of these patients and are most often presented in patients with undetectable FV levels [5, 7]. FV deficiency is suspected in the presence of a positive family history, bleeding episodes, and prolonged prothrombin time (PT) and activate thromboplastin time (APTT). For confirmation of diagnosis, the measurement of activity and antigen level of FV is necessary. In cases with low factor activity and antigen level, the measurement of FVIII antigen and activity is performed to exclude FV deficiency from combined FV and FVIII deficiency [7].

Acquired FV deficiency is a rare condition that mostly results from previous exposure to bovine thrombin, antibiotic administration, surgery, or malignancies. Acquired FV deficiency is associated with prolonged PT and aPTT without correction of a mixing study. In this condition, the presence of an inhibitor is confirmed by the Bethesda method [8, 9]. Since there is no FV concentrate available, fresh frozen plasma (FFP) is the only available treatment option for patients affected by FV deficiency. The dose of FFP depends on the kind of treatment (on demand or prophylaxis) and on the severity of bleeding episodes. Platelet transfusion in some situations is another option for treatment of patients with FV deficiency but the risk alloimmunization should be considered [10].

8.2 Factor V Structure and Function

The FV gene (*F5*) is composed of 25 exons and 24 introns covering a ~ 80 kb region mapped on long arm of chromosome 1(1q24.2) and is not far from antithrombin gene [11]. Cloning of the human coagulation FV cDNA led to the identification of a 6672-bp transcript encoding a protein with 2224 amino acids. Following removal of a signal sequence, FV circulates in plasma as a single chain precursor protein consisting of 2196 amino acids with a molecular weight of approximately 330 kDa. FV contains a 28-residue leader peptide and 6 different domains oriented in an A1, A2, B, A3, C1, and C2 conformation [1]. FV and FVIII have ~40% homology in domains A and C [12]. The 3 A domains are homologous to the copper-binding protein ceruloplasmin while the C domains belong to lipid-binding discoidin-like protein family [13, 14]. The B domain has no similarity to other known proteins and contains 2 tandem repeat with 17 amino acids and 31 tandem repeat with 9 amino acids which are not conserved among species [15–17]. FV has 19 cysteines of which 14 of them are involved in disulfide bridges. FV precursor undergoes post translational modifications including N- and O-linked glycosylation as well as sulfaction [1]. Structural information derived from inactivated bovine FVa and snake FV indicate that the three A domains are arranged in a triangular fashion sitting atop the two C domains [18].

8.3 Factor V Activation and Inactivation

FV circulates in blood in an inactive form and is converted to an active cofactor (FVa) by thrombin or activated FX (FXa) following proteolysis at arginine (Arg)^{709}, Arg^{1018}, and Arg^{1545}. Subsequently, the large central B-domain is entirely removed leaving the active cofactor which is comprised of a 105-kDa heavy chain (A1 and A2) and a 74/71-kDa light chain (A3, C1, and C2). The heavy chain interacts with FXa and prothrombin, whereas, the light chain interacts with phospholipids located in the membrane. These 2 fragments are stabilized by non-covalent association by Ca^{+2} ion and also hydrophobic interaction (Fig. 8.1a, b) [19, 20]. A key aspect of this procofactor to cofactor transition is the removal of important B-domain sequences, comprised of a basic and acidic sequence that are essential for keeping FV inactive [18]. Downregulation of FV/FVa is mediated by activated protein C (APC). APC is a vitamin K-dependent serine protease which inhibits FVa and FVIIIa through limited proteolysis. APC with its cofactor (protein S) cleaves FV/FVa at 3 residues including Arg^{506}, Arg^{306}, and Arg^{679} or Lys^{994} (Fig. 8.1a). The first cleavage occurs at Arg^{506} leads to decreased FV cofactor activity and also the affinity of this protein to FXa which results in partial FVa inactivation. The next

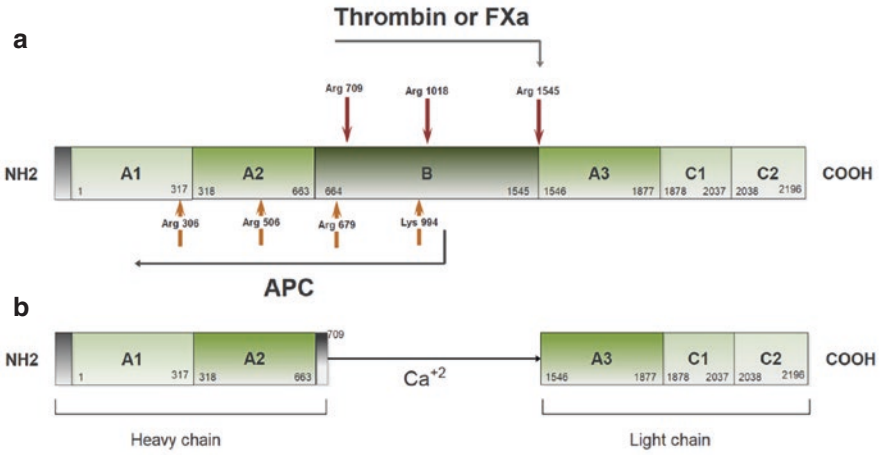

Fig. 8.1 Structure and activation of factor V. (A) The schematic presentation of factor V protein. Factor V precursor protein consists of 2224 amino acids and 6 different domains including A1, A2, B, A3, C1, and C2. The dark box located at the N-terminus is the 28-residue signal peptide. The A1, A2, and A3 domains are homologous to copper-binding protein ceruloplasmin whereas the 2 B domain belongs to lipid-binding discoidin-like protein family. The positions of proteolytic cleavage sites responsible for the activation and inactivation of factor V are shown. The arrows on the top represent activation of factor V by thrombin or activated FX (FXa) and arrows on the bottom represent inactivation sites by APC. (B) The activated form of factor V is shown. Factor V activation occurs by cleavage by thrombin or activated factor X in Arg ^{709}, Arg ^{1018}, and Arg^{1545}. After the cleavages of the B domain are removed, FVa consists of 2 fragments including 105-kDa heavy chain and 74- or 71-kDa light chain, which is stabilized by Ca^{+2} ion and also hydrophobic interaction

cleavage taking place in Arg^{306} leads to complete FVa inactivation. The functional implications of cleavage of FV/FVa at Arg^{679}/Lys ^{994} are not well understood [21]. Following cleavage of FV/FVa by APC, the A2 domain, divided into two fragments, dissociates from the rest of the molecule. The remaining FV molecule (FVai) consists of A1 domain, which is noncovalently associated with the light chain (A3, C1, and C2) [22]. The significance of FV/FVa inactivation by APC is illustrated by prothrombotic clinical manifestations in FV Leiden (Arg^{506} to Gln) which is resistant to APC cleavage at 506 site [23].

Inactivation of FVa also can be performed by other proteases such as thrombin or the fibrinolytic protease plasmin. Thrombin cleavage of FVa at Arg^{643} reduces the affinity between the two chains [24]. Plasmin is another inactivator of FVa and the amino acid region of 307–348 of FV plays critical role for this inactivation [25]. However, the physiological significance of these reactions is not clear.

8.4 Role of Factor V in the Coagulation Cascade

FV is synthesized by hepatocytes and circulates in plasma at a concentration of 7–10 μg/mL. Of the total amount of FV in blood, ~80% is in the plasma compartment while the remaining 20% is found in the α-granules of platelets (4600–14,000 molecules/platelet) in association with multimerin 1(MMRN) and originates from the plasma pool via endocytosis by megakaryocytes. Platelet FV appears to be processed by certain enzymes (e.g. FXa and APC) differently compared to its plasma counterpart. This appears to contribute to the platelet FV having some different functional properties following its release from α-granules upon platelet activation [26–28].

FVa is a procoagulant cofactor for the serine protease FXa in the prothrombinase complex, which consists of FVa, calcium, phospholipids, and activated FX. This complex rapidly converts prothrombin to thrombin at the platelet membrane surface or on other activated cells which expose negatively charged phospholipids. FVa accelerates the rate of conversion of prothrombin to thrombin by several orders of magnitude. Since FXa alone is not potent activator of prothrombin and because there is no alternative pathway to generate thrombin, FVa is essential for rapid thrombin generation (Fig. 8.2) [11]. In addition, following platelet activation, platelet FV(a) is disassociated from the MMRN and then exposed on platelet membrane surface. The exposing FV(a), enhances assembly and activity of prothrombinase complex [29]. In addition to procoagulant role of FV, FV protein is thought to have an **anticoagulant role**. The anticoagulant capacity of FV is related to APC-mediated inactivation of FVIII. FV appears to act as an APC cofactor to stimulate the inactivation of FVIIIa by APC/protein S complex (Fig. 8.2) [29].

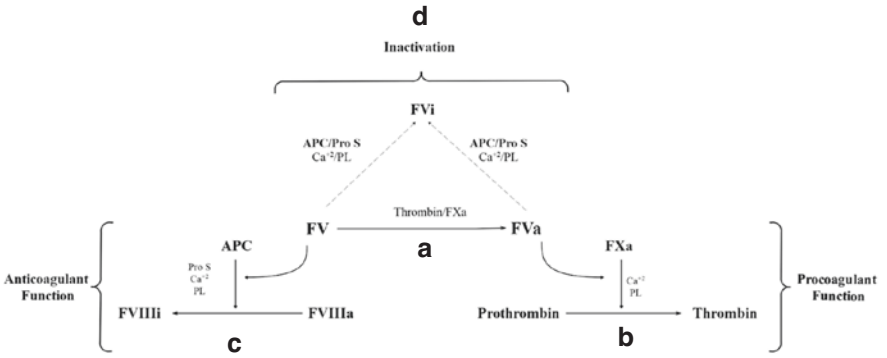

Fig. 8.2 Different functions of factor V. (**a**) Factor V (FV) is activated via proteolysis mediated by activated factor X (FXa) or thrombin. (**b**) After activation of FV, this factor acts as a cofactor for conversion of prothrombin to thrombin by FXa in presence of calcium ion (Ca^{+2}) and phospholipid (PL) in prothrombinase complex. (**c**) FV (before activation), acts as a cofactor for activated protein C (APC) in presence of protein S (ProS), calcium ion, and phospholipid in order to inactive FVIII (anticoagulant function). (**d**) Activated FV (FVa) and FV inactivated by APC and in presence of ProS, calcium, and phospholipid

8.5 Congenital Factor V Deficiency

Congenital FV deficiency is an RBD with population frequency of about 1 per one million in the general population [30]. This disorder, also named Owren parahemophilia, was first described in 1947 by Paul Owern in Norwegian women who suffered from lifelong epistaxis and menorrhagia [2]. FV deficiency, like other RBDs, is most common in regions with a high frequency of parental consanguinity. FV deficiency is inherited in an autosomal recessive pattern and results from near complete absence of FV in plasma or platelets [30, 31].

FV deficiency can be classified as either a type I deficiency or cross-reacting material negative (CRM−) which presents with low or undetectable antigen level (quantitative defect) and type II deficiency or cross-reacting material positive (CRM+) with normal or mildly reduced antigen level but reduced coagulant activity (qualitative defect) [6]. Based on the residual factor level, the severity of FV deficiency is classified as follows: mild (FV level ≥ 10%), moderate (FV level < 10%), or severe (FV level: undetectable) [32]. Individuals with homozygous or compound heterozygous deficiency typically have FV levels less than 10 iu/dL, while patients affected by heterozygote state have mild or moderate FV deficiency with FV level of 30–30 iu/dL [33].

8.6 Clinical Manifestations

FV deficiency is characterized by mild to severe bleeding features that mostly start before the age of six. Several studies have evaluated the clinical aspects of patients affected by FV deficiency. Based on these studies, mucocutaneous (epistaxis and menorrhagia in women), soft-tissue, and post-traumatic are the most common bleeding features (Table 8.1). Hematoma and hemarthrosis are present in one quarter of patients which are mostly related to trauma rather than spontaneous development. There have been occasional reports of spontaneous life-threatening bleeding episodes including CNS bleeding, umbilical cord bleeding (UCB), and GI bleeding [34–36]. In fact, spontaneous minor bleeding including epistaxis, bruising, oral cavity bleeding, minor bruising, ecchymosis, and menorrhagia are the most common bleeding diathesis and account for 34% of all clinical manifestations. About 32% of FV deficient patients are asymptomatic. Spontaneous major bleedings including CNS bleeding, UCB, GI bleeding, hematoma and hemarthrosis and provoked bleedings including post trauma bleeding, post-surgical bleeding, and antiplatelet or anticoagulant therapy account for 14% and 20% of all bleeding features, respectively [37]. Furthermore, the risk of postpartum hemorrhage (PPH) is estimated high (55–75%).

Patients with FV deficiency develop a bleeding diathesis early in life. Although most patients with a mild deficiency present no clinical features, they can manifest later in life and may be accidentally diagnosed in routine coagulation laboratory tests [6]. Homozygous or compound heterozygous patients which have moderate to severe bleeding features based on residual factor level while the heterozygote cases mostly present with mild degree of clinical manifestations [33]. Based on a European network of rare bleeding disorders (EN-RBD), there is a weak association between

Table 8.1 Clinical manifestations of patients affected by congenital factor V deficiency

Bleeding episodes	Iran (%)[a]	Iran (%)[a]	USA and Canada (%)		German (%) N:39
			Severe	Mild	
	$N = 35$	$N = 16$	$N = 18$	$N = 19$	
Epistaxis	57	68.7	42	62	25
Oral cavity bleeding		31.25			48
Menorrhagia	50	83.3			8
Gastrointestinal bleeding	6	6.25	6	–	4
Genitourinary bleeding	6	12.5	19	19	–
Hemarthrosis	26	18.75	%23	19	44
Hematoma	29	–	–	–	–
Central nervous bleeding	6	–	8	–	25
Umbilical cord bleeding	3	–	–	–	35.8
Post-operative bleeding/post-partum bleeding	43	6.25			–

[a]The severity of factor V deficiency is not mentioned in these studies

residual FV and clinical bleeding severity [38]. It can be concluded that undetectable FV level does not necessarily result in life-threatening bleeding episodes and also higher level of FV does not guarantee patients from presentation of life-threatening episodes. Whether these observations are related to the potential protective effect of having low TFPI levels in some of these patients is currently not known; however, from a biochemical perspective low TFPI should provide a compensatory mechanism to limit severe bleeding. Despite an overall benign phenotype of FV deficiency, different cases affected by neonatal and prenatal and neonatal intracranial hemorrhage (ICH) have been reported. In addition, there is an Iranian study conducted on 6 patients with moderate FV deficiency which reported 20 episodes of miscarriage. The significant point is that recurrent miscarriage is not reported as a clinical manifestation of FV deficiency in available clinical studies [39]. Therefore, different types of bleeding diathesis at different FV levels could be observed.

8.7 Quebec Platelet Disorder

The Quebec platelet disorder (QPD) is an inherited disorder which transmitted in an autosomal dominant pattern. QPD results from increased expression (more than 100 fold) of urokinase-type plasminogen activator (u-PA) which leads to plasmin generation and degradation of alpha granules proteins [40]. Affected patients experience a wide range of bleeding features including easy bruising to joint bleeding. However, the most common bleeding episode in these patients is delayed-onset bleeding following surgery or trauma. The bleeding episodes in QPD are hardly distinguishable from bleedings in patients affected in platelet function disorders (PFD). However, patients with PFD present with immediate bleeding and delayed bleeding is not typical in these patients. The laboratory features of these patients are normal bleeding time (BT), normal PT and APTT, normal to reduced platelet count, defective response to platelet aggregation test, and reduced FV levels but normal plasma FV level [40, 41]. The definite diagnosis of QBD is molecular analysis of tandem duplication of a 78 kb region located in chromosome 10 containing PLAU and C10 or f55. QPD is treated with antifibrinolytic agents such as tranexamic acid and aminocaproic acid. The only platelet FV deficiency characterized is $FV_{NewYork}$ which result from defective activity of platelet prothrombinase without proteolysis in platelet granules proteins [42].

8.8 Acquired Factor V Deficiency

Acquired FV deficiency is a rare disorder which presents with different bleeding tendencies ranging from complete absence of bleeding symptoms to life-threatening bleeding episodes [43]. In acquired FV deficiency, antibodies are directed against FV and lead to enhanced clearance or neutralization of procoagulant activity. Inhibitors directed against FV can occur at any age but generally are very rare;

however, they represent a clinical challenge. There are two forms of inhibitors: (i) inhibitors which develop in patients with congenital FV deficiency who are exposed to exogenous FV (FFP) and (ii) inhibitors which form in individuals due to autoimmune or alloimmune events [9]. By comparison, the first type is rarely present in patients with FV deficiency. The second type of inhibitors mostly present in elderly patients and have high titers. There are different risk factors which lead to development of inhibitors against FV including surgical procedure, exposure to bovine thrombin, antibiotics administration, blood transfusion, and malignancy [44–47]. Among all these risk factors, exposure to bovine thrombin represents the most prevalent risk. Bovine thrombin which contains trace amount of bovine FV is used as a hemostatic agent in different surgical procedures. Bovine FV is a potent stimulus that leads to development of autoantibodies direct against it (anti-bovine FV). These antibodies cross-react with human FV and result in acquired FV deficiency [48, 49]. The clinical manifestations and outcome of this disorder vary depending upon antibody titers, epitopes of the antibodies, and the access of antibody to platelet FV [50, 51]. Inhibitors against the C2 domain which is responsible for binding of this factor to phospholipid are associated with bleeding manifestations [52]. In cases which inhibitors lead to inactivation of anticoagulant function of FV, thrombotic manifestations are more common compared to hemorrhagic manifestations [53]. Although FV inhibitors generally spontaneously resolve in months, in presence of acute bleeding episodes, administration of FFP and platelet concentrate is recommended [54]. In addition, follow-up treatment including intravenous immunoglobulin injection, immunosuppression, and plasmapheresis in order to decrease the inhibitors titer is also recommended [6].

8.9 Molecular Basis of Congenital Factor V Deficiency

Although the first patient affected by FV deficiency was described in 1943, it took half of century before the first causative mutation in *F5* gene was uncovered [2]. Currently, over 200 different mutations have been reported in the *F5* gene (Human Genome Mutation Database, www.hgmd.cf.ac.uk; https://f5-db.eahad.org) [55]. These mutations include missense (46.8%), nonsense (11.7%), splicing mutations (10.7%), and small insertions/deletions (25.2) covering the whole *F5* gene except promoter region which has not investigated enough. The only two large reported deletions were chromosome 1q and p.Ser234Trp mutations in a 15-year-old girl and Exon 1–7 deletion (2.5 kbp) of *F5* in a South-African baby. Figure 8.3 shows the diversity of mutations in FV deficiency [37]. Furthermore, the only mutation which results in partial deficiency of FV (type II FV deficiency) is FV New Brunswick (Ala 221 Val) that occurred in domain A1 which reported in 1995 [56]. The first true null mutation reported in a patient who were pseudo homozygous for activated protein C resistance in 1997 in *F5* [57]. In addition to FV deficiency-casing mutations, different single nucleotide polymorphisms (SNP) in *F5* are reported which have been assembled in http://www.ncbi.nlm.nih.gov/SNP. These alterations of sequence may be (missense mutation) associated with changes in activity and antigen levels

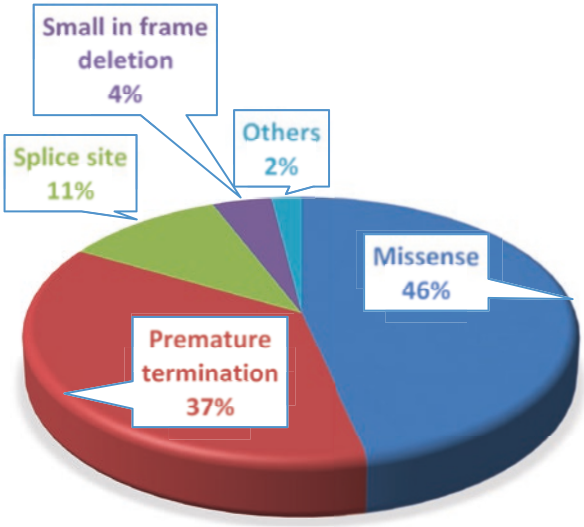

Fig. 8.3 The diversity of mutations in congenital factor V deficiency. Premature termination includes small deletion (18.4%), small insertion (6.8%), and nonsense mutation (11.7%). Others include major rearrangement (1%) and gross deletion (1%)

of FV [58]. Figures 8.3 and 8.4 show most of missense FV deficiency causing mutations.

The distinction between innocuous polymorphisms and true protein altering mutations represents a significant challenge. The difference between these two is rarely obvious and a careful family history investigation is needed as well as biochemical work. The availability of different databases assembling all known polymorphisms (innocuous polymorphism and true mutation) is clearly helpful to make distinctions. Most FV causing mutations have been reported as private mutations (the mutations reported in unique families or one patient) and few of them representing as recurrent [19]. P.Tyr1702Cys reported as a mutation which detected in several European and Asiatic patients [59]. The heterogeneity of mutations required the full genomic screening for molecular diagnosis. Almost one third of all mutations cause premature termination codons (PTCs). Investigation at mRNA level showed degradation of corresponding transcript by nonsense-mediated mRNA decay pathway (NMD). It appears that mutations predicting a premature stop codon are overrepresented compared to other genes. Most of the nonsense mutations detected in exon 13 (domain B) while the majority of missense mutations are clustered in the A and C domains, while domain B is free of this type of mutation [37]. Expression of missense mutations in mammalian cells showed that these mutations lead to secretion defects or early intracellular degradation. Splicing mutations consist of ~10% of total mutations, but different studies showed that this frequency was probably underestimated because splicing mutations outside the canonical splice sites are not easily recognized. Splicing mutations disrupt splice sites and therefore

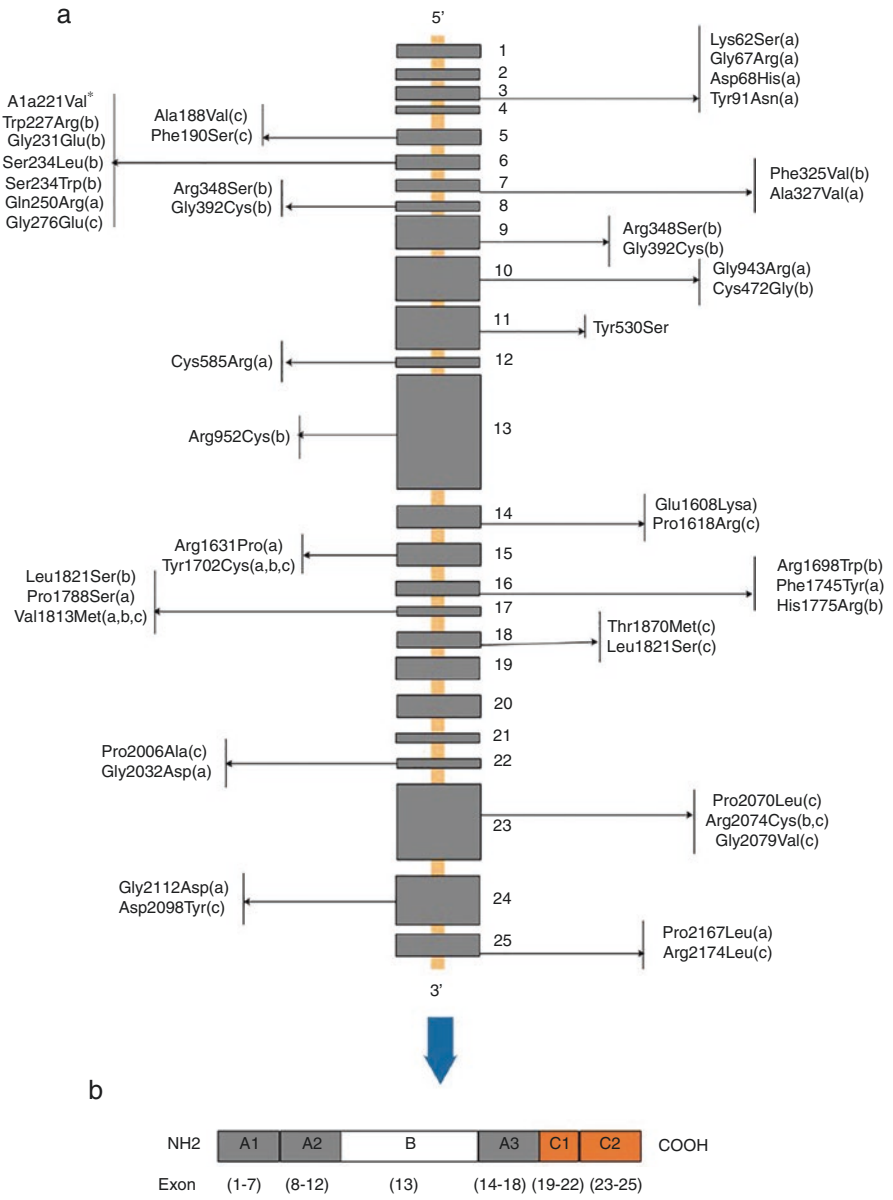

Fig. 8.4 (**a**) The mutational spectrum of factor V deficiency. Some missense mutations for type I and type II deficiency are represented. Exons and Intron are shown by boxes and lines, respectively. Mutations are named at protein level and the protein domains are also indicated. The mutation indicated by * is the only mutation of type II deficiency. The mutations indicated by a, b, c detected in heterozygous, compound heterozygous, and homozygous patients, respectively. (**b**) The factor V domains and the number of the exons are shown [37]

result in mRNA degradation and exon skipping in final protein [60, 61]. There are few studies which show the relation between type of mutations and the severity of bleeding episodes. However, the study which conducted in 2016 presents the association between *F5* splicing mutations and severe hemorrhagic diathesis, which is related to mRNA degradation by nonsense-mediated decay pathway [55].

8.10 Diagnosis

Like other RBDs, the diagnosis of FV deficiency, should include several key factors including bleeding episodes, family history and routine, and specific coagulation laboratory tests [34]. FV deficiency is suspected when routine coagulation laboratory tests including PT and aPTT are prolonged. Since different coagulation disorders result in prolongation of one or both of these tests, specific tests including, mixing studies and measurement of FV antigen and activity level may be necessary. In situations with prolonged PT and aPTT, addition of normal plasma to patient's plasma should correct these prolonged tests [6, 8]. In severe FV deficiency, bleeding time (BT) is also prolonged which can be related to platelet FV [8]. In the presence of low FV, measurement of FVIII in order to exclude combined FV and FVIII deficiency is necessary. In addition, the low activity of FV should distinguish from liver disease and consumptive coagulopathy [8].

8.10.1 Factor V Antigen Assay

To quantitate FV levels in plasma, a FV antigen assay can be performed. The principle of test is a sandwich enzyme immunoassay (ELISA). In this method, a monoclonal or polyclonal antibody against FV is coated on a microplate. Dilutions of the patient sample containing FV or standard are added to the plate followed by a secondary antibody directed against FV (monoclonal or polyclonal), which is conjugated to horseradish peroxidase (HRP) and is added to each plate. A substrate for HRP is added and the color change is spectrophotometrically assessed at 450 nm. The concentration of FV in the sample is determined by comparing the optical density (OD) of sample to a standard curve [62].

8.10.2 Factor V Activity Assay

Several manual and automated methods including PT-based assay, Lewis and Ware method, EDTA method, microplate activity assay were described in order to measure the activity of FV. These methods have different advantages and disadvantages; however, a one-stage FV-specific PT-based clotting assay is typically used.

8.10.3 One Stage PT-Based Assay

The principle of this assay is based on the ability of patient's plasma to correct the PT of factor deficient plasma (substrate). The diluted patient's plasma is mixed with equal volume of FV deficient plasma for correction of PT. Then the factor activity in patient's plasma is determined in a standard curve by using standard plasma. In this assay, serial dilution of standard plasma (1 in 5, 10, 20, and 40) and test plasma in buffered saline is prepared. Then, the equal amount of each dilution and deficient (substrate) plasma is mixed in another tube and warm to 37 °C. By adding the equal amount of diluted thromboplastin, the clotting time is recorded. For calculation of the activity of FV, the clotting time of each dilution of test plasma and standard plasma is plotted against concentration of FV.

In 2012, the rapid and convenient microplate assay for measurement of FV activity was introduced. In this method, a kinetic microplate reader is used to measure the increase in turbidity of plasma [63]. In fact, this assay monitors the absorbance change at 405 nm during fibrin formation at 0.35–0.45 unit. Then between starting and maximal absorbance before and after addition of thromboplastin and calcium chloride, the activity of FV is measured. This method, unlike previously reported techniques, requires small amount of plasma, also measures the initial time, rate and extends of fibrin formation in both not activated (FV1-stage assay) or have been activated with thrombin (FV2-stage assay) human plasma samples [64, 65]. These parameters provide more accurate information about the statue of FV during clot formation. Compared to manual and automated methods, this assay has higher sensitivity and detection limit (20–80 pM) and also has comparable clot times ranges (25–75 s) and corresponding FV levels in the sample (0.5–0.005 units/mL). In addition, this technique is a quantitative method and accurately measured the time, rate, and extent of clotting, while manual techniques work based on visual assessment of clot formation. The microplate assay compared to automated assay is inexpensive which can provide the required reagents commercially or made-in house. Another significant advantage of these methods is simultaneous assessment of about 12 samples [64, 65].

This method is done by using a multichannel pipet in order to add FV deficient plasma and diluted test or normal plasma in immunomodule strip wells, simultaneously. After adding thromboplastin and 1 min incubation at 25 °C, the calcium chloride is added to initiate clotting. The clotting time is determined as a time between the minimum and maximum absorbance at 405 nm after addition of chloride calcium. The initial clotting rate is measured by using a curve in which absorbance plotted versus time and defined as a rate of increasing in absorbance at 405 nm (first 5 time points of clot formation) in linear portion. In addition, the extent of clot formation was calculated by measuring the difference between maximal and minimal absorbance at 405 nm during clot formation [64, 65].

The FV activity of diluted test plasma in one stage assay is determined by use of standard curve of initial rate of clot formation or clot times versus the activity of serial dilution of normal reference plasma. One unit of FV is defined as an activity of FV in 1 milliliter of normal human plasma prior activation by thrombin [64, 65].

The two-stage FV assay initially requires a pre-incubation of the plasma sample with thrombin to convert FV to FVa. After incubation, the plasma is further diluted and **re-assayed** as mentioned above. The two-stage FV activity is calculated from one-stage standard curve. The total FV activity is calculated as a two-stage FV activity−one-stage FV activity [64, 65].

8.10.4 Factor V Inhibitor Assay

In the initial investigation, the prolonged PT and aPTT were further investigated by mixing patients' plasma with normal pooled plasma (50:50). The inhibitor was suspected when PT and PTT were not corrected by mixing studies. Inhibitors confirmed and titrated by Bethesda method.

8.10.5 Mixing Study

For this method, the reagents including normal plasma, patient's plasma, and aPTT reagents are required. The normal plasma which can be a commercial lyophilized or a plasma pool of at least 20 donors used for this purpose (Fig. 8.5).

In the first step, 4 tubes are prepared as follows:

1. Normal plasma.
2. Patient's plasma.
3. Equal mixture of patient and normal plasma (50:50) (time-dependent inhibitor: incubate 2 h at 37 °C).
4. Equal mixture of patient and normal plasma (50:50) (immediate inhibitor).

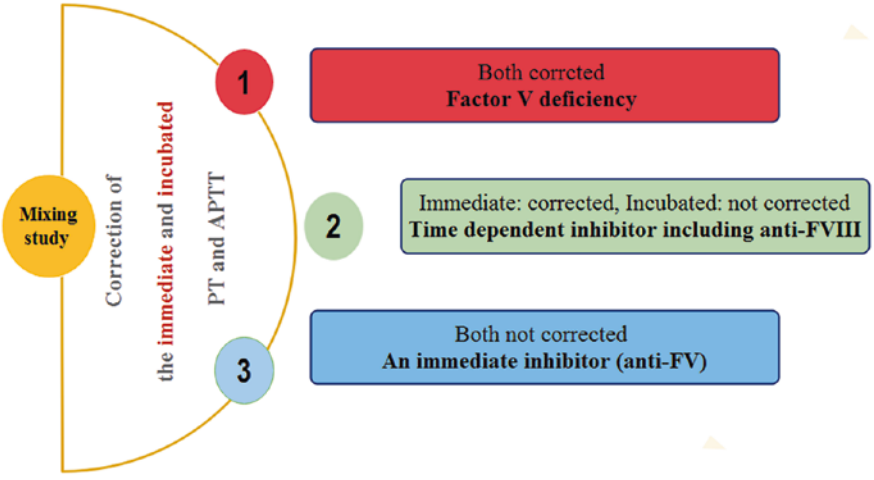

Fig. 8.5 The principle of mixing study

APTT and PT should be performed in duplicate for all tubes.

Interpretation:

1. If both immediate and incubated PT and aPTT tests show correction, the FV deficiency or multiple factor deficiencies are suspected.
2. If neither immediate nor incubated PT/aPTT shows correction, the presence of inhibitor is suspected.
3. If the immediate PT/aPTT results show correction, but the result of incubated tubes shows no correction, the slow-acting inhibitor including anti-FVIII is suspected [9, 62].

An important issue that must be considered is that, the activity of coagulation factors including FV and FVIII in potassium EDTA samples is factitiously decreased. Therefore, the mixing studies in these samples do not correct and the results of Bethesda assays are spuriously positive. Finding the inhibitors directly against FV and FVIII is a clue of potassium EDTA samples [66, 67].

8.10.6 Bethesda Assay

For quantitative measurement of FV inhibitor titer, The Bethesda method is used. The Bethesda unit is the amount of inhibitor which neutralized 50% of 1 unit of FV in normal plasma [68]. Dilution of patient's plasma with an equal volume of normal plasma (normally contains 100% FV) incubated at 37 °C for 0 hour (h), 1 h, or 2 h at 37 °C. FV inhibitors contrast to FVIII inhibitors that need 1–2 h incubation to fully inactivated FVIII, immediately neutralize FV [69]. However, incubation up to 2 h in order to accurate quantification of FV inhibitors level is recommended [69]. Dilutions of control plasma that contain no inhibitors are treated as the same way (A control consist of an equal volume of normal plasma with buffer). After incubation, the residual FV for each mixture assay using one-stage PT based assay is measured. The dilution with residual FV activity of about 50% is considered for determination of inhibitor strength. The strength of FV inhibitor is calculated by using standard graph of residual FV activity versus inhibitor units. The inhibitor titers from graph multiple by dilution in order to give the final titer (Fig. 8.6) [9, 62].

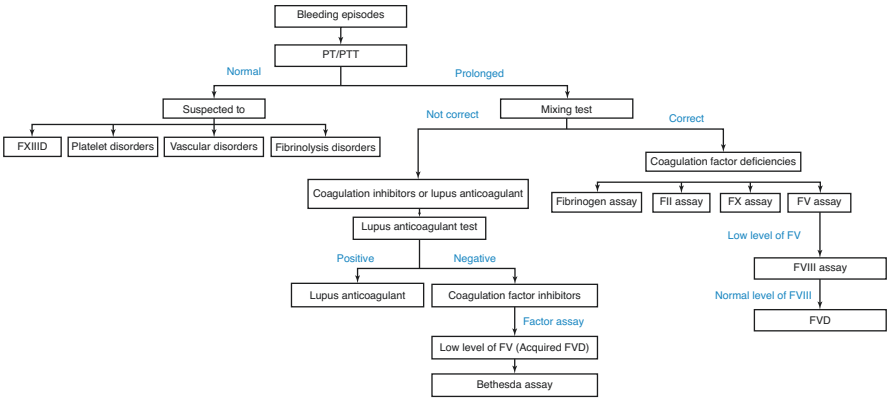

Fig. 8.6 An algorithmic approach for diagnosis of congenital and acquired factor (F) V deficiency. This algorithm has been developed by authors based on different studies. The algorithm started by presentation of bleeding diathesis and routine coagulation laboratory test including PT and PTT. In presence of normal PT and PTT along with bleeding episode, FXIII deficiency can be suspected and further specific tests are required (for more information refer to Chap. 13). If these two tests are prolonged, mixing test is indicated. The mixing test should be performed by using a mixture of equal volume of patient's plasma and normal plasma. **If correction achieved**, there is a factor deficiency or multiple factor deficiencies. Therefore, performance of factor assays for coagulation factors in common pathway is mandatory. In presence of low level of FV, FVIII assay in order to distinguish FVD from combined FV and FVIII deficiency is necessary. A normal level of FVIII indicates FV deficiency. **If the mixing study does not correct**, inhibitors direct against coagulation factors of common pathway or lupus anticoagulant are suspected. A negative lupus anticoagulant test indicated the presence of coagulation factors inhibitors. Factor assay should be performed in order to find the coagulation factor for which the inhibitor is directly against. Low level of FV indicted acquired FV deficiency. The Bethesda assay should be performed to do quantification of the inhibitor titer. (*FXIIID* Factor XIII deficiency, *FVD* factor V deficiency)

8.11 Treatment

The mainstay of treatment in patients with congenital FV deficiency is on-demand therapy to alleviate the hemorrhage as soon as possible. Since there is no commercially available *FV concentrate*, the only source of FV for patients affected by FV deficiency is virus inactivated FFP or the use of Octaplas® (an alternative solvent/detergent pharmaceutical product). In addition, because platelets contain FV, platelet transfusion can be helpful in some situations [70]. The required dosage for coagulation factor deficiencies depends on the kind of treatment (on demand and prophylaxis), the minimal hemostatic level, plasma half-life, and most importantly, the type of bleeding episode [10]. Plasma half-life of FV is 36 h, and based on the EN-RBD study, 10% of FV is the minimum level need for patients to be asymptomatic [38]. Table 8.2 lists the doses and therapeutic target levels in different kinds of treatments and major surgeries [71].

A new FV concentrate has currently been developed for clinical trials and related preclinical studies are being conducted for the orphan drug designation application [72]. A novel engineered FV which is known as superFVa has been

Table 8.2 Doses and target therapeutic levels in patients with factor V deficiency

Kind of treatment	Recommend level	Dosage of therapeutic level
On demand therapy	10%	15–25 mL/kg: FFP Platelet transfusion
Long term prophylaxis	10%	20 mL/kg 2 times/week
Major surgery	>15–20% (maintaining level)	15–25 mL/kg)before surgery) 10 mL/kg every 12 h Platelet transfusion (if required)
Minor surgery Cesarean delivery	–	15–20 mg/kg or 1 g four times daily: Tranexamic acid 15–20 mg/kg Maintain the level until wound healing achieved (5–7 days)

recently tested in preclinical studies against severe bleeding in FV deficiency and hemophilia A with inhibitors.

Although the treatment of the congenital FV deficiency is based on the administration of the FFP, advance therapies including genes and cell therapies can be replaced as curative treatments. Gene therapies could correct the underlying mutations, whereas in cell therapy, the functional healthy cells are generated and take over the function of defective cells. In the study conducted in 2021, Serrano et al. proposed methods which induce differentiation of the mesenchymal stem cells of human placenta to FV producing hepatospheres that could be a preliminary step of cell therapy of congenial FV deficiency.

References

1. Mann KG, Kalafatis M. Factor V: a combination of Dr Jekyll and Mr Hyde. Blood. 2003;101(1):20–30. https://doi.org/10.1182/blood-2002-01-0290.
2. Owren P. Parahaemophilia: haemorrhagic diathesis due to absence of a previously unknown clotting factor. Lancet. 1947;249(6449):446–8.
3. Rosing J, Tans G, Govers-Riemslag J, Zwaal R, Hemker HC. The role of phospholipids and factor Va in the prothrombinase complex. J Biol Chem. 1980;255(1):274–83.
4. Shen L, Dahlbäck B. Factor V and protein S as synergistic cofactors to activated protein C in degradation of factor VIIIa. J Biol Chem. 1994;269(29):18735–8.
5. Bolton-Maggs P, Perry D, Chalmers E, Parapia L, Wilde J, Williams M, et al. The rare coagulation disorders–review with guidelines for management from the United Kingdom Haemophilia Centre Doctors' Organisation. Haemophilia. 2004;10(5):593–628.
6. Duckers C, Simioni P, Rosing J, Castoldi E. Advances in understanding the bleeding diathesis in factor V deficiency. Br J Haematol. 2009;146(1):17–26.
7. Tabibian S, Kazemi A, Dorgalaleh A. Laboratory diagnosis of congenital factor V deficiency, routine, specific coagulation tests with molecular methods. J Cell Mol Anesth. 2016;1(2):87–90.
8. Duckers C, Simioni P, Spiezia L, Radu C, Gavasso S, Rosing J, et al. Low plasma levels of tissue factor pathway inhibitor in patients with congenital factor V deficiency. Blood. 2008;112(9):3615–23.
9. Favaloro EJ, Posen J, Ramakrishna R, Soltani S, McRae S, Just S, et al. Factor V inhibitors: rare or not so uncommon? A multi-laboratory investigation. Blood Coagul Fibrinolysis. 2004;15(8):637–47.

10. Peyvandi F, Garagiola I, Biguzzi E. Advances in treatment of bleeding disorders. J Thromb Haemost. 2016;14(11):2095–106.
11. Cripe LD, Moore KD, Kane WH. Structure of the gene for human coagulation factor V. Biochemistry. 1992;31(15):3777–85.
12. William H, Earl W. Blood coagulation factor V and VIII: structural and functional similarities and their relationship to hemorrhagic and thrombotic disorders. Blood. 1988;71(71):539–55.
13. Ortel TL, Takahashi N, Putnam FW. Structural model of human ceruloplasmin based on internal triplication, hydrophilic/hydrophobic character, and secondary structure of domains. Proc Natl Acad Sci. 1984;81(15):4761–5.
14. Baumgartner S, Hofmann K, Bucher P, Chiquet-Ehrismann R. The discoidin domain family revisited: new members from prokaryotes and a homology-based fold prediction. Protein Sci. 1998;7(7):1626–31.
15. Nicolaes GA, Dahlbäck B. Factor V and thrombotic disease. Arterioscler Thromb Vasc Biol. 2002;22(4):530–8.
16. Macedo-Ribeiro S, Bode W, Huber R, Quinn-Allen MA. Crystal structures of the membrane-binding C2 domain of human coagulation factor V. Nature. 1999;402(6760):434.
17. Villoutreix BO, Dahlbäck B. Structural investigation of the A domains of human blood coagulation factor V by molecular modeling. Protein Sci. 1998;7(6):1317–25.
18. Lechtenberg BC, Murray-Rust TA, Johnson DJ, Adams TE, Krishnaswamy S, Camire RM, et al. Crystal structure of the prothrombinase complex from the venom of Pseudonaja textilis. Blood. 2013;122(16):2777–83.
19. Asselta R, Tenchini M, Duga S. Inherited defects of coagulation factor V: the hemorrhagic side. J Thromb Haemost. 2006;4(1):26–34.
20. Camire R, Bos M. The molecular basis of factor V and VIII procofactor activation. J Thromb Haemost. 2009;7(12):1951–61.
21. Kalafatis M, Rand MD, Mann KG. The mechanism of inactivation of human factor V and human factor Va by activated protein C. J Biol Chem. 1994;269(50):31869–80.
22. Mann KG, Hockin MF, Begin KJ, Kalafatis M. Activated protein C cleavage of factor Va leads to dissociation of the A2 domain. J Biol Chem. 1997;272(33):20678–83.
23. Thorelli E, Kaufman RJ, Dahlbäck B. Cleavage of factor V at Arg 506 by activated protein C and the expression of anticoagulant activity of factor V. Blood. 1999;93(8):2552–8.
24. Hockin M, Kalafatis M, Cawthern K, Simoni P, Mann K. A novel cellular mechanism for factor Va inactivation. In: 40th Annual Meeting of the American Society of Hematology Blood; 1998.
25. Kalafatis M, Mann KG. The role of the membrane in the inactivation of factor Va by plasmin amino acid region 307–348 of factor v plays a critical role in factor Va cofactor function. J Biol Chem. 2001;276(21):18614–23.
26. Christella M, Thomassen L, Castoldi E, Tans G, Magdeleyns E, Delaunoit C, et al. Endogenous factor V synthesis in megakaryocytes contributes negligibly to the platelet factor V pool. Haematologica. 2003;88(10):1150–6.
27. Gould WR, Silveira JR, Tracy PB. Unique in vivo modifications of coagulation factor V produce a physically and functionally distinct platelet-derived cofactor characterization of purified platelet-derived factor V/Va. J Biol Chem. 2004;279(4):2383–93.
28. Camire RM, Kalafatis M, Cushman M, Tracy RP, Mann KG, Tracy PB. The mechanism of inactivation of human platelet factor Va from normal and activated protein C-resistant individuals. J Biol Chem. 1995;270(35):20794–800.
29. Lippi G, Favaloro EJ, Montagnana M, Manzato F, Guidi GC, Franchini M. Inherited and acquired factor V deficiency. Blood Coagul Fibrinolysis. 2011;22(3):160–6.
30. Naderi M, Tabibian S, Alizadeh S, Hosseini S, Zaker F, Bamedi T, et al. Congenital factor V deficiency: comparison of the severity of clinical presentations among patients with rare bleeding disorders. Acta Haematol. 2015;133(2):148–54.
31. Mannucci PM, Duga S, Peyvandi F. Recessively inherited coagulation disorders. Blood. 2004;104(5):1243–52.
32. Lippi G, Pasalic L, Favaloro EJ. Detection of mild inherited disorders of blood coagulation: current options and personal recommendations. Expert Rev Hematol. 2015;8(4):527–42.

33. Asselta R, Peyvandi F. Factor V deficiency. In: Seminars in thrombosis and hemostasis. © Thieme Medical Publishers; 2009.
34. Acharya S, Coughlin A, Dimichele DM. Rare Bleeding Disorder Registry: deficiencies of factors II, V, VII, X, XIII, fibrinogen and dysfibrinogenemias. J Thromb Haemost. 2004;2(2):248–56.
35. Mansouritorghabeh H, Manavifar L, Mobalegh A, Shirdel A. Haemorrhagic manifestations and prevalence of factor V deficiency in north-eastern Iran. Haemophilia. 2010;16(2):376–80.
36. Lak M, Sharifian R, Peyvandi F, Mannucci F. Symptoms of inherited factor V deficiency in 35 Iranian patients. Br J Haematol. 1998;103:1067–9.
37. Thalji N, Camire RM. Parahemophilia: new insights into factor v deficiency. In: Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2013.
38. Peyvandi F, Palla R, Menegatti M, Siboni S, Halimeh S, Faeser B, et al. Coagulation factor activity and clinical bleeding severity in rare bleeding disorders: results from the European Network of Rare Bleeding Disorders. J Thromb Haemost. 2012;10(4):615–21.
39. Naderi M, Tabibian S, Shamsizadeh M, Dorgalaleh A. Miscarriage and recurrent miscarriage in patients with congenital factor V deficiency: a report of six cases in Iran. Int J Hematol. 2016;103(6):673–5.
40. Diamandis M, Adam F, Kahr W, Wang P, Chorneyko K, Arsenault A, et al. Insights into abnormal hemostasis in the Quebec platelet disorder from analyses of clot lysis. J Thromb Haemost. 2006;4(5):1086–94.
41. Blavignac J, Bunimov N, Rivard GE, Hayward CP. Quebec platelet disorder: update on pathogenesis, diagnosis, and treatment. In: Seminars in thrombosis and hemostasis. © Thieme Medical Publishers; 2011.
42. Weiss HJ, Lages B, Zheng S, Hayward CP. Platelet factor V New York: a defect in factor V distinct from that in factor V Quebec resulting in impaired prothrombinase generation. Am J Hematol. 2001;66(2):130–9.
43. Franchini M, Lippi G. Acquired factor V inhibitors: a systematic review. J Thromb Thrombolysis. 2011;31(4):449–57.
44. Miesbach W, Voigt J, Peetz D, Scharrer I. Massive bleeding symptoms in two patients with factor V inhibitor and antiphospholipid antibodies after treatment with ciprofloxacin. Medizinische Klinik (Munich, Germany: 1983). 2003;98(6):339–43.
45. Lucia J, Aguilar C. A case of an asymptomatic idiopathic inhibitor to coagulation factor V. Haemophilia. 2005;11(2):178–80.
46. Takahashi H, Fuse I, Abe T, Yoshino N, Aizawa Y. Acquired factor V inhibitor complicated by Hashimoto's thyroditis, primary biliary cirrhosis and membranous nephropathy. Blood Coagul Fibrinolysis. 2003;14(1):87–93.
47. Koyama T, Saito T, Kusano T, Hirosawa S. Factor V inhibitor associated with Sjögren's syndrome. Br J Haematol. 1995;89(4):893–6.
48. Streiff MB, Ness PM. Acquired FV inhibitors: a needless iatrogenic complication of bovine thrombin exposure. Transfusion. 2002;42(1):18–26.
49. Ortel TL, Mercer MC, Thames EH, Moore KD, Lawson JH. Immunologic impact and clinical outcomes after surgical exposure to bovine thrombin. Ann Surg. 2001;233(1):88–96.
50. Nesheim ME, Nichols WL, Cole TL, Houston JG, Schenk RB, Mann KG, et al. Isolation and study of an acquired inhibitor of human coagulation factor V. J Clin Investig. 1986;77(2):405.
51. Ajzner E, Balogh I, Haramura G, Boda Z, Kalmar K, Pfliegler G, et al. Anti-factor V autoantibody in the plasma and platelets of a patient with repeated gastrointestinal bleeding. J Thromb Haemost. 2003;1(5):943–9.
52. Ortel TL, Moore KD, Quinn-Allen MA, Okamura T, Sinclair AJ, Lazarchick J, et al. Inhibitory anti–factor V antibodies bind to the factor V C2 domain and are associated with hemorrhagic manifestations. Blood. 1998;91(11):4188–96.
53. Kalafatis M, Simioni P, Tormene D, Beck DO, Luni S, Girolami A. Isolation and characterization of an antifactor V antibody causing activated protein C resistance from a patient with severe thrombotic manifestations. Blood. 2002;99(11):3985–92.

54. de Raucourt E, Barbier C, Sinda P, Dib M, Peltier JY, Ternisien C. High-dose intravenous immunoglobulin treatment in two patients with acquired factor V inhibitors. Am J Hematol. 2003;74(3):187–90.
55. Nuzzo F, Beshlawi I, Wali Y, Castoldi E. High incidence of intracranial bleeding in factor V-deficient patients with homozygous F5 splicing mutations. Br J Haematol. 2017;179(1):163–6.
56. Murray JM, Rand MD, Egan J, Murphy S, Kim H, Mann K. Factor VNew Brunswick: Ala221-to-Val substitution results in reduced cofactor activity. Blood. 1995;86(5):1820–7.
57. Guasch J, Lensen R, Bertina R. Molecular characterization of a type I quantitative factor V deficiency in a thrombosis patient that is "pseudo homozygous" for activated protein C resistance. Thromb Haemost. 1997;77(2):252–7.
58. Vos H. An online database of mutations and polymorphisms in and around the coagulation factor V gene. J Thromb Haemost. 2007;5(1):185–8.
59. Castoldi E, Lunghi B, Mingozzi F, Muleo G, Redaelli R, Mariani G, et al. A missense mutation (Y1702C) in the coagulation factor V gene is a frequent cause of factor V deficiency in the Italian population. Haematologica. 2001;86(6):629–33.
60. Nuzzo F, Bulato C, Nielsen B, Lee K, Wielders S, Simioni P, et al. Characterization of an apparently synonymous F5 mutation causing aberrant splicing and factor V deficiency. Haemophilia. 2015;21(2):241–8.
61. Castoldi E, Bulato C, Nuzzo F, Nielsen B, Lee K, Key N, et al. Characterisation of an apparently synonymous F5 mutation causing aberrant splicing and factor V deficiency. J Thromb Haemost. 2013;11:195.
62. Bain BJ, Bates I, Laffan MA. Dacie and Lewis practical haematology. Elsevier Health Sciences; 2016.
63. Bloom JW, Nesheim ME, Mann KG. A rapid technique for the preparation of factor V deficient plasma. Thromb Res. 1979;15(5–6):595–9.
64. Tilley D, Levit I, Samis JA. Measurement of factor V activity in human plasma using a microplate coagulation assay. J Vis Exp. 2012;(67):3822.
65. Tilley D, Levit I, Samis JA. Development of a microplate coagulation assay for factor V in human plasma. Thromb J. 2011;9(1):11.
66. Favaloro EJ, Bonar R, Duncan E, Earl G, Low J, Aboud M, et al. Mis-identification of factor inhibitors by diagnostic haemostasis laboratories: recognition of pitfalls and elucidation of strategies. A follow up to a large multicentre evaluation. Pathology. 2007;39(5):504–11.
67. Favaloro EJ, Bonar R, Duncan E, Earl G, Low J, Aboud M, et al. Identification of factor inhibitors by diagnostic haemostasis laboratories. A large multi-centre evaluation. Thromb Haemost. 2006;96(1):73–8.
68. Knöbl P, Lechner K. Acquired factor V inhibitors. Baillieres Clin Haematol. 1998;11(2):305–18.
69. Ang AL, Kuperan P, Ng CH, Ng HJ. Acquired factor V inhibitor. A problem-based systematic review. Thromb Haemost. 2009;101(5):852–9.
70. Salooja N, Martin P, Khair K, Liesner R, Hann I. Severe factor V deficiency and neonatal intracranial haemorrhage: a case report. Haemophilia. 2000;6(1):44–6.
71. Peyvandi F, Menegatti M. Treatment of rare factor deficiencies in 2016. ASH Education Program Book. 2016;2016(1):663–9.
72. Lawrie AS, Berbenni C, Menegatti M, Nardini I, Mackie I, Machin S, et al. In vitro characterizations of the first therapeutic factor V concentrate. Haemophilia. 2014;20:25–6.

Combined Factor V and Factor VIII Deficiency, Diagnosis, and Management

9

Elena Yakovleva

9.1 Comparative Characteristics of Coagulation Factors V and VIII

Blood clotting factors V and VIII have a lot in common. They belong to proteins with high molecular weight. They do not have catalytic activity but are cofactors in the coagulation cascade. Factor (F) V is a cofactor of the prothrombinase complex and FVIII is a cofactor of the tenase complex. Factors V and VIII share similar domain structure (A_1-A_2-B-A_3-C_1-C_2) with high sequence identities between the A and C domains (~40% amino acid sequence homology) and undergo similar extensive post-translational modifications [1–4]. The functionally dispensable B domains of FV and FVIII share few sequence similarities, but both are heavily glycosylated [5]. Approximately, 80% of total blood FV circulates in plasma, whereas the remaining 20% is concentrated within platelet alpha granules. Plasma FV is produced in the liver while platelet FV is synthesized in the megakaryocytes or absorbed from plasma and then sequestered in the alpha granules [6]. Liver is the predominant site of FVIII synthesis by liver sinusoidal endothelial cells and hepatocytes. The main activators of FV and FVIII are thrombin and factor Xa. FV and FVIII have different half-lives. For FVIII it is 10–14 h, for factor V, on the average, 20 h, although according to various data, the time may vary between 6–36 h. These factors differ slightly in concentration and activity in plasma. The genes of blood clotting factors V and VIII are located on different chromosomes. This determines different inheritance paths for isolated FV or FVIII deficiencies. The FV gene is composed of 25 exons spanning a region of about 80 kb in the 1q24.2 chromosomal region [7]. The FVIII gene is located on the long arm of the X chromosome, occupies a region of the order of 186 bp, long and consists of 26 exons. The underlying cause of combined FV and FVIII deficiency (F5F8D) is in specific genetic mutations while

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_9

Table 9.1 Comparative characteristics of coagulation factors V and VIII

Characteristics	FV	FVIII
Molecular weight	330 kDa	310 kDa
Biochemical function	cofactor	cofactor
Role in the coagulation cascade	prothrombinase complex	tenase complex
Structure	A_1–A_2–B–A_3–C_1–C_2	A_1–A_2–B–A_3–C_1–C_2
Synthesis	Hepatocytes, megakaryocytes	Hepatocytes, liver sinusoidal endothelial cells
Activators	FIIa, FXa	FIIa, FXa
Half-life	6–36 h	10–14 h
Location of the gene	1q24	Xq28
Plasma activity	70–120%	50–150%

isolated congenital deficiency of FV or FVIII is due to a defect in *F5* or *F8* genes, respectively. Comparative characteristics of FV and FVIII are presented in Table 9.1.

9.2 Combined Factor V and Factor VIII Deficiency

F5F8D (OMIM #227300 and #61362522) was first described in a pair in Swiss siblings by Oeri et al. in 1954 [8]. F5F8D is a rare bleeding disorder (RBD) that accounts for about 3% of all RBD and is the most common form of familial multiple coagulation factor deficiency (MCFD). F5F8D has been reported throughout the world. The prevalence in the general population is 1:1,000,000. Although the rate is higher (1:10,000) in populations where consanguineous marriages are acceptable. Most cases described are from Iran, Italy, Pakistan, Iraq, and India [9–14].

The disease has an autosomal recessive inheritance pathway, and men and women are equally affected. All homozygous individuals manifest the disease, this means complete penetrance. Heterozygous ones are usually asymptomatic [15].

F5F8D is characterized by simultaneous decrease of plasma FV and FVIII, usually between 5% and 30%. F5F8D is usually accompanied by mild to moderate bleeding tendency. Cases have been reported, however, with the activity of the factors to be as low as less than 5% with severe bleeding [10].

9.3 Etiology and Pathogenesis

Orei suggested that F5F8D is the result of a defect in a gene that encodes a common precursor of both blood clotting factors [8]. This hypothesis was refuted by Saito et al., who did not receive the effect of plasma transfusions from patients with hemophilia [16]. In what followed different genes encoding FVIII and FV were identified, in 1984 and 1992, respectively [17–19].

It became then obvious that the underlying cause of F5F8D was different from the one which causes isolated deficiency of FV or FVIII. The isolated FV or FVIII

deficiency results from defects in *F5* or *F8* genes, respectively. For many years, the mechanism by which a gene defect could cause the deficiency of these two different coagulation factors was unclear. In 1981, Soft and Levin suggested a common pathway of metabolism of both factors, disturbance of which leads to bleeding manifestations [20]. It was not until 1998 that Nichols et al. introduced null mutations in the *ERGIC53* (currently known as *lectin mannose binding 1-LMAN1*) gene, as the responsible genetic defect causing F5F8D [15, 21, 22]. LMAN1 protein was previously identified in the intermediate compartment between the endoplasmic reticulum (ER) and Golgi complex, but its function was not known [23]. Nichols et al. found out that LMAN1 was involved in transport of factors V and VIII from the ER to Golgi complex [22]. Mutations in *LMAN1* gene were identified in 70% of patients with F5F8D. This finding led to further genetic analysis. Finally, in 2003, Zhang et al. detected a second defect, referred to as multiple coagulation factor deficiency 2 (*MCFD2*) [24]. LMAN1 and MCFD2 form a receptor complex to transport specific secreted proteins, including FV and FVIII. In vitro studies suggest that LMAN1 acts as a cargo receptor for 2 lysosomal proteins, cathepsin C (CatC) and cathepsin Z (CatZ), and for α1-antitrypsin. FV and FVIII are synthesized on the rough ER and translocated into the ER lumen, where both proteins undergo N-linked glycosylation, proper folding, and quality control. Subsequently, proteins are packaged into coat protein complex-II (COPII) vesicles. These vesicles detach from the ER and connect to the endoplasmic reticulum—Golgi intermediate compartment (ERGIC). Retrieval (or retrograde transport) in coat protein I (COPI) vesicles returns many of the lost ER resident proteins back to the endoplasmic reticulum [25]. ERGIC is an organelle in eukaryotic cells which was first identified in 1988 using an antibody to the protein that has since been named ERGIC-53 [26]. This compartment mediates trafficking between the ER and Golgi complex, facilitating the sorting of cargo [27, 28].

The LMAN1–MCFD2 complex is the first known example of a specific ER-to-Colgi cargo receptor, discovered largely through the studies of F5F8D. The structure of the LMAN1-MCFD2 protein complex and **cargo transport by the LMAN1–MCFD2 complex** are shown in Fig. 9.1 [5, 25]. FV, FVIII, CatC, and CatZ bind the LMAN1–MCFD2 complex and are packaged into COPII vesicles. COPII vesicles bud from the ER membrane and fuse together forming the ERGIC. Anterograde transport of cargo protein to the Golgi occurs along microtubules. The LMAN1–MCFD2 complex is recycled back to the ER through COPI vesicles. Posttranslational modification of the cargo proteins occurs in the Golgi. CatC and CatZ are transported to the lysosomes. FV and FVIII are secreted outside of the cells.

LMAN1 is a homohexameric 53-kD type-1 transmembrane protein. MCFD2 is 16-kDa soluble protein-cofactor. They have similar half-lives. They form a stable, Ca^{2+}-dependent complex with 1:1 stoichiometry [29]. Separately, they do not provide transport FV and FVIII. The interaction of this complex with FV or FVIII is provided first of all by their B domains. Thus a qualitative or quantitative defect in LMAN1 or in MCDF2 leads to disorders in the secretion pathway

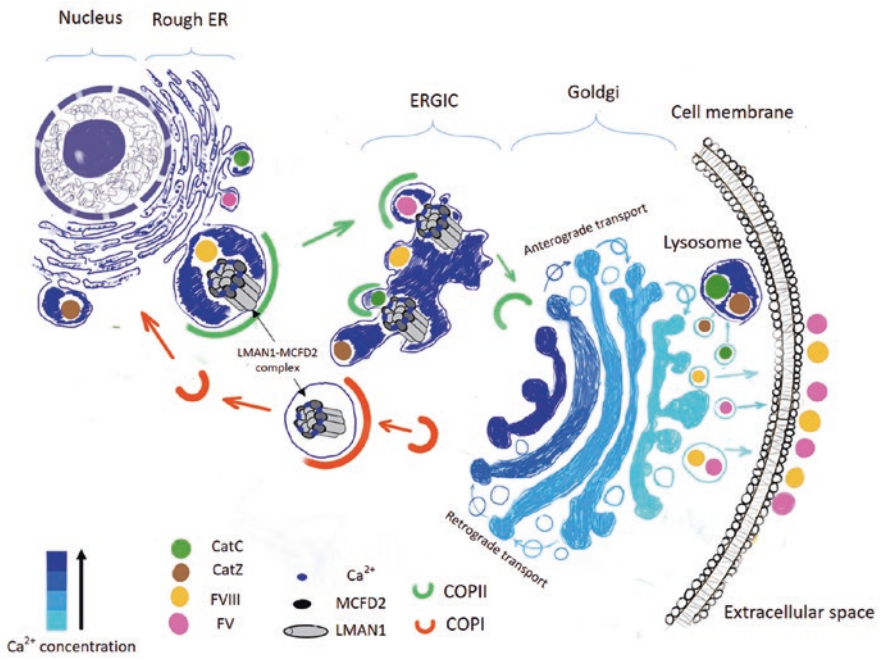

Fig. 9.1 Cargo transport by the LMAN1–MCFD2 complex

of FV and FVIII and impaired release into the circulation. A large number of different mutations have been described in *LMAN1* and *MCDF2* genes.

9.4 Clinical Manifestations

Patients with F5F8D are not usually manifested by severe bleeding events. Common spontaneous bleeding symptoms include epistaxis, gum bleeding, easy bruising, and menorrhagia. The most frequent clinical manifestations of F5F8D are bleeding from trauma, surgery, tooth extraction, and childbirth. Hemarthrosis and gastrointestinal bleeding (GI) may also occur in these patients but with much less frequency. Hematuria, muscle hematoma, and central nervous system (CNS) hemorrhages have been only described in few cases [9–11, 30, 31]. The spectrum and frequency of clinical manifestations in patients with F5F8D in different populations are presented in Table 9.2.

Women with congenital bleeding disorders, including F5F8D, are more susceptible to severe course of the disease due to the physiological causes of bleeding due to menstruation, ovulation, and childbirth. Gynecological and obstetric problems in women with F5F8D require special attention. Spiliopoulos et al. analyzed clinical manifestations in 86 women suffering from F5F8D [32]. The most common

Table 9.2 Clinical manifestations in patients with combined factor V and factor VIII deficiency

Clinical manifestations	Incidence (number of patients with symptom/total number of patients)				
	Seligsohn et al. (n = 14)	Peyvandi et al. (n = 27)	Shetty et al. (n = 9)	Mansouritorghabeh et al. (n = 19)	Viswabandya et al. (n = 37)
Epistaxis	57%	77.8%	–	69.2%	18.9%
Gingival	64.3%	–	44.4%	–	48.6%
Ecchymosis/easy bruising	28.6%	–	44.4%	–	29.7%
Menorrhagia	100%	58.3%	50%	33.3%	66.7%
Post-circumcision bleeding	–	66.7%	–	46.1%	–
Excessive post-dental extraction bleeding	92.3%	82.3%	33.3%	92.3%	56.7%
Excessive post-surgical bleeding	75%	75%	–	83.3%	62.2%
Excessive post-partum hemorrhage	100%	75%	–	50%	–
After lacerations	42.8%	–	–	–	–
After abortion	80%	–	–	–	–
After cutting	–	–	77.8%	57.9%	–
Hemarthrosis	–	26%	–	36.8%	13.5%
Gastrointestinal bleeding	21.4%	7.4%	–	10.5%	2.7%
Hematuria	14.3%	–	–	–	–
Muscle hematoma	–	7.4%	–	–	–
Intracranial hemorrhage	–	3.7%	–	–	–

bleeding symptom is menorrhagia (49%). Other common manifestations are epistaxis (34%), easy bruising (19%), postoperative bleeding (17%), post-traumatic bleeding (15%), bleeding after dental extraction (11%), recurrent ovulation bleeding (4%). In total, 19 pregnancies were reported in 18 women. There was no miscarriage reported. The mode of delivery was mentioned in nine pregnancies, including four cesarean sections required for obstetric reasons and five spontaneous vaginal deliveries. Postpartum hemorrhage was reported in six women (32%). In one case, a newborn presented with spontaneous cephalohaematoma [33].

In comparison with isolated FV or FVIII deficiencies, F5F8D is not associated with a higher bleeding tendency [15]. A possible reason for this mild clinical phenotype has been provided by Shao et al., who suggested that low FV levels in F5F8D may ameliorate the bleeding tendency in these patients [34]. This finding was based on an in vitro thrombin-generation assay which surprisingly showed that low FV

level concomitant with low free tissue factor pathway inhibitor α (TFPIα) in F5F8D was associated with optimal procoagulant activity and addition of FV to plasma only leads to anticoagulation. Here, the anticoagulant activity of FV is attributed to the interaction with TFPIα as its carrier in circulation which consequently results in lower TFPIα in FV deficiency. TFPIα is a natural anticoagulant that regulates not only TF/FVIIa complex but also FXa. It has been indicated that FV also acts as a cofactor for TFPIα in the inhibition of FXa, owing to defective anticoagulation in case of low FV level.

9.5 Diagnosis

F5F8D typically manifests as prolongation of both the prothrombin time (PT) and activated partial thromboplastin time (APTT) and approximately concordant reduction in FV and FVIII activities. APTT-based activity assays and antigen assays reveal levels of between 5% and 20% for both FV and FVIII and rarely less than 5%. Factor antigen assays are not strictly necessary for diagnosis. PT and APTT are corrected by mixing studies using normal plasma. Mixing study helps rule out presence of inhibitors [2, 35].

Bleeding phenotype of F5F8D is relatively similar to the clinical picture of isolated FV or FVIII deficiency, therefore some cases may be misdiagnosed as mild hemophilia A or FV deficiency and one of the factor defects may be overlooked [5]. Such errors revealed that there may be a bias in the exact number of patients with F5F8D in some countries specially where there are poor laboratory and genetic assays.

The concomitant presence of FV deficiency with either hemophilia A or von Willebrand disease (VWD) is the main differential diagnosis of F5F8D. These cases are extremely rare, but are described in the literature [36–39].

Based on VWD-specific tests, F5F8D and coinheritance of FV deficiency and VWD can be distinguished. Differentiation of F5F8D from coinheritance of FV deficiency and hemophilia A is mainly based on family history. In F5F8D, there may be no evidence of a positive family history; however, if present, the inheritance pattern is autosomal recessive [39].

Finally, molecular analysis of *MCFD2* and *LMAN1* genes can be used for confirmation of F5F8D, although it is not routinely performed [1]. Molecular analysis should start with the *LMAN1* gene, because 70% of mutations in patients with F5F8D are identified in this gene except in the Indian population [2].

9.6 Molecular Basis and Diagnosis

The gene encoding for LMAN1 with about 29 kb length and 13 exons (Fig. 9.2a) is located on the long arm of chromosome 18 (18q21.32). MCFD2 is encoded by a 19 kb gene on the short arm of chromosome 2 (2p21), containing 4 exons (Fig. 9.3a).

According to Human Gene Mutation Database (HGMD) 38 mutations in *LMAN1* and 20 mutations in *MCFD2* genes have been described so far (Tables 9.3 and 9.4)

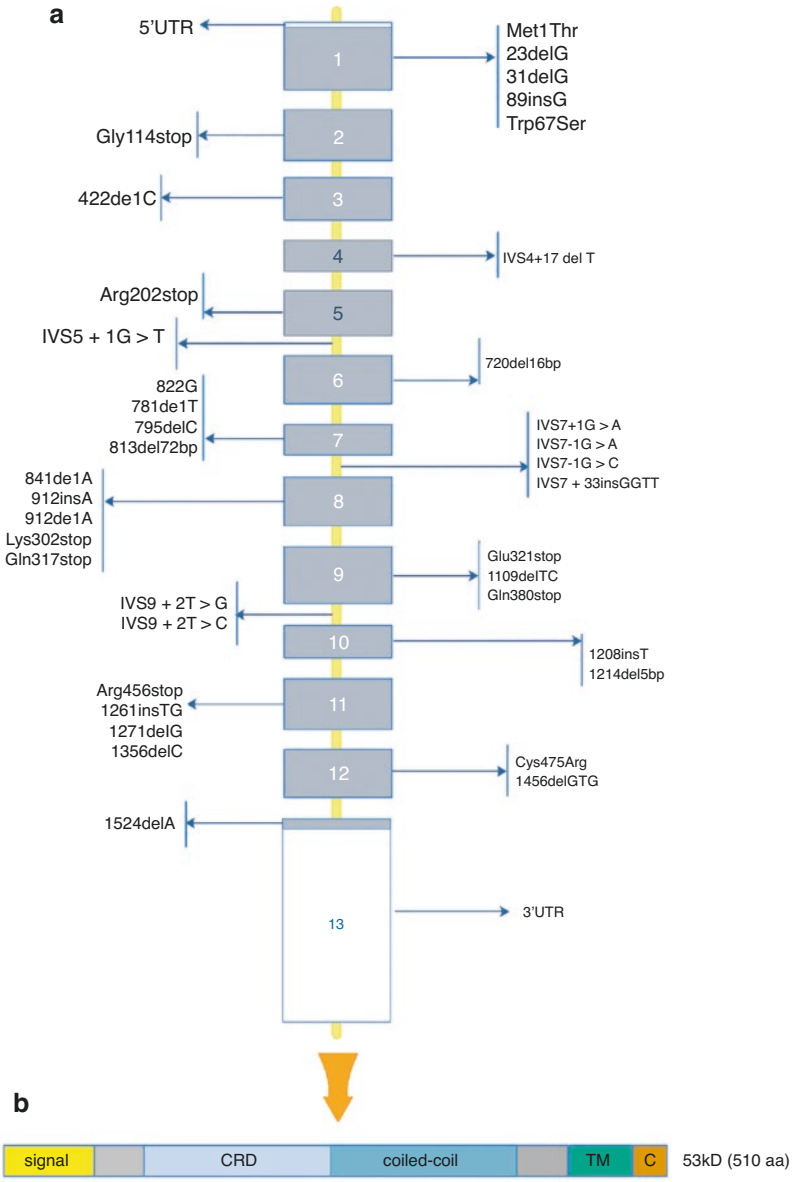

Fig. 9.2 (**a**) *LMAN1* gene and F5F8D causing mutations. *LMAN1* gene comprises 13 exons. Exons are shown in gray rectangles and are drawn to scale, and introns are indicated by yellow lines and are not to scale. White rectangles indicate 3′ UTR and 5′ UTR of gene. (**b**) Domains of LMAN1 protein. Signal peptide of LMAN1 contributes in translocation of LMAN1 into ER. The protein consists of 3 domains: luminal, transmembrane (TM), and short cytoplasmic (**c**). The luminal domain divides into two sub-domains, an N-terminal CRD and a membrane-proximal α-helical coiled domain, known as stalk domain. *LMAN1* lectin mannose binding 1, *UTR* untranslated region, *ER* endoplasmic reticulum, *CRD* carbohydrate recognition domain

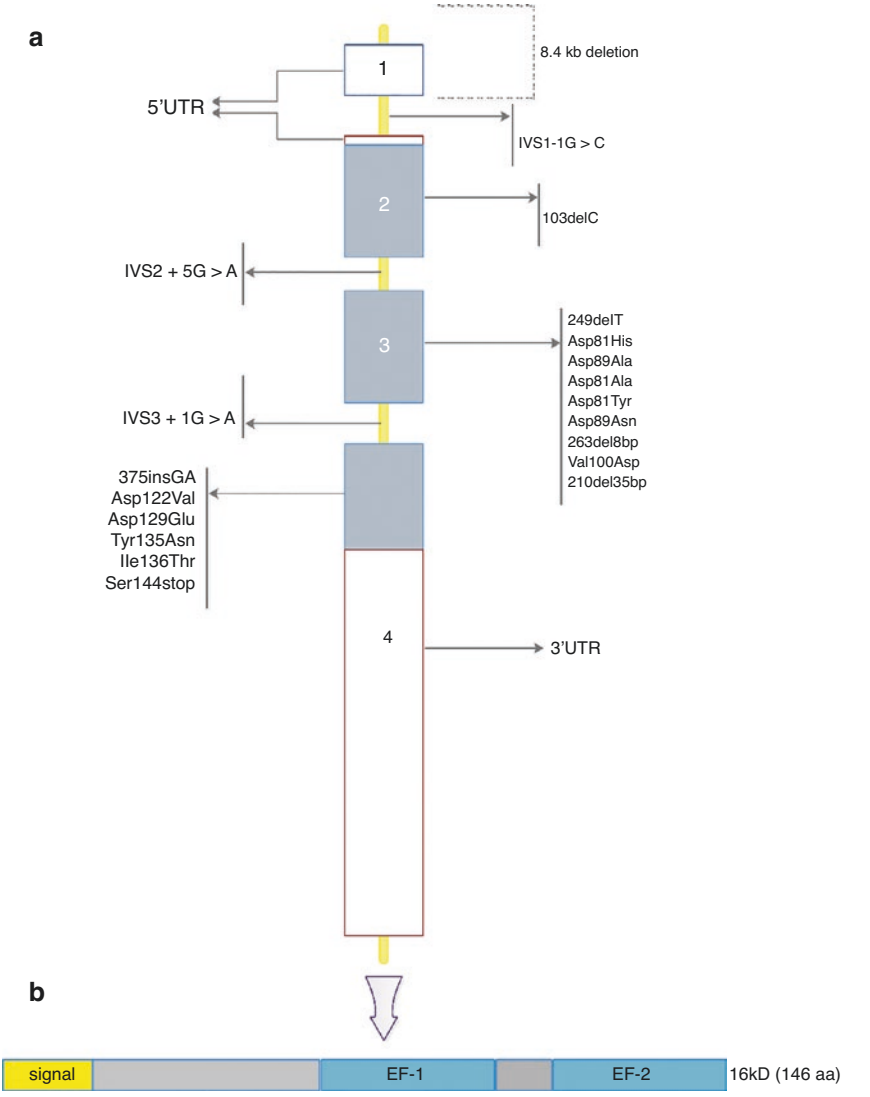

Fig. 9.3 (**a**) *MCFD2* gene and F5F8D causing mutations. Exons are indicated by gray rectangles and are drawn to scale. Introns are indicated by yellow lines and are not to scale. White rectangles indicate 3′ UTR and 5′ UTR of gene. (**b**) Domains of MCFD2 protein. MCFD2 is a small protein with 166 amino acids and three domains including a signal sequence for direction of MCFD2 into ER and two EF-hand motifs which probably can bind to Ca^{2+} ions. *MCFD2* multiple coagulation factor deficiency 2, *UTR* untranslated region, *ER* endoplasmic reticulum EF-hand motif: calcium-binding motif composed of two helixes (E and F) joined by a loop (helix-loop-helix)

(Figs. 9.2a and 9.3a) [1, 40, 41–45]. The most common mutations in *LMAN1* gene are insertion/deletion, nonsense, and splice site mutations, which lead to complete destruction of protein function (Table 9.3). The majority of mutations involving *MCFD2* gene is insertion/deletion, missense, and splice site (Table 9.4).

Table 9.3 *LMAN1* gene mutations in patients with combined factor V and factor VIII deficiency (F5F8D)

Type of mutation	Nomenclature	Gene location
Initiation codon	Met1Thr	Exon 1
Frameshift	23delG	Exon 1
Frameshift	31delG	Exon 1
Frameshift	89insG	Exon 1
Missense	Trp67Ser	Exon 1
Non-sense	Gly114stop	Exon 2
Frameshift	422delC	Exon 3
Frameshift	IVS4 + 17 del T	Exon 4
Non-sense	Arg202stop	Exon 5
Splicing	IVS5 + 1G > T	Intron 5
Frameshift	720del16bp	Exon 6
Frameshift	781delT	Exon 7
Frameshift	795delC	Exon 7
Frameshift	813del72bp	Exon 7
Splicing	822G > A	Exon 7
Splicing	IVS7 + 1G > A	Intron 7
Splicing	IVS7-1G > A	Intron 7
Splicing	IVS 7–1G > C	Intron 7
Splicing	IVS7 + 33insGGTT	Intron 7
Non-sense	Lys302stop	Exon 8
Non-sense	Gln317stop	Exon 8
Frameshift	841delA	Exon 8
Frameshift	912insA	Exon 8
Frameshift	912delA	Exon 8
Non-sense	Glu321stop	Exon 9
Frameshift	1109delTC	Exon 9
Non-sense	Gln380stop	Exon 9
Splicing	IVS9 + 2 T > G	Intron 9
Splicing	IVS9 + 2 T > C	Intron 9
Frameshift	1208insT	Exon 10
Frameshift	1214del5bp	Exon 10
Frameshift	1261insTG	Exon 11
Non-sense	Arg456stop	Exon 11
Splicing	1271delG	Exon 11
Frameshift	1356delC	Exon 11
Missense	Cys475Arg	Exon 12
Deletion	1456delGTG	Exon 12
Frameshift	1524delA	Exon 13

LMAN1 lectin mannose binding 1

The most common mutation in the MCFD2 gene is IVS2 + 5G > A, which is identified in patients F5F8D from different geographical regions (India, Italy, USA, Serbia, and Germany). Some mutations such as 89insG (frameshift), IVS9 + 2 T > C (splicing), and M1T (c.2 T > C) (abolish initiation codon), which are exclusively seen in Middle Eastern Jewish, Tunisian Jewish and Italian origin, respectively, are

Table 9.4 *MCFD2* gene mutations in patients with combined factor V and factor VIII deficiency (F5F8D)

Type	Nomenclature	Location
Large deletion	8.4 kb deletion	Promoter and Exon 1
Splicing	IVS1–1G > C	Intron 1
Frameshift	103delC	Exon 2
Splicing	IVS2 + 5G > A	Intron 2
Frameshift	249delT	Exon 3
Frameshift	263del8bp	Exon 3
Frameshift	210del35bp	Exon 3
Missense	Asp81Tyr	Exon 3
Missense	Asp81His	Exon 3
Missense	Asp81Ala	Exon 3
Missense	Asp89Asn	Exon 3
Missense	Asp89Ala	Exon 3
Missense	Val100Asp	Exon 3
Splicing	IVS3 + 1G > A	Intron 3
Frameshift	375insGA	Exon 4
Missense	Asp122Val	Exon 4
Missense	Asp129Glu	Exon 4
Missense	Tyr135Asn	Exon 4
Missense	Ile136Thr	Exon 4
Nonsense	Ser144stop	Exon 4

MCFD2 multiple coagulation factor deficiency 2

suggestive of founder effect [1, 39]. Missense mutations in patients with F5F8D are significantly more often found in the *LMAN1* gene than *MCFD2* [46, 47].

The case of a compound patient with F5F8D is known. Nyfeler et al. studied a patient who was found to be a compound heterozygote for 2 novel mutations in MCFD2: a large deletion of 8.4 kb eliminating the 5′UTR of the gene and a nonsense mutation resulting in the deletion of only 3 amino acids (DeltaSLQ) from the C-terminus of MCFD2 [48].

9.7 Genotype-Phenotype Correlation

The study of the relationship between genotype and plasma activity of factors and clinical manifestations is an important aspect. This knowledge helps to predict the prognosis of the disease, the levels of activity of the coagulation factor that determine protection from bleeding episodes, in order to determine appropriate prevention and management strategies.

According to a study by Zhang et al. it was suggested that mutations involving *MCFD2* are associated with lower levels of FV and FVIII, compared with *LMAN1* mutations (mean values of 9.6% vs. 13.7% for FV and 10.0% vs. 16.0% for FVIII). This finding supports a genotype-phenotype relationship in patients with F5F8D [47]. But FV and FVIII plasma activity cannot be used to predict a defective gene.

On the other hand, there is a correlation between the activity of FV and FVIII, regardless of which gene the mutation is in. This confirms damage to the common secretion pathway of molecules FV and FVIII. No significant differences were observed between male and female patients for either FV or FVIII levels. Another interesting fact is lack of any significant difference in FV and FVIII levels in patients with blood group O compared with non-O blood groups.

As mentioned earlier circulating FV exists in both plasma and platelets. Zhang et al. showed that platelet FV levels are reduced to the same extent as FV levels in plasma, for both the *LMAN1* group and the *MCFD2* group [47].

According to The European Network of Rare Bleeding Disorders (EN-RBD) data there is a strong association between coagulation factor activity level and clinical bleeding severity for combined FV and FVIII, as well as for fibrinogen, FVIII and FXIII deficiencies. The study group consisted of 18 F5F8D patients. The mean value of factors activity for asymptomatic patients is 44%, for patients with bleeding that occurred after trauma or drug ingestion (antiplatelet or anticoagulant therapy) is 34%, for patients with spontaneous minor bleeding is 24% and for patients with spontaneous major bleeding is 15% [49].

9.8 Management

The bleeding tendency in F5F8D is not more severe than isolated FV or FVIII defect. Clinical management of F5F8D does not rely on molecular diagnosis [1]. Clinical presentations are usually mild, thus on-demand therapy is usually preferred for management of bleeding in surgical procedures or in time of delivery and in cases of traumatic bleeding events. In F5F8D, there is no need for prophylaxis except for specific cases with severe recurrent bleeding events [50].

The treatment of bleeding episodes is dependent on the nature of the bleed and the affected individual's FV and FVIII levels. Mild bleeding or moderate episode usually respond to anti-fibrinolytic agents, like aminocapronic acid 15–20 mg/kg intravenously or 1 g per os 3–4 times daily. Severe spontaneous or post-traumatic/postoperative bleeding, as well as preparation for major surgical interventions, requires hemostatic replacement therapy. A large number of products that replace FVIII are available. In contrast, there are no FV concentrates accessible. The extreme lability of human FV in plasma, used in preparing FV-deficiency plasma by aging, is the main reason why isolated procedures yielding an undegraded product have not been reported previously. The lability can presumably be explained by the high susceptibility of FV to proteolysis during blood collection as well as during the initial purification steps [51–53].

In 2018, Bulato et al. published data on the development of a novel plasma-derived FV concentrate. This concentrate was tested for the in vitro correction of severe FV deficiency in patients with FV deficiency and both, standard tests and all thrombin generation parameters normalized [54]. FV concentrates, however, are currently still unavailable as a therapeutic option. Available therapy includes virus-inactivated fresh frozen plasma (FFP) which contains FV and FVIII. The

therapeutic goal is to achieve 20–25% FV activity and at least 50% FVIII. FFP infusion is usually sufficient to restore FV activity to hemostatic level, but may not be enough for FVIII activity. Additional transfusion is required of plasma-derived or recombinant FVIII concentrate. The initial dose of FFP usually is 15–25 mg/kg and FVIII concentrate is 20–40 IU/kg (depending on the intensity of the hemorrhagic syndrome and basic plasma activity FV and FVIII) [2, 15, 35, 55]. Repeated administration with a reduction in dosage is recommended after 12 h. Analysis of APTT, PT, FV, and FVIII activities is necessary to assess the effectiveness of therapy and prescribe the next transfusion regimen. After major surgical interventions, it is recommended to maintain the activity of FV at least 20–25% and FVIII at least 50% within 72 h. Replacement therapy should be continued until the wound heals. Screening coagulation tests (APTT, PT) are routine and are performed in laboratories everywhere. Not all laboratories are equipped to assess the activity of blood clotting factors. Hence, relying only on screening tests for the laboratory control of hemostatic replacement therapy is a challenge. In the described clinical observation by Yakovleva et al., the analysis of hemostasis parameters during the perioperative period in F5F8D patient allowed to conclude that full compensation of screening parameters is not required, and the maintenance of APTT within 50 s and the quick prothrombin within 50% is sufficient to prevent hemorrhagic complications in the perioperative period [56]. It is necessary to remember about possible complications of this therapy, such as volume overload, allergic reaction, and the production of alloantibodies when using FFP and the development of inhibitors when using FVIII concentrates. Such cases are rare, but they are described in the literature [2, 35, 57, 58].

There is experience with the use of desmopressin (DDAVP) in F5F8D patients. It promotes a short-term (60–120 min) doubling of FVIII activity. DDAVP can be used in F5F8D patients in whom the activity of FVIII is slightly reduced. In this case, it can be an alternative to FVIII concentrates. The dosage DDAVP is 260 μg intranasal or 0.3 μg/kg subcutaneous. It is necessary to remember about the depletion of the effect with prolonged use [59–64].

Recombinant activated coagulation factor VIIa (rFVIIa) has been introduced to improve hemostasis in hemophilia A/B patients with inhibitors, patients with Glanzmann's thrombasthenia, but many reports describe an off-label use in other bleeding conditions. The literature describes cases of successful use of rFVII in patients with F5F8D. In one case, rFVII was used due to uncontrolled hemorrhagic syndrome, despite of the ongoing therapy of FFP and FVIII concentrate [65]. In another case, hemostatic therapy with rFVII was carried out due to the development of anaphylactic reaction to the transfusion of FFP [66].

Also, an alternative option is the use of platelet transfusions, the alpha granules of which contain FV [2]. Several cases of platelet concentrate use have been described in patients with F5F8D and FV deficiency, including with the presence of FV inhibitor [58, 67, 68].

There is no information in the literature about the use of cryoprecipitate in F5F8D patients. Most authors believe that the cryoprecipitate does not contain FV. However, Yakovleva et al. had a positive experience of using cryoprecipitate to

control bleeding syndrome in F5F8D patients. Experimental research evaluated the activity of clotting factors in cryoprecipitate samples from different donors immediately after defrosting and after 1 h showed sufficient FV activity [69].

Nonsteroidal anti-inflammatory drugs, antiplatelet drugs, psychotropic drugs and a number of other medicines can aggravate the hypocoagulation condition in patients. Their appointment should be strictly justified and, if necessary, combined with hemostatic therapy.

9.9 Family Planning, Pregnancy, Childbirth

When planning a family and genetic counseling, it is necessary to take into account that the disease is autosomal recessive and inheritance does not depend on gender. If one of the parents suffers from F5F8D (i.e. is homozygous), then the risk of having a child with a heterozygous carrier is 50%. The birth of a child with F5F8D has to be expected if both parents are F5F8D patients or with a risk of 25% if both parents are heterozygous carriers.

Throughout pregnancy, the level of FV does not change, while the level of FVIII increases [64, 70]. Thus, the diagnosis of F5F8D is difficult during pregnancy. In this case, a family history is important if F5F8D was established in relatives. Factor assays should be performed in the third trimester and used to develop a hemostatic plan for delivery. Women with F5F8D have to be considered potentially at risk for developing postpartum hemorrhage [32, 65]. F5F8D is not a contraindication to normal vaginal delivery. The method of delivery is determined by obstetric indications [71]. For delivery, it is necessary to reach a level of FV more than 20% and FVIII more than 50%. Initial dosage FFP is 15–25 ml/kg once in established labor or before cesarean section, then 10 ml/kg once every 12 h for at least 3 days with supplementary with FVIII concentrate [2, 15, 35, 72].

Due to the mild bleeding phenotype of F5F8D, prenatal diagnosis is not usually recommended. However, if wish to be performed, both parents should be carriers of the disorder and it may be known by already having an affected child. Chorionic villus sampling (CVS) has to be performed at 10–12 gestational weeks. Then fetal DNA is evaluated for the specific mutations, which were found in parents and affected child [2]. Currently, Non-Invasive Prenatal Testing (NIPT) is being introduced into clinical practice, based on the analysis of extracellular fetal DNA circulating in the blood of a pregnant woman after 10 weeks of pregnancy. In the future NIPT may become a common tool for prenatal diagnosis of monogenic diseases [73, 74].

F5F8D can be diagnosed in the neonatal period using cord or peripheral blood. According to Andrew et al. FV and FVIII activities in healthy term neonates are 36–108 IU/ml and 61–139 IU/ml, respectively, with FV activity increasing further within 1 week [75]. Also, a recent study by Mitsiakos et al. showed that in healthy newborns, FV and FVIII activity positively correlates with gestational age and birth weight [76]. F5F8D very rarely presents with bleeding in neonates. Neonatal intracranial hemorrhage has not been described in this condition. There are no reports of prophylaxis in F5F8D newborns [15].

References

1. Zheng C, Zhang B. Combined deficiency of coagulation factors V and VIII: an update. Semin Thromb Hemost. 2013;39(6):613–20. https://doi.org/10.1055/s-0033-1349223].
2. Spreafico M, Peyvandi F. Combined FV and FVIII deficiency. Haemophilia. 2008;14(6):1201–8. https://doi.org/10.1111/j.1365-2516.2008.01845.x.
3. Childers KC, Peters SC, Spiegel PC Jr. Structural insights into blood coagulation factor VIII: Procoagulant complexes, membrane binding, and antibody inhibition. J Thromb Haemost. 2022;20(9):1957–70. https://doi.org/10.1111/jth.15793.
4. Zhang B, Ginsburg D. Familial multiple coagulation factor deficiencies: new biologic insight from rare genetic bleeding disorders. J Thromb Haemost. 2004;2(9):1564–72. https://doi.org/10.1111/j.1538-7836.2004.00857.x.
5. Zhang B. Recent developments in the understanding of the combined deficiency of FV and FVIII. Br J Haematol. 2009;145(1):15–23. https://doi.org/10.1111/j.1365-2141.2008.07559.x.].
6. Mann KG, Kalafatis M. Factor V: a combination of Dr Jekyll and Mr Hyde. Blood. 2003;101(1):20–30. https://doi.org/10.1182/blood-2002-01-0290.
7. Vos HL. Inherited defects of coagulation Factor V: the thrombotic side. J Thromb Haemost. 2006;4(1):35–40. https://doi.org/10.1111/j.1538-7836.2005.01572.x.
8. Oeri J, Matter M, Isenschmid H, Hauser F, Koller F. Angeborener Mangel an Faktor V (Parahaemophilie) verbunden mit echter Haemophilie A bei zwei Brüdern [Congenital factor V deficiency (parahemophilia) with true hemophilia in two brothers]. Bibl Paediatr German. 1954;58:575–88.
9. Seligsohn U, Zivelin A, Zwang E. Combined factor V and factor VIII deficiency among non-Ashkenazi Jews. N Engl J Med. 1982;307(19):1191–5. https://doi.org/10.1056/NEJM198211043071907.
10. Peyvandi F, Tuddenham EG, Akhtari AM, Lak M, Mannucci PM. Bleeding symptoms in 27 Iranian patients with the combined deficiency of factor V and factor VIII. Br J Haematol. 1998;100(4):773–6. https://doi.org/10.1046/j.1365-2141.1998.00620.x.
11. Mansouritorgabeh H, Rezaieyazdi Z, Pourfathollah AA, Rezai J, Esamaili H. Haemorrhagic symptoms in patients with combined factors V and VIII deficiency in north-eastern Iran. Haemophilia. 2004;10(3):271–5. https://doi.org/10.1111/j.1365-2516.2004.00890.x.
12. Shetty S, Shelar T, Mirgal D, Nawadkar V, Pinto P, Shabhag S, Mukaddam A, Kulkarni B, Ghosh K. Rare coagulation factor deficiencies: a countrywide screening data from India. Haemophilia. 2014;20(4):575–81. https://doi.org/10.1111/hae.12368.
13. Palla R, Peyvandi F, Shapiro AD. Rare bleeding disorders: diagnosis and treatment. Blood. 2015;125(13):2052–61. https://doi.org/10.1182/blood-2014-08-532820.
14. Naderi M, Tabibian S, Alizadeh S, Hosseini S, Zaker F, Bamedi T, Shamsizadeh M, Dorgalaleh A. Congenital factor V deficiency: comparison of the severity of clinical presentations among patients with rare bleeding disorders. Acta Haematol. 2015;133(2):148–54. https://doi.org/10.1159/000363598.
15. Mumford AD, Ackroyd S, Alikhan R, Bowles L, Chowdary P, Grainger J, et al. Guideline for the diagnosis and management of the rare coagulation disorders. Brit J Haematol. 2014;167(3):304–26. https://doi.org/10.1111/bjh.13058.
16. Saito H, Shioya M, Koie K, Kamiya T, Katsumi O. Congenital combined deficiency of factor V and factor 8. A case report and the effect of transfusion of normal plasma and hemophilic blood. Thromb Diath Haemorrh. 1969;22(2):316–25.
17. Toole JJ, Knopf JL, Wozney JM, Sultzman LA, Buecker JL, Pittman DD, Kaufman RJ, Brown E, Shoemaker C, Orr EC, et al. Molecular cloning of a cDNA encoding human antihaemophilic factor. Nature. 1984;312(5992):342–7. https://doi.org/10.1038/312342a0.
18. Gitschier J, Wood WI, Goralka TM, Wion KL, Chen EY, Eaton DH, Vehar GA, Capon DJ, Lawn RM. Characterization of the human factor VIII gene. Nature. 1984;312(5992):326–30. https://doi.org/10.1038/312326a0.

19. Cripe LD, Moore KD, Kane WH. Structure of the gene for human coagulation factor V. Biochemistry. 1992;31(15):3777–85. https://doi.org/10.1021/bi00130a007.
20. Soff GA, Levin J. Familial multiple coagulation factor deficiencies. I. Review of the literature: Differentiation of single hereditary disorders associated with multiple factor deficiencies from coincidental concurrence of single factor deficiency states. Semin Thromb Hemost. 1981;7(2):112–48. https://doi.org/10.1055/s-2007-1005073.
21. Nichols WC, Seligsohn U, Zivelin A, Terry VH, Arnold ND, Siemieniak DR, et al. Linkage of combined factors V and VIII deficiency to chromosome 18q by homozygosity mapping. J Clin Investig. 1997;99(4):596.
22. Nichols WC, Seligsohn U, Zivelin A, Terry VH, Hertel CE, Wheatley MA, et al. Mutations in the ER–Golgi intermediate compartment protein ERGIC-53 cause combined deficiency of coagulation factors V and VIII. Cell. 1998;93(1):61–70.
23. Ginsburg D. Identifying novel genetic determinants of hemostatic balance. J Thromb Haemost. 2005;3(8):1561–8. https://doi.org/10.1111/j.1538-7836.2005.01461.x.
24. Zhang B, Cunningham MA, Nichols WC, Bernat JA, Seligsohn U, Pipe SW, McVey JH, Schulte-Overberg U, de Bosch NB, Ruiz-Saez A, White GC, Tuddenham EG, Kaufman RJ, Ginsburg D. Bleeding due to disruption of a cargo-specific ER-to-Golgi transport complex. Nat Genet. 2003;34(2):220–5. https://doi.org/10.1038/ng1153.
25. Khoriaty R, Vasievich MP, Ginsburg D. The COPII pathway and hematologic disease. Blood. 2012;120(1):31–8. https://doi.org/10.1182/blood-2012-01-292086.
26. Schweizer A, Fransen JA, Bächi T, Ginsel L, Hauri HP. Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubulo-vesicular compartment at the cis-side of the Golgi apparatus. J Cell Biol. 1988;107(5):1643–53. https://doi.org/10.1083/jcb.107.5.1643.
27. Zheng C, Liu HH, Yuan S, Zhou J, Zhang B. Molecular basis of LMAN1 in coordinating LMAN1-MCFD2 cargo receptor formation and ER-to-Golgi transport of FV/FVIII. Blood. 2010;116(25):5698–706. https://doi.org/10.1182/blood-2010-04-278325.
28. Appenzeller-Herzog C, Hauri HP. The ER-Golgi intermediate compartment (ERGIC): in search of its identity and function. J Cell Sci. 2006;119(Pt 11):2173–83. https://doi.org/10.1242/jcs.03019.
29. Zhang B, Kaufman RJ, Ginsburg D. LMAN1 and MCFD2 form a cargo receptor complex and interact with coagulation factor VIII in the early secretory pathway. J Biol Chem. 2005;280(27):25881–6. https://doi.org/10.1074/jbc.M502160200.
30. Shetty S, Madkaikar M, Nair S, Pawar A, Baindur S, Pathare A, et al. Combined factor V and VIII deficiency in Indian population. Haemophilia-Oxford. 2000;6:504–7.
31. Viswabandya A, Baidya S, Nair SC, Lakshmi KM, Mathews V, George B, et al. Clinical manifestations of combined factor V and VIII deficiency: a series of 37 cases from a single center in India. Am J Hematol. 2010;85(7):538–9.
32. Spiliopoulos D, Kadir RA. Congenital factor V and VIII deficiency in women: a systematic review of literature and report of two new cases. Blood Coagul Fibrinol. 2016;27(3):237–41. https://doi.org/10.1097/MBC.0000000000000407.
33. Abdullah WZ, Ismail R, Nasir A, Mohamad N, Hassan R. Developmental haemostasis for factor V and factor VIII levels in neonates: a case report of spontaneous cephalhaematoma. Fetal Pediatr Pathol. 2013;32(2):77–81. https://doi.org/10.3109/15513815.2012.671447.
34. Shao Y, Wu W, Xu G, Wang X, Ding Q. Low factor V level ameliorates bleeding diathesis in patients with combined deficiency of factor V and factor VIII. Blood. 2019;134(20):1745–54. https://doi.org/10.1182/blood.2018886069.
35. Bolton-Maggs PH, Perry DJ, Chalmers EA, Parapia LA, Wilde JT, Williams MD, Collins PW, Kitchen S, Dolan G, Mumford AD. The rare coagulation disorders—review with guidelines for management from the United Kingdom Haemophilia Centre Doctors' Organisation. Haemophilia. 2004;10(5):593–628. https://doi.org/10.1111/j.1365-2516.2004.00944.x.
36. Castaman G, Linari S. Diagnosis and treatment of von Willebrand disease and rare bleeding disorders. J Clin Med. 2017;6(4):45. https://doi.org/10.3390/jcm6040045.

37. Seidizadeh O, Ahmadinejad M, Homayoun S, Mannucci PM, Peyvandi F. Von Willebrand disease combined with coagulation defects in Iran. Blood Transfus. 2021;19(5):428–34. https://doi.org/10.2450/2021.0078-21.
38. Suzuki S, Nakamura Y, Suzuki N, Yamazaki T, Takagi Y, Tamura S, Takagi A, Kanematsu T, Matsushita T, Kojima T. Combined deficiency of factors V and VIII by chance coinheritance of parahaemophilia and haemophilia A, but not by mutations of either LMAN1 or MCFD2, in a Japanese family. Haemophilia. 2018;24(1):e13–6. https://doi.org/10.1111/hae.13360.
39. Akutsu Y, Mori K, Suzuki S, Sugai K, Ishikawa M, Sakai H, Endo E, Yasuda H. A family of congenital combined deficiency of factor V and von Willebrand factor. Rinsho Ketsueki. 1990;31(3):365–70.
40. Karimi M, Cairo A, Safarpour MM, Haghpanah S, Ekramzadeh M, Afrasiabi A, et al. Genotype and phenotype report on patients with combined deficiency of factor V and factor VIII in Iran. Blood Coagul Fibrinol. 2014;25(4):360–3. https://doi.org/10.1097/MBC.0000000000000046.
41. Hejer E, Adnen L, Asma J, Ibtihel M, Benammar-Elgaaied A, Gouider E. Identification of a novel mutation in the MCFD2 gene in a Tunisian family with combined factor V and VIII deficiency. La Tunisie Med. 2012;90(4):343–4.
42. Wang A, Liu X, Wu J, Cai X, Zhu W, Sun Z. Combined FV and FVIII deficiency (F5F8D) in a Chinese family with a novel missense mutation in MCFD2 gene. Haemophilia. 2014;20(6) https://doi.org/10.1111/hae.12549.
43. Genotypes of patients with combined factor V and VIII deficiency [Internet]. 2011. Available from: https://c.ymcdn.com/sites/www.isth.org/resource/resmgr/publications/fv_and_viii_mutations-2011.pdf.
44. Wang A, Duan Q, Ding K, Liu X, Wu J, Sun Z. Successful abdominal operation without replacement therapy in a patient with combined factor V (FV) and FVIII deficiency due to novel homozygous mutation in LMAN1. Haemophilia. 2015;21(6) https://doi.org/10.1111/hae.12756.
45. Elmahmoudi H, Wigren E, Laatiri A, Jlizi A, Elgaaied A, Gouider E, et al. Analysis of newly detected mutations in the MCFD2 gene giving rise to combined deficiency of coagulation factors V and VIII. Haemophilia. 2011;17(5) https://doi.org/10.1111/j.1365-2516.2011.02529.x.
46. Ivaskevicius V, Windyga J, Baran B, Bykowska K, Daugela L, Watzka M, Seifried E, Oldenburg J. The first case of combined coagulation factor V and coagulation factor VIII deficiency in Poland due to a novel p.Tyr135Asn missense mutation in the MCFD2 gene. Blood Coagul Fibrinol. 2008;19(6):531–4. https://doi.org/10.1097/MBC.0b013e3283061103.
47. Zhang B, Spreafico M, Zheng C, Yang A, Platzer P, Callaghan MU, Avci Z, Ozbek N, Mahlangu J, Haw T, Kaufman RJ, Marchant K, Tuddenham EG, Seligsohn U, Peyvandi F, Ginsburg D. Genotype-phenotype correlation in combined deficiency of factor V and factor VIII. Blood. 2008;111(12):5592–600. https://doi.org/10.1182/blood-2007-10-113951.
48. Nyfeler B, Kamiya Y, Boehlen F, Yamamoto K, Kato K, de Moerloose P, Hauri HP, Neerman-Arbez M. Deletion of 3 residues from the C-terminus of MCFD2 affects binding to ERGIC-53 and causes combined factor V and factor VIII deficiency. Blood. 2008;111(3):1299–301. https://doi.org/10.1182/blood-2007-09-112854.
49. Peyvandi F, Palla R, Menegatti M, Siboni SM, Halimeh S, Faeser B, Pergantou H, Platokouki H, Giangrande P, Peerlinck K, Celkan T, Ozdemir N, Bidlingmaier C, Ingerslev J, Giansily-Blaizot M, Schved JF, Gilmore R, Gadisseur A, Benedik-Dolničar M, Kitanovski L, Mikovic D, Musallam KM, Rosendaal FR, European Network of Rare Bleeding Disorders Group. Coagulation factor activity and clinical bleeding severity in rare bleeding disorders: results from the European Network of Rare Bleeding Disorders. J Thromb Haemost. 2012;10(4):615–21. https://doi.org/10.1111/j.1538-7836.2012.04653.x.
50. Latif A, Aledort L. Inherited combined factor deficiency states. Hemost Thromb 2014:127–36. doi: https://doi.org/10.1002/9781118833391.ch10.
51. Colman RW, Weinberg RM. Factor V. Methods Enzymol. 1976;45:107–22. https://doi.org/10.1016/s0076-6879(76)45015-2.

52. Rosenberg JS, Beeler DL, Rosenberg RD. Activation of human prothrombin by highly purified human factors V and X-a in presence of human antithrombin. J Biol Chem. 1975;250(5):1607–17.
53. Dahlbäck B. Human coagluation factor V purification and thrombin-catalyzed activation. J Clin Invest. 1980;66(3):583–91. https://doi.org/10.1172/JCI109890.
54. Bulato C, Novembrino C, Anzoletti MB, Spiezia L, Gavasso S, Berbenni C, Tagariello G, Farina C, Nardini I, Campello E, Peyvandi F, Simioni P. "In vitro" correction of the severe factor V deficiency-related coagulopathy by a novel plasma-derived factor V concentrate. Haemophilia. 2018;24(4):648–56. https://doi.org/10.1111/hae.13465.
55. Peyvandi F, Menegatti M. Treatment of rare factor deficiencies in 2016. ASH Educ Prog Book. 2016;2016(1):663–9. https://doi.org/10.1182/asheducation-2016.1.663.
56. Yakovleva EV, Efimov IV, Kostin AI, Gasanov AM, Azimova MK, Orel EB, Lavrova PS, Konyashina NI, Surin VL, Pshenichnikova OS, Sats NV, Zozulya NI. Diagnosis and choice of haemostatic therapy during surgery in patients with combined coagulation factor V and VIII deficiency. Russian J Hematol Transfus. 2021;66(1):79–87. (In Russ.). https://doi.org/10.35754/0234-5730-2021-66-1-79-87.
57. Lee WS, Chong LA, Begum S, Abdullah WA, Koh MT, Lim EJ. Factor V inhibitor in neonatal intracranial hemorrhage secondary to severe congenital factor V deficiency. J Pediatr Hematol Oncol. 2001;23(4):244–6. https://doi.org/10.1097/00043426-200105000-00013.
58. Buckner TW, Nielsen BI, Key NS, Ma A. Factor VIII inhibitory antibody in a patient with combined factor V/factor VIII deficiency. Haemophilia. 2015;21(1):e77–80. https://doi.org/10.1111/hae.12557.
59. Bauduer F, Guichandut JP, Ducout L. Successful use of fresh frozen plasma and desmopressin for transurethral prostatectomy in a French Basque with combined factors V +VIII deficiency. J Thromb Haemost. 2004;2(4):675. https://doi.org/10.1111/j.1538-7836.2004.00714.x.
60. Chuansumrit A, Mahaphan W, Pintadit P, Chaichareon P, Hathirat P, Ayuthaya PI. Combined factor V and factor VIII deficiency with congenital heart disease: response to plasma and DDAVP infusion. Southeast Asian J Trop Med Public Health. 1994;25(1):217–20.
61. Garcia VV, Silva IA, Borrasca AL. Response of factor VIII/von Willebrand factor to intranasal DDAVP in healthy subjects and mild haemophiliacs (with observations in patients with combined deficiency of factors V and VIII). Thromb Haemost. 1982;48(1):91–3.
62. Devecioğlu O, Eryilmaz E, Celik D, Unüvar A, Karakaş Z, Anak S, Ağaoğlu L. Circumcision in a combined factor V and factor VIII deficiency using desmopressin (DDAVP). Turk J Pediatr. 2002;44(2):146–7.
63. Guglielmone H, Minoldo S, Jarchum G. Response to the DDAVP test in a patient with combined deficiency of factor V and factor VIII. Haemophilia. 2009;15(3):838–9. https://doi.org/10.1111/j.1365-2516.2009.02011.x.
64. Oukkache B, El Graoui O, Zafad S. Combined factor V and VIII deficiency and pregnancy. Int J Hematol. 2012;96(6):786–8. https://doi.org/10.1007/s12185-012-1201-z.
65. Di Marzio I, Iuliani O, Malizia R, Rolandi G, Sanna S, Castaman G, Dragani A. Successful use of recombinant FVIIa in combined factor V and FVIII deficiency with surgical bleeding resistant to substitutive treatment. A case report. Haemophilia. 2011;17(1):160–1. https://doi.org/10.1111/j.1365-2516.2010.02368.x.
66. Lechner D, Eichinger S, Wanivenhaus A, Kyrle PA. Peri-interventional control of haemostasis in a patient with combined coagulation factor V- and factor VIII-deficiency and anaphylaxis to fresh frozen plasma—a rare indication for recombinant factor VIIa. Haemophilia. 2010;16(4):704–5. https://doi.org/10.1111/j.1365-2516.2010.02240.x.
67. Chediak J, Ashenhurst JB, Garlick I, Desser RK. Successful management of bleeding in a patient with factor V inhibitor by platelet transfusions. Blood. 1980;56(5):835–41.
68. Yakovleva EV, Konyashina NI, Gorgidze LA, Surin VL, Pshenichnikova OS, Polevodova OA, Spirin MV, Galstyan GM, Zozulya NI. Congenital factor V deficiency: case reports. Russian J Hematol Transfus (Gematol Transfuz). 2019;64(4):489–503. (in Russian). https://doi.org/10.35754/0234-5730-2019-64-4-489-503.

69. Yakovleva E, Gorgidze L, Konyashina N, Bulgakov A, Gaponova T, Kumskova M, Likhacheva E, Zozulya N. Use of cryoprecipitate in treatment of inherited factor V deficiency and inherited combined factor V and factor VIII deficiency. Haemophilia. 2020;26(2):149. https://doi.org/10.1111/hae.13911.
70. Fogarty H, Doyle MM, Campbell R, Keenan C, White B, Ryan K, O'Donnell JS, Slevin J, O'Keeffe D, O'Connell NM, Lavin M. Management of combined factor V and factor VIII deficiency in pregnancy. J Obstet Gynaecol. 2019;39(2):271–2. https://doi.org/10.1080/01443615.2018.1448766.
71. Hoffmann C, Falzone E, Mihai A, Gitz L, Itzhar-Baikian N, Martel-Jacob S, Mercier FJ. Combined factor V and VIII deficiency and pregnancy—need for an early protocol-based multidisciplinary management. Ann Fr Anesth Reanim. 2013;32(11):e163–5. https://doi.org/10.1016/j.annfar.2013.08.014.
72. Ueno H, Asami M, Yoneda R, Muraoka A, Oribe T, Suzuki K, Maeda M, Chinzei T. Management of cesarean section under replacement therapy with factor VIII concentrates in a pregnant case with congenital combined deficiency of factor V and factor VIII. Rinsho Ketsueki. 1991;32(9):981–5.
73. Horn R. NIPT and the concerns regarding 'routinisation'. Eur J Hum Genet. 2022;30(6):637–8. https://doi.org/10.1038/s41431-022-01053-6.
74. Alyafee Y, Al Tuwaijri A, Umair M, Alharbi M, Haddad S, Ballow M, Alayyar L, Alam Q, Althenayyan S, Al Ghilan N, Al Khaldi A, Faden MS, Al Sufyan H, Alfadhel M. Non-invasive prenatal testing for autosomal recessive disorders: a new promising approach. Front Genet. 2022;13:1047474. https://doi.org/10.3389/fgene.2022.1047474.
75. Andrew M, Paes B, Milner R, Johnston M, Mitchell L, Tollefsen DM, Powers P. Development of the human coagulation system in the full-term infant. Blood. 1987;70(1):165–72.
76. Mitsiakos G, Katsaras GN, Pouliakis A, Papadakis E, Chatziioannidis I, Mitsiakou C, Gialamprinou D, Papacharalampous E, Kioumi A, Athanasiou M, Athanassiadou F, Sfoungaris D, Nikolaidis N. Neonatal haemostatic parameters in correlation to gestational age and birth weight. Int J Lab Hematol. 2022;44(5):952–8. https://doi.org/10.1111/ijlh.13932.

Vitamin K-Dependent Coagulation Factors Deficiency, Diagnosis, and Management

10

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10.1 Introduction

Vitamin K-dependent coagulation factors deficiency (VKCFD) is a rare bleeding disorder and literature is only restricted to a few case reports and small case series [1], even if the progress of genotyping techniques has recently allowed to improve the knowledge of the disease. VKCFD is an autosomal recessive disorder, which arises from defects in either γ-glutamyl carboxylase (*GGCX*) or subunit 1 of vitamin K epoxide reductase complex (*VKORC1*) genes. If there is a mutation in the former enzyme it will be considered as type I disorder, and if the responsible mutation involves the latter, it will be considered as type II [2]. These genes are encoding proteins that are involved in the γ-carboxylation of the glutamate (Glu) residues of several vitamin K-dependent (VKD) proteins including a number of coagulation factors (FII, FVII, FIX, and FX), and also some non-hemostatic proteins entangled in mineralization or cell signaling. VKCFD is usually symptomatic since infancy with life-threatening bleeding events [3]. VKCFD can be diagnosed by prolongation of PT, APTT with normal TT, and parallel reduction of VKD coagulation factors, usually around 1–30%. However, the definite diagnosis is based on molecular analysis of GGCX and VKORC1 genes. Management of the disorder is mainly through administration of vitamin K1 or four-factor prothrombin complex concentrate (PCC) [4].

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_10

10.2 Structure and Function of GGCX and VKOR

GGCX is an integral trans-membrane protein, which is located on the ER membrane. It consists of 758 amino acids with a disulfide bond between cysteines 99 and 450 [3]. There is limited information on the structure of GGCX. However, it seems that GGCX contains 5 trans-membrane domains. The amino terminal of this enzyme is located in cytoplasm and the carboxyl terminal is exposed to ER lumen [5]. Based on the studies on the structure and mechanism of action, different binding and catalytic sites are considered for GGCX. Accordingly, GGCX contains a propeptid binding site, a glutamate binding site, a vitamin K binding site, a carboxylation active site, an epoxidation active site, and probably, a CO_2 binding site; however, the information on the exact location of these functional regions is limited [6].

The VKOR protein is also an integral protein in the ER membrane with 163 amino acids [3]. There are two topology models which consider three or four trans-membrane domains for the protein. According to the different studies, three trans-membrane model is more reasonable. In this model, the amino terminal of VKOR is located in the ER lumen and the carboxy terminal is located in the cytoplasm [5]. For a long period, it was assumed that the VKOR was a multi-enzyme complex, a theory, which is now questioned [5].

10.2.1 Vitamin K Cycle

VKD carboxylation is a post-translational modification, which is critical for proper function of VKD proteins. The most important VKD proteins are coagulation factors (F) FII, FVII, FIX, and FX, natural anticoagulants protein C, protein S, and protein Z, and non-hemostatic proteins including osteocalcin (also known as bone Gla protein, BGP), matrix Gla protein (MGP), growth arrest-specific protein 6 (Gas6), and Gla rich protein (GRP) [7].

In VKD carboxylation, specific glutamate (Glu) residues are modified to gamma-carboxyglutamate (Gla). Each VKD factor contains 10–12 Gla residues in the amino terminus which is called Gla domain. The responsible enzyme for this conversion is GGCX which requires reduced vitamin K (KH_2), CO_2, and O_2 as cofactors. When each Glu is modified, one KH_2 molecule is oxidized to vitamin K 2, 3 epoxide (KO). For restoration of KH_2, this KO molecule needs to be converted back to the reduced form (Fig. 10.1). This conversion occurs in a two-step reaction, first KO is reduced to vitamin K using VKOR and then vitamin K is reduced to KH_2 using vitamin K reductase [5, 8].

Recognition of protein substrates by GGCX is mediated via binding to the N-terminal propeptide of the substrate which plays a pivotal role in the carboxylation of clotting factors and determines the carboxylation efficiency. The propeptide of coagulation factors exerts various affinities towards GGCX. Although naturally occurring mutations involving the propeptide of coagulation factors do not have a

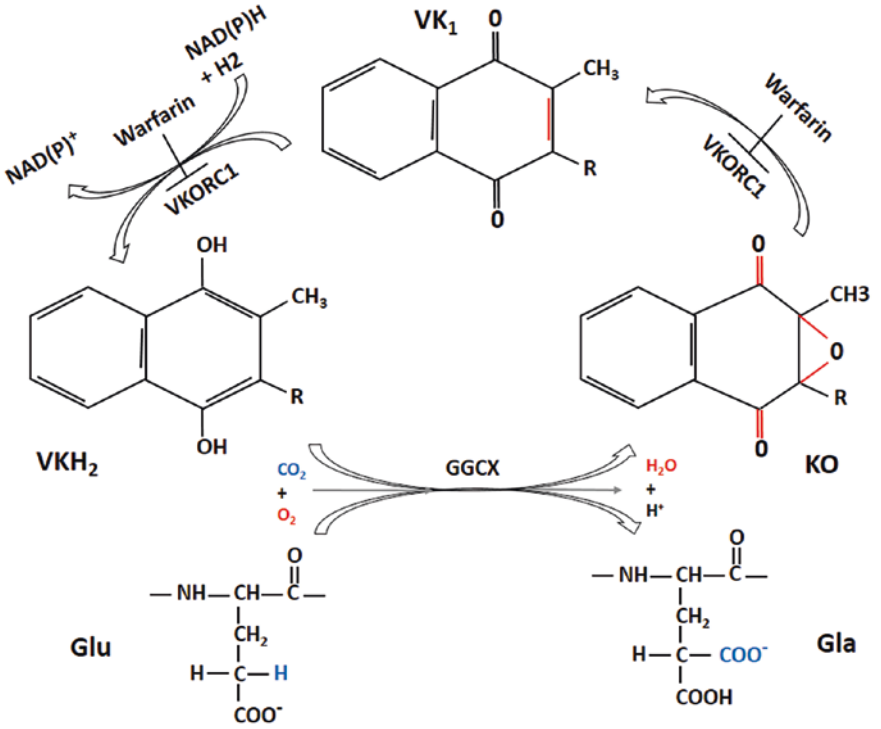

Fig. 10.1 Carboxylation of glutamate (Glu) to carboxyglutamate (Gla) residues by GGCX is necessary for activation of vitamin K-dependent coagulation factors. VKH_2 is the cofactor of this conversion. In this process, VKH_2 is oxidized into KO and then reduced to vitamin K quinone and VKH_2, respectively by VKORC1. Warfarin can block the VKORC1. Another reductase, NQO1 can also convert VK to VKH_2. Warfarin can inhibit VKORC1 but not NQO1 [9]. *GGCX* γ-glutamyl carboxylase; *VKH_2* vitamin K hydroquinone; *KO* vitamin K epoxide; *VKORC1* vitamin K epoxide reductase complex subunit 1; *NQO1* NADPH quinone oxidoreductase; *VK* vitamin K

considerable impact on the carboxylation and the normal levels of active VKD clotting factors, it has been recently suggested that such mutations may predispose to warfarin hypersensitivity during anticoagulant treatment [10].

Gamma-carboxylation of the Glu residues is necessary for binding of calcium ions which then allows binding of VKD factors to phospholipid membranes such as surface of activated platelets or damaged endothelium. This phenomenon leads to concentration of clotting factors at the site of vascular injury [5].

Non-hemostatic VKD proteins also possess several biological functions for which carboxylation is necessary, such as vascular calcification, bone metabolism, and signal transduction. Therefore, disrupted carboxylation of VKD proteins may also lead to comorbid phenotypes in VKCFD patients including skeletal, dermatological or cardiac abnormalities [11].

10.3 Vitamin K-Dependent Coagulation Factors Deficiency

VKCFD (OMIM #277450 and #607473) was first described in 1966 by McMillan and Robert in a 4-month-old girl [12]. She presented with several bruises and bleeding events, a prolonged PT and APTT, and undetectable levels of FII, FVII, FIX, and FX by clotting assays. However, neither liver disease nor malabsorption had been detected. Low level of coagulation factors showed a partial recovery following administration of high doses of vitamin K [12]. The patient was further investigated at age of 15 years and the clotting factors were reevaluated by immunologic assays [13]. However, the molecular mechanism remained unclear.

Now, VKCFD is known as an autosomal recessive bleeding disorder that arises from defects in either *GGCX* or *VKORC1* genes. Plasma level of VKD coagulation factors in VKCFD may be around 1–30% [14].

10.4 Clinical Manifestations

VKCFD usually manifests in infancy, although it may also remain latent for a short time. Severity of clinical manifestations depends on the level of reduced coagulation factors [15]. However, the clinical picture is not closely correlated to the activity of VKD coagulation factors [4]. Severe bleeding such as intra cranial hemorrhage (ICH) or umbilical cord bleeding has been described in affected neonates [16–18]. Mucocutaneous, soft tissue bleeding and post traumatic hemorrhages are also reported in these patients (Table 10.1) [17, 19]. Less commonly, VKCFD may present with hemorrhagic events in adulthood or even may be found incidentally [4].

Some affected individuals may also suffer from mental retardation, skin, cardiac and skeletal abnormalities which are attributed to the impaired γ-carboxylation of other VKD proteins [16, 20]. Skeletal abnormalities including nasal hypoplasia, distal digital hypoplasia, and epiphyseal stippling are similar to those seen in warfarin embryopathy [21]. Pseudoxanthoma elasticum-like (PXE-like) disorders have also been reported in patients affected by VKCFD with GGCX mutations [22]. Recently, the effect of GGCX variants on their ability to γ-carboxylate non-hemostatic VKD proteins in the presence of different concentrations of vitamin K

Table 10.1 Clinical manifestations of vitamin K-dependent coagulation factors deficiency

Clinical manifestations	Incidence
Intracranial hemorrhage	34%
Ecchymoses/easy bruising	21%
Skeletal abnormalities/growth or developmental retardation	21%
Umbilical cord bleeding	17%
Post-trauma/post-operative	17%
Epistaxis	17%
Gingival/oral cavity	12%
Hemarthrosis	4%

has been explored; variants with a markedly reduced ability to γ-carboxylate the upper zone of the growth plate and cartilage matrix-associated protein (UCMA/GRP) have been identified in patients with a PXE-like phenotype [23].

Some natural anticoagulants including protein C, protein S, and protein Z also require Glu residues to be modified into γ-carboxyglutamate (Gla) residues and therefore there are also low levels of protein C and protein S in the deficiencies of GGCX or VKOR. The fact that no cases with thrombosis have been reported in the literature so far, may suggest the dominant effect of these two enzymes in procoagulant activities [1, 24].

10.5 Molecular Basis

The gene encoding for GGCX with 13 kb length is located on chromosome 2p11.2 and comprises 15 exons. The responsible gene for VKORC1 protein that is called *VKORC1* is located on chromosome 16p11.2. It is a small gene with 5126 bp length and includes 3 exons [15]. Defect in GGCX is known as type I VKCFD. Another enzyme that plays an important role in this cycle is VKORC1. VKORC1 catalyzes reconversion of vitamin K epoxide (KO), which is produced during the last reaction, to KH2. Defect in VKORC1 is known as type II VKCFD [4, 7]. It seems that a missense mutation that leads to the substitution of tryptophan to arginine at amino acid number 98 is the only reported mutation involving *VKORC1* (Table 10.2).

To date, at least 34 mutations have been reported in the *GGCX* gene, which are associated with VKCFD and the majority of them are point mutations (Table 10.2) [1, 15]. The mutations can be observed in homozygous or compound heterozygous. Jin et al. showed that 1657delA and IVS13-6G>A are the underlying mutations of the first case of VKCFD which was reported by McMillan and Robert [25].

Considering various VKD proteins as substrates of GGCX, different mutations of GGCX have been linked to distinct bleeding and non-bleeding phenotypes. A number of these mutations lead to PXE-like disorder combined with reduced activity of VKD coagulation factors with or without abnormal bleeding tendency. The variable clinical picture of patients harboring GGCX mutations, regarding both hemostatic and non-hemostatic features, may be explained by the recent findings of Hao et al. and Gosh et al. The study of the first group revealed that GGCX mutations have differential impacts on the carboxylation efficiency of structurally/functionally discrete VKD proteins, including FIX, BGP, and MGP in a cell-based study. Furthermore, the effect of administrating vitamin K as a cofactor of carboxylation was affected differentially, a finding which also explains why vitamin K administration improves bleeding but not non-bleeding disorders/manifestations [26]. Another in vitro study by Gosh et al. was indicative of the differential impacts of GGCX mutations on carboxylation of VKD coagulation factors. According to this study, different GGCX mutations also have variable impacts on the responsiveness to vitamin K. Such findings can be useful in predicting the risk of bleeding and also the effectiveness of vitamin K therapy based on the molecular defect [27].

Table 10.2 Vitamin K-dependent coagulation factors deficiency causing mutations in the *GGCX* and *VKORC1* genes

	Type	Nomenclature	Gene region
Mutations in *GGCX* gene (type I disorder)	Undefined[a]	IVS1del14bp	Intron 1
	Splicing	IVS1-1G>A	Intron 1
	Missense	Asp31Asn	Exon 2
	Splicing	IVS2+1G>T	Intron 2
	Splicing	IVS2-1G>T[b]	Intron 2
	Missense	Pro80Leu	Exon 3
	Missense	Arg83Pro	Exon 3
	Missense	Arg83Trp	Exon 3
	Missense	Cys139Trp	Exon 4
	Missense	Cys139Tyr	Exon 4
	Missense	Asp153Gly	Exon 4
	Missense	Trp157Arg	Exon 4
	Missense	Met174Arg	Exon 4
	Missense	Asp183Val	Exon 5
	Missense	Arg204Cys	Exon 5
	Missense	Val255Met	
	Missense	Ser284Pro	
	Missense	Phe299Ser	
	Missense	Ser300Phe	
	Nonsense	Trp315Ter	
	Nonsense	Gln374X	Exon 8
	Missense	Gly386Val	
	Missense	Leu394Arg	Exon 9
	Missense	His404Pro	Exon 9
	Missense	Arg476Cys	Exon 10
	Missense	Arg476His	Exon 10
	Missense	Arg485Pro	Exon 11
	Missense	Trp493Cys	Exon 11
	Missense	Trp501Ser	Exon 11
	Missense	Arg513Lys	Exon 11
	Missense	Ile532Thr	Exon 11
	Missense	Gly537Ala	Exon 11
	Missense	Gly558Arg	Exon 11
	Splicing	IVS11+3A>G	Intron 11
	Frameshift	1657delA	Exon 12
	Missense	Thr591Lys	Exon 13
	Splicing	IVS13-6G>A	Intron 13
	Nonsense	Arg704X	Exon 15
	Missense	Ser741Leu	Exon 15
Mutations in *VKORC1* gene (type II disorder)	Missense	Arg98Trp	Exon 3

GGCX γ-glutamyl carboxylase; *VKORC1* vitamin K epoxide reductase complex subunit 1

[a]It seems that the deleted region in intron 1 is probably associated with cis-acting elements and thereby is involved in gene regulation [28]

[b]This splice site mutation in intron 2 results in a loss of exon 3 (Gly72-Leu124del)

Most recently, Rishavy et al. reported the significance of GGCX processivity and complete carboxylation for appropriate function of VKD proteins. The assay was based on comparing the carboxylase activity of wild type and mutant (V255M) GGCX enzymes in the presence of a VKD protein (FIX or MGP) and a challenge protein as an interfering agent for VKD protein carboxylation. In the presence of wild type enzyme, the VKD protein became fully carboxylated before the initiation of Challenge protein carboxylation, while both VKD protein and Challenge protein became carboxylated simultaneously but not completely in the presence of mutant carboxylase, indicating that GGCX V255M mutant has lost its processivity in carboxylation which subsequently results in poor clotting activity of FIX. the latter was obtained by an in vitro-study using FIX-HEK293 cells, a finding that explains the impaired hemostasis in patients with mutant GGCX [28].

10.6 Diagnosis

There is a weak relationship between severity of clinical manifestations and laboratory results of VKCFD [4]. VKCFD can be diagnosed by prolongation of PT, APTT with normal TT, and parallel reduction of FII, FVII, FIX, and FX activity [4, 14]. Although both PT and APTT are prolonged in VKCFD, PT test is slightly more affected. Factor activities are usually around 0.2–0.6 IU/mL and less commonly are <0.1 IU/mL at baseline [4]. Presence of inhibitors can be excluded by mixing study.

Differentiation of VKCFD and acquired vitamin K deficiency must be intentioned with normal fasting serum KH_2 (reduced vitamin K) concentration in VKCFD. Acquired vitamin K deficiency may arise from intestinal malabsorption of vitamin K in the inflammatory bowel disease or celiac disease, liver cirrhosis, and exposure to coumarin anticoagulants [4]. The differential diagnosis of the disorder in neonates mainly includes vitamin K deficiency. In healthy newborns the levels of VKD coagulation factors gradually increase up to the age of 6 months. Therefore, the diagnosis in neonates has to be confirmed by repeating the test at 6 months of age [4, 29].

In the subject of type I and II of the disorder, it should be considered that oxidized vitamin K (KO) is typically undetectable in VKCFD type I, even after vitamin K supplementation, but in VKCFD type II, an elevation of KO level can be observed following vitamin K supplementation [2, 4]. In addition, VKCFD must be distinguished from congenital factor deficiency including FII deficiency, isolated FVII and FIX deficiencies, and FX deficiency, as well as combined FVII and FX deficiency. In this setting, inhibitor against FIX (acquired hemophilia B) and FVII must be assayed [2]. For definite diagnosis, molecular analysis for mutations of *VKORC* or *GGCX* is necessary. Recently, a cell-based system for the detection of GGCX activity has been developed, it may thus be adopted for the diagnosis of VKCFD1 caused by GGCX variants [30].

10.7 Management

Management of the disorder is mainly through administration of vitamin K1 (phytomenadion), but in severely affected patients and in major surgeries, four-factor prothrombin complex concentrate (PCC) (containing prothrombin, FVII, FIX, and FX) is also required (Table 10.3) [31]. Most of the cases (not all) may show a partial restoration in the level of deficient factors with high doses of vitamin K [15].

As VKCFD may lead to life-threatening hemorrhagic events, prophylaxis at diagnosis is highly recommended. Dose of treatment for long-term prophylaxis is 5–20 mg/day of oral vitamin K1 and when response is poor, 5–20 mg/week of parenteral vitamin K1 is recommended [4, 31]. In the subject of mild bleeding or minor surgery, 15–20 mL/kg tranexamic acid or 1 g four times daily alone is recommended [4]. In connection with severe bleeding or major surgery, 20–30 IU/kg of four-factor PCC combined with 5–20 mg of vitamin K1 is recommended. In the lack of four-factor PCC, 15–25 mL/kg of virus inactivated FFP can be administered [4, 31].

In normal pregnancy, the levels of FVII and FX usually increase, while FII and FIX levels do not alter. The level of FVII in some pregnant women may elevate even up to tenfold [32]. However, in some pregnant women affected by VKCFD, the physiologic increase in the deficient factors may be inadequate for an uneventful delivery. Pregnant women in whom each of the VKD factors has an activity below 20 IU/mL in the third trimester must be managed carefully at the time of delivery. In this condition, one dose of four-factor PCC 20–30 IU/kg before delivery or before cesarean section is recommended, treatment with PCC should be continued for at least 72 h [4].

It currently seems that prenatal diagnosis of VKCFD is not preferred, taking into account the potential bleeding risk of the procedure but also because major hemorrhagic events in the affected newborn can be prevented by the administration of vitamin K in the third trimester of pregnancy in mothers which are prone to have a

Table 10.3 Different therapeutic choices for management of patients with vitamin K-dependent coagulation factors deficiency (VKCFD) in different conditions

Condition	Recommended dosages
Long-term prophylaxis	Oral vitamin K1 (5–20 mg/day) *If response is poor:* Parenteral vitamin K1 (5–20 mg/week)
Mild bleeding events/minor surgeries	Tranexamic acid (15–20 mL/kg or 1 g four times daily)
Severe bleeding events/major surgeries	Four-factor PCC (20–30 IU/kg) + Vitamin K1 (5–20 mg) *Four-factor PCC can be substituted by virus inactivated FFP (15–25 mL/kg)*
Pregnancy (each of the vitamin K-dependent factors activity <20 IU/mL in the third trimester)	Four-factor PCC (20–30 IU/kg), one dose at the time of delivery or before cesarean section and continued for at least 3 days

PCC prothrombin complex concentrate

child with VKCFD. Antenatal management and timing of Vitamin K administration for prophylaxis of intrauterine bleeding need, however, to be better defined also in order to prevent bone and skin manifestations of the disease [2, 33].

References

1. Brenner B, Kuperman AA, Watzka M, Oldenburg J, editors. Vitamin K-dependent coagulation factors deficiency. Seminars in thrombosis and hemostasis, vol. 35; 2009. p. 439.
2. Napolitano M, Mariani G, Lapecorella M. Hereditary combined deficiency of the vitamin K-dependent clotting factors. Orphanet J Rare Dis. 2010;5(1):21.
3. Stafford D. The vitamin K cycle. J Thromb Haemost. 2005;3(8):1873–8.
4. Mumford AD, Ackroyd S, Alikhan R, Bowles L, Chowdary P, Grainger J, et al. Guideline for the diagnosis and management of the rare coagulation disorders. Br J Haematol. 2014;167(3):304–26.
5. Tie JK, Stafford DW. Structural and functional insights into enzymes of the vitamin K cycle. J Thromb Haemost. 2016;14(2):236–47.
6. Hosseini MS, Shams M, Dorgalaleh A, Mansouritorghabeh H. Multiple coagulation factor deficiency. Congenital bleeding disorders. Springer; 2018. p. 219–38.
7. Oldenburg J, Marinova M, Müller-Reible C, Watzka M. The vitamin K cycle. Vitamins Hormones. 2008;78:35–62.
8. Oldenburg J, Bevans CG, Müller CR, Watzka M. Vitamin K epoxide reductase complex subunit 1 (VKORC1): the key protein of the vitamin K cycle. Antioxid Redox Signal. 2006;8(3–4):347–53.
9. Gallieni M, Fusaro M. Vitamin K and cardiovascular calcification in CKD: is patient supplementation on the horizon? Kidney Int. 2014;86(2):232.
10. Hao Z, Jin D-Y, Stafford DW, Tie J-K. Vitamin K-dependent carboxylation of coagulation factors: insights from a cell-based functional study. Haematologica. 2020;105(8):2164.
11. Watzka M, Geisen C, Scheer M, Wieland R, Wiegering V, Dörner T, et al. Bleeding and non-bleeding phenotypes in patients with GGCX gene mutations. Thromb Res. 2014;134(4):856–65.
12. McMillan CW, Roberts HR. Congenital combined deficiency of coagulation factors II, VII, IX and X: report of a case. N Engl J Med. 1966;274(23):1313–5.
13. Chung K-S, Bezeaud A, Goldsmith JC, McMillan CW, Menache D, Roberts HR. Congenital deficiency of blood clotting factors II, VII, IX, and X. Blood. 1979;53(4):776–87.
14. Castaman G, Linari S. Diagnosis and treatment of von Willebrand disease and rare bleeding disorders. J Clin Med. 2017;6(4):45.
15. Zhang B, Ginsburg D. Familial multiple coagulation factor deficiencies: new biologic insight from rare genetic bleeding disorders. J Thromb Haemost. 2004;2(9):1564–72.
16. Oldenburg J, Von Brederlow B, Fregin A, Rost S, Wolz W, Eberl W, et al. Congenital deficiency of vitamin K-dependent coagulation factors in two families presents as a genetic defect of the vitamin K-epoxide-reductase-complex. Throm Haemost. 2000;84(6):937–41.
17. Brenner B, Sánchez-Vega B, Wu S-M, Lanir N, Stafford DW, Solera J. A missense mutation in γ-glutamyl carboxylase gene causes combined deficiency of all vitamin K-dependent blood coagulation factors. Blood. 1998;92(12):4554–9.
18. Spronk HM, Farah RA, Buchanan GR, Vermeer C, Soute BA. Novel mutation in the γ-glutamyl carboxylase gene resulting in congenital combined deficiency of all vitamin K–dependent blood coagulation factors. Blood. 2000;96(10):3650–2.
19. Lunghi B, Redaelli R, Caimi T, Corno A, Bernardi F, Marchetti G. Novel phenotype and γ-glutamyl carboxylase mutations in combined deficiency of vitamin K-dependent coagulation factors. Haemophilia. 2011;17(5):822–4.
20. Hauschka PV, Lian JB, Cole D, Gundberg CM. Osteocalcin and matrix Gla protein: vitamin K-dependent proteins in bone. Physiol Rev. 1989;69(3):990–1047.

21. Pauli R, Lian J, Mosher D, Suttie J. Association of congenital deficiency of multiple vitamin K-dependent coagulation factors and the phenotype of the warfarin embryopathy: clues to the mechanism of teratogenicity of coumarin derivatives. Am J Hum Genet. 1987;41(4):566.
22. Vanakker OM, Martin L, Gheduzzi D, Leroy BP, Loeys BL, Guerci VI, et al. Pseudoxanthoma elasticum-like phenotype with cutis laxa and multiple coagulation factor deficiency represents a separate genetic entity. J Investig Dermatol. 2007;127(3):581–7.
23. Ghosh S, Kraus K, Biswas A, Müller J, Forin F, Singer H, et al. GGCX variants leading to biallelic deficiency to γ-carboxylate GRP cause skin laxity in VKCFD1 patients. Hum Mutat. 2022;43(1):42–55. https://doi.org/10.1002/humu.24300.
24. Palla R, Peyvandi F, Shapiro AD. Rare bleeding disorders: diagnosis and treatment. Blood. 2015;125:2052.
25. Jin D-Y, Ingram BO, Stafford DW, Tie J-K. Molecular basis of the first reported clinical case of congenital combined deficiency of coagulation factors. Blood. 2017;130(7):948–51.
26. Hao Z, Jin D-Y, Chen X, Schurgers LJ, Stafford DW, Tie J-K. γ-Glutamyl carboxylase mutations differentially affect the biological function of vitamin K–dependent proteins. Blood. 2021;137(4):533–43.
27. Ghosh S, Kraus K, Biswas A, Müller J, Buhl AL, Forin F, et al. GGCX mutations show different responses to vitamin K thereby determining the severity of the hemorrhagic phenotype in VKCFD1 patients. J Thromb Haemost. 2021;19(6):1412–24.
28. Rishavy MA, Hallgren K, Wilson LA, Hiznay JM, Runge KW, Berkner KL. GGCX mutants that impair hemostasis reveal the importance of processivity and full carboxylation to VKD protein function. Blood. 2022;140:1710.
29. Thomas A, Stirling D. Four factor deficiency. Blood Coagul Fibrinolysis. 2003;14:S55–S7.
30. Gao W, Liu H, Su G, Xu Y, Wang Y, Cui L, et al [Development of a cell-based diagnostic system for vitamin K-dependent coagulation factor deficiency 1]. Zhonghua Yi Xue Yi Chuan Xue Za Zhi. 2020;37(8):811–4. https://doi.org/10.3760/cma.j.issn.1003-9406.2020.08.002.
31. Peyvandi F, Menegatti M. Treatment of rare factor deficiencies in 2016. ASH Educ Program Book. 2016;2016(1):663–9.
32. Prisco D, Ciuti G, Falciani M, editors. Hemostatic changes in normal pregnancy. Hematology Meeting Reports (formerly Haematologica Reports); 2009.
33. Guzzardo GM, Ghosh S, Pezeshkpoor B, Pavlova A, Czogalla-Nitsche K, et al. Novel gamma-Glutamyl carboxylase mutation causing hemorrhagic and non-hemorrhagic VKCFD1 phenotypes. Blood. 2022;140(Supplement 1):8480–2. https://doi.org/10.1182/blood-2022-169105.

Congenital Factor VII Deficiency, Diagnosis, and Management

11

Mahmood Shams

11.1 Introduction

Congenital factor (F) VII deficiency is a rare autosomal recessive bleeding disorder with an estimated prevalence of 1 per 500,000 in the general population, with ethnic or gender predilection [1, 2]. However, the prevalence is higher in regions with a high rate of consanguinity marriage [1, 3, 4]. Clinical pictures in these patients ranging from asymptomatic condition to severe, life-threatening hemorrhages [5, 6]. There is a relatively poor correlation between FVII coagulant activity (FVII:C) and bleeding tendency, as well as mutation profile in congenital FVII deficiency [3, 7]. Severe clinical symptoms usually present in patients with less than 1% FVII:C; however, some patients with severe deficiency do not experience severe bleeding episodes. The complete absence of functional FVII in knock-out mice is incompatible with life, suggesting FVII deficiency is not associated with complete absence of functional FVII. In line with this issue, patients with residual FVII level can survive and able to prevent lethal bleeding [8, 9]. The disorder is accompanied with a wide spectrum of bleeding problems, including mild symptoms such as mucous membranes and skin hemorrhages and life-threatening hemorrhages such as central nervous system (CNS) bleeding. Iron deficiency due to menorrhagia is common in women with FVII deficiency [3]. In aggregate, it seems that patients with FVII deficiency experience substantial psychosocial impact due to the high disease burden, however, the generally held view is that the majority of patients maintain optimism regards to their disease management and thereby the quality of their living [10]. This disorder can be managed by different therapeutic options including fresh frozen plasma (FFP), prothrombin complex concentrate (PCC), plasma-derived FVII (pd-FVII) products, and recombinant activated FVII (rFVIIa).

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_11

11.2 Factor VII Structure and Function

FVII is a low molecular weight protein (50 kDa) composed of 406 amino acids, synthesized as single-chain molecule in the endoplasmic reticulum of hepatocytes. FVII has homology with FIX, FX, and protein C at the catalytic site and the amino-terminal region [11]. Within hepatocytes, FVII has a signal peptide that is required for secretion and a propeptide (removed intracellularly) that is necessary for γ-carboxylation of all glutamate residues within ~45 amino acids at the N-terminus site of the FVII protein [12]. Coagulation FVII consists four domains, including a gamma-carboxyglutamate acid (**Gla**) domain on the N-terminal with ten glutamic acid residues (at residues 6, 7, 14, 16, 19, 20, 25, 26, 29, and 35). These ten glutamic acid residues undergo post-translational modification; convert to γ-carboxyglutamic acid (Gla) with calcium-binding capacity. Binding of calcium to the Gla domain induces conformational changes and exposure of new epitopes that facilitate its subsequent binding to tissue factor and phospholipid [13]. The other FVII domains are two epidermal growth factor-like domains (**EGF1**, **EGF2**) and a catalytic serine protease (**SP**) domain at the C-terminal of the FVII protein [1, 12] (Fig. 11.1).

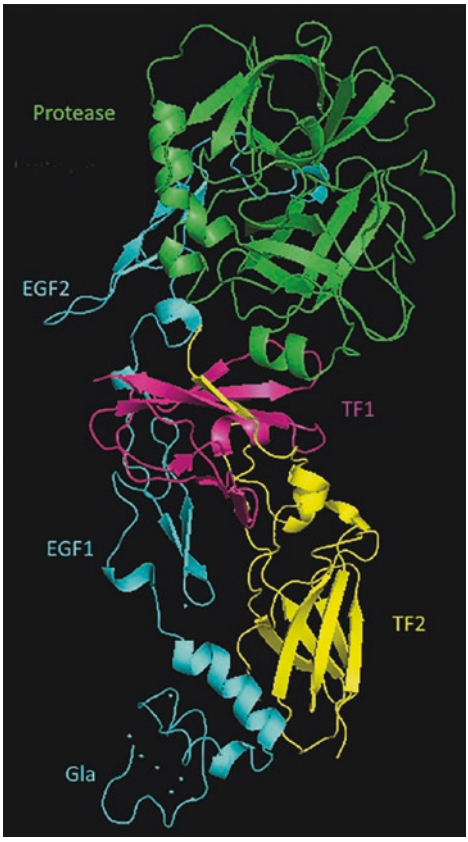

Fig. 11.1 The cartoon representation of activated factor VII (FVIIa)/soluble tissue factor (sTF) complex. FVII has four domains including a gamma-carboxyglutamate acid (Gla), two epidermal growth factor-like (EGF1, EGF2) domains, and serine protease domain. sTF contains two fibronectin type III domains (TF1 and TF2 represent of N- and C-terminal of sTF, respectively)

Zymogen form of FVII with the plasma half-life of 5 h has the shortest half-life among the clotting factors; however, the half-life of free activated FVII (FVIIa) is 2 h, whereas the plasma half-life of most other activated coagulation factors is very short [1, 12].

FVII reversibly in a Ca2+ dependent manner can bind to membranes with negatively charged phospholipids such as phosphatidylserine or phosphatidic via Gla domain [14, 15]. The majority of plasma FVII circulates as the single-chain inert zymogen (10 nmol/L (0.5 μg/mL)) and the minority circulates in the plasma as two-chain active protein ~10–110 pmol/L) [1, 3, 12, 16]. The key event in the activation of FVII is proteolysis of a single peptide bond between Arg-c15 (amino acid 152) and Ile-c16 (amino acid 153) in the connecting region of EGF2 and SP domains. This results in formation of two polypeptide chains: heavy chain with 254 amino acids (30 kDa) (residues 153–406), comprised of serine protease domain with Trypsin homology at C terminus, and light chain with 152 amino acids (20 kDa) (residues 1–152), composed of a Gla and two EGF-like domains [1, 12, 17–19].

FVII, chiefly interacts with TF via the Gla and EGF1 domains, however, it's worth noting that two other domains can also interact with TF [18, 20]. FVII/TF complex is necessary for restructuring the active site and achieving full enzymatic activity of FVIIa, because free FVIIa exhibits very weak catalytic activity [17, 18]. In addition to FVII/TF complex, several other coagulation factors, including FXa, FIIa, FIXa, FXIIa, and FIXa contribute to FVII activation, however it seems that membrane-bound FXa is the most effective activator [21]. Once the TF/FVIIa complex is formed, it results in proteolytic activation of FIX and FX to FIXa and FXa, respectively, generating few amount of thrombin that able to produce a strong feedback amplification of coagulation cascade [12, 22].

Tissue factor pathway inhibitor (TFPI) and antithrombin (AT) are inhibitors of FVIIa, but they can only inhibit FVIIa after the formation of the FVIIa/TF complex [23, 24]. TFPI is a Kunitz-type proteinase inhibitor that attaches to the membrane surface via glycophosphatidylinositol (GPI)-linkage. TFPI is mainly expressed by endothelial cells and, to a lesser extent, by platelets [25]. The TFPI/FXa complex can inhibit the FVIIa/TF complex and prevents further FX activation via the formation of inactivated tetra-molecular (TF-FVIIa-TFPI-FXa) complex, causing rapid inhibiting of the extrinsic coagulation pathway [23, 25]. Inhibitory function of TFPI/FXa, at last partly, is inducing TF-expressing cells to internalize the TF/FVIIa complexes, leading to degradation of the majority of FVIIa [26]. TFPI is synthesized by microvascular endothelial cells, megakaryocytes, and the liver [3, 27]. Heparin and various platelet agonists can increase the release of TFPI from the surface of endothelial cells. AT reaction is heparin-dependent and its reactivity with FVIIa is increased after FVIIa/TF complex formation. Following the binding of AT to the FVIIa/TF complex, the affinity of FVIIa to TF decreases, and the FVIIa/AT complex is then released into the blood-stream [3, 23] (Fig. 11.2). The FVIIa/AT complex is increased in many prothrombotic situations and seems to be an early marker of coagulation cascade activation [23].

FVII with the initiation of coagulation pathway following complex formation with TF at injury site has a critical role in the coagulation cascade. This complex is an important activator of both extrinsic and intrinsic coagulation pathways by activation of FVII, FIX, and FX [5]. It was shown that complete deletion of FVII gene

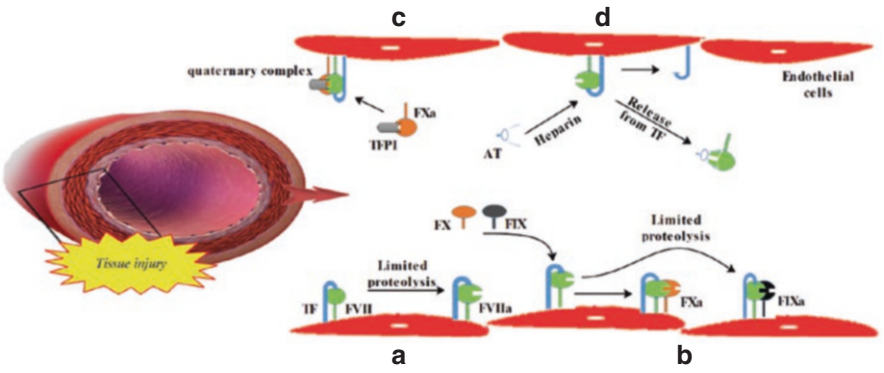

Fig. 11.2 In vivo activation and inhibition of factor VII (FVII). (**a**, **b**) FVII binds to tissue factor and is converted to its active form, FVIIa, by minor proteolysis and then activates factor X (FX) and factor IX (FIX). (**c**, **d**) Tissue factor pathway inhibitor (TFPI) and antithrombin (AT) are inhibitors of FVII/TF complex. TFPI/FXa complex can form an inactive tetra-molecular (TF-FVIIa-TFPI-FXa) complex that results in extrinsic coagulation pathway inhibition. In addition to direct deactivation of FXa, TFPI can prevent further FX activation after TFPI/TF/FVIIa complex formation. TFPI/FXa can induce internalization of TF/FVIIa complex, resulting in degradation of the majority of FVIIa (not shown). TFPI can also inhibit the early forms of prothrombinase (Not shown). AT directly attaches to TF/FVIIa complex and causes segregation of FVIIa from TF by losing affinity of FVIIa for TF, resulting in the release of FVIIa/AT into blood-stream and therefore causes extrinsic coagulation pathway inhibition. AT activity can be strengthened by heparin

leads to mouse perinatal death, while mice and humans with very low FVII levels could be survived [8, 28, 29]. Although the normal perinatal course was observed in FVII knockout mice, major abdominal and intracranial hemorrhages (ICH) lead to death in such cases at birth or shortly after birth [9]. Generally, it is accepted that the absence of FVII is incompatible with life [3, 22, 30].

TF known as thromboplastin, coagulation FIII, or CD142 is a glycosylated, transmembrane protein that doesn't require proteolysis for activation [12]. It is well known that normal hemostasis process in some tissues with high TF-expression such as the brain, bowel, uterus, placenta, lungs, and heart depends mainly on the extrinsic pathway, therefore, reduced or absence of FVII can result in bleeding in some of these tissues [5, 8]. In addition to well-known role of TF in the coagulation process, in complex with FVIIa other functions including embryonic angiogenesis, oncogenic angiogenesis, tumor progression, leukocyte diapedesis, and regulation of inflammation and sepsis are described. This complex can also change cellular physiology in the TF-expression cells [31, 32].

11.3 Congenital Factor VII Deficiency

Congenital FVII deficiency (OMIM 227500) is an autosomal recessive bleeding disorder, for the first time described by Alexander in 1951 in a 4-years old white girl who experienced prolonged umbilical cord bleeding at birth [33]. This bleeding disorder with a prevalence of 1 per 500,000 individuals is the most common among rare

Table 11.1 Classification of congenital factor VII deficiency and results of coagulation tests

	APTT	PT	FVII:C	FVII:Ag
Normal people	Normal	Normal	Normal	Normal
FVII deficiency (type I)	Normal	Prolonged	Decreased	Decreased
FVII deficiency (type II)	Normal	Prolonged	Decreased	Normal or nearly normal

APTT activated partial thromboplastin time; *PT* prothrombin time; *FVII:C* factor VII coagulant activity; *FVII:Ag* factor VII antigen

bleeding disorders (RBDs) [1, 6, 33]. While this disorder is distributed worldwide, it is more frequent in some areas such as the United Kingdom, United States, Brazil, Turkey, Italy, Slovak Republic, and Iran, as reported in the annual global survey of World Federation of Hemophilia (WFH). Although consanguinity is the main cause for high rate of disorder, in some countries like the United Kingdom, this increae has also been attributed to noticeable grow up of hygienic surveillance and enhanced overall quality of life. The number of patients with congenital FVII deficiency might be underestimated probably due to undiagnosed asymptomatic patients and those with the only mild bleeding tendencies. FVII deficiency is categorized into two groups, including type I (quantitative deficiencies), which is characterized by simultaneous decreases in FVII activity and antigen levels, and type II (qualitative defects) with only decreases in FVII activity with normal or near normal FVII antigen level (Table 11.1) [6]. It should be noted that type II is divided into two distinct groups including cases with no variation in the FVII:C levels, regardless of the type of thromboplastin used, and those with different FVII:C levels when different origins of the thromboplastin are used. Clinical manifestations of the disorder are highly variable both in severity and type of bleeding, with poor correlation between residual plasma factor activity and severity of bleeding [5, 34]. The FVII reference range is between 70% and 140% and usually, less than 2% FVII activity (FVII:C) is related to an increased risk of severe bleeds during the newborn and young childhood periods [1, 7]. The disorder is due to mutations in *F7* gene, and a wide spectrum of mutations has been identified within this gene. Most of the identified mutations are new and restricted to the special area or specific family and could be used for carrier detection, precise diagnosis, and prenatal diagnosis in affected families.

The presence of abnormal bleeding, accompanied by isolated prolonged PT, serve as the first clue for suspicion to FVII deficiency, but in general, a combination of clinical presentations, physical examination, family history, and laboratory assessments can be used for precise diagnosis of the disorder. Several therapeutic options such as fresh-frozen plasma (FFP), plasma-derived FVII (pd-FVII), prothrombin complex concentrate (PCC), activated PCC (aPCC), and more recently recombinant FVIIa (rFVIIa) are available for patients with FVII deficiency.

11.4 Acquired Factor VII Deficiency

Acquired FVII deficiency can be present either in isolation or as a part of a deficiency in vitamin-K dependent coagulation factors [1, 35, 36]. Acquired isolated FVII deficiency is an extremely rare condition, with only few reported cases, so far

[37]. However, the frequency was underestimated, because clinical symptoms of the disorder can be moderate with only slightly prolonged PT [35, 36, 38]. Isolated acquired FVII deficiency should be considered a potential medical condition in patients with prolonged PT and no personal or family history of bleeding tendency. In such cases, FVII:C should be repeated after resolving the acute phase of the illness. If persistently high levels of FVII are observed, genetic testing is indicated to investigate the possibility of congenital FVII deficiency [39]. Different conditions such as malignancies, severe systemic sepsis, infectious agents, drugs (penicillin), aplastic anemia, stem cell transplantation, and presence of an inhibitory antibodies, may be accompanied by acquired isolated FVII deficiency [1, 36, 40, 41]. In this setting, an interesting study showed a mild FVII deficiency (<60%, ranged 35–56%) with no bleeding tendency in a majority of patients (89.6%) who received chronically the packed cell transfusions [42]. In some cases, no underlying condition of FVII deficiency (idiopathic) was identified [36]. The exact pathophysiological basis and the possible mechanism of FVII deficiency are not clear in these situations [36].

Simultaneous deficiency of FVII with other coagulation factors may arise in different conditions including [1]:

- Problem in synthesis, particular in liver failure that leads to decrease of all coagulation factors.
- A defective synthesis, especially during hypovitaminosis K syndrome caused by insufficient intake, malabsorption, or anticoagulant therapy with vitamin K antagonists such as Warfarin, Acenocoumarol (Sinthromin), and Phenprocoumon (Marcoumar). These conditions only lead to vitamin-K dependent coagulation factors deficiency (FII, FVII, FIX, and FX) and the decrease of protein C and protein S levels. Warfarin inhibits the vitamin K-dependent reductase and the vitamin K-dependent quinone reductase and leads to disturbing in the recycling of vitamin K to its enzymatically active form and its carboxylation activity.
- Consumption syndromes, especially disseminated intravascular coagulation (DIC) or hyperfibrinolysis that leads to consumption of all coagulation factors.

As mentioned, FVII has the shortest plasma half-life among clotting factors, thus, the decrease in plasma level of FVII occurs faster than other coagulation factors. Therefore, the diagnosis of isolated FVII deficiency should be made with caution [1].

11.5 Clinical Manifestations

Patients with congenital FVII deficiency have variable bleeding diathesis with poor correlation between FVII activity and bleeding tendency [1, 36]. The clinical phenotype is very heterogeneous and ranging from asymptomatic conditions to life-threatening diathesis. The clinical phenotype of these patients could be categorized into two main categories [1, 3, 43]:

- Asymptomatic composed about one-third of patients.
- Symptomatic with two subgroups:
  - Nonsevere: Mild to moderate with mucocutaneous bleeding (mimic platelet disorders) including approximately two-third of affected patients. These patients usually don't require medical intervention.
  - Severe with life- or limb-threatening hemorrhages (central nervous system (CNS) bleeding, gastrointestinal (GI) bleeding, or hemarthrosis) that composed about 10–15% of patients [1, 3, 43].

Asymptomatic patients might be randomly diagnosed or identified during family studies, especially in cases with other affected family member (s). According to a large study, 71% of homozygous and 50% of compound heterozygous patients are symptomatic, while only 19% of heterozygous subjects are symptomatic [2]. Based on another large study, the most common bleeding features among patients with FVII deficiency are epistaxis, easy bruising, gum bleeding, hematoma, hemarthrosis, postoperative bleeding and menorrhagia, and less common bleeding features are hematuria, GI bleeding, and CNS bleeding (Table 11.2) [2, 7, 30, 43–46]. Severe clinical presentations generally occur at young ages (soon after birth or when they are toddler) in severely affected patients [11, 43]. Severe chronic iron deficiency due to menorrhagia is common in women with FVII deficiency [3]. In this regard, due to the high rate of FVII deficiency in patients with unexplained heavy menstrual bleeding, alongside evaluation of the von Willebrand factor, evaluation of FVII

Table 11.2 Clinical manifestations of patients with congenital factor VII deficiency

	Mariani et al. (n:174[a])	G Mariani et al. (n:139[b])	G Mariani et al. (n:228)	F. H. Herrmann et al. (n:217)	F. Peyvandi et al. (n:28)	G Mariani et al. (n:24)
CNS bleeding	4.6%	6.5	7%	1%	17%	n.r.
GI bleeding	13.8%	14.4	14%	9%	n.r.	17%
Hemarthrosis	16.1%	21.6	22%	12%	21%	67%
Epistaxis	56.3%	66.2	83%	58%	64%	62%
Easy bruising	47.7%	43.2	62%	37%	32%	29%
Gum bleeding	33.9%	25.9	42%	25%	n.r.	33%
Menorrhagia	62.9%	–	15.6	57% (of 106 female)	60% (of 10 female)	90% (of 10 female)
Hematomas	16.1%	20.9	21%	20%	12%	46%
Hematuria	5.2%	12.2	12%	7%	10%	29%
Postoperative bleeding	29.8%	30.4	34%	–	55%	–
Thrombosis	3%	–	–	–	–	–

CNS central nervous system; *GI* gastrointestinal

[a] The incidence of menorrhagia has been reported in females aged >10 and <50 years and all of patients are female

[b] Only male

deficiency as part of the initial congenital bleeding disorders investigations is recommended [47]. Patients with plasma FVII levels <2% may present severe bleeding, while those with >20% are generally asymptomatic. Interestingly, bleeding can be observed among patients with plasma levels between 20% and 50%, while asymptomatic subjects with plasma levels <1% were also reported [11]. Prediction of hemorrhagic risk may not be possible, even in presence of laboratory assays such as thrombin generation test, FVIIa, and FVII antigen level (FVII:Ag) assays, and TFPI measurement [48]. CNS bleeding is a less common condition and is an important problem, mainly in children under 6 months with severe FVII deficiency and is associated with a high rate of morbidity and mortality [3, 11]. Bleeding episodes in FVII deficiency may mimic hemophilia (hemophilia type) with the presence of hemarthrosis and hematoma or may mimic primary hemostasis defects with menorrhagia, epistaxis or ecchymosis [11]. Based on available data, FVII deficiency does not provide protection against thrombotic events [49]. Accordingly, in addition to bleeding episodes, thrombotic events with the unknown mechanism (particularly deep vein thrombosis) also may occur in ~3% of patients with severe FVII deficiency, especially in the presence of prothrombotic risk factor such as recent surgical interventions, replacement therapy, immobilization, or trauma; however spontaneous thrombosis also may occur [49–51]. Although severe clinical events have been observed in homozygous or compound heterozygotes, the heterozygous are usually asymptomatic [2, 30].

11.6 Laboratory Approach to Congenital Factor VII Deficiency

The first case with congenital FVII deficiency was described by prolonged PT in 1951 [33]. The diagnosis is based on clinical presentations, physical examination, family history and laboratory assessments [44]. Occasionally, the disorder could be identified during routine work up. For a more precise diagnosis and to confirm the presence of the disease, the molecular diagnosis is strongly recommended [1].

11.6.1 Primary Tests for Diagnosis of FVII Deficiency

FVII deficiency is usually suspected by the presence of isolated prolonged PT which is corrected by 50:50 mixing of patient's plasma with normal pooled plasma. In this setting, the activated partial thromboplastin time (APTT), thrombin time (TT), fibrinogen concentration and platelet count are usually normal. Evaluation of FVII coagulant activity (FVII:C) (with twice repetition) leads to confirmation of disorder [3, 44]. In addition, unlike FX, the normal result of Russell viper venom (RVV) clotting time is also observed. Nonetheless, the normal result of RVV in FX Friuli (a variant of FX deficiency) should be considered [30, 52]. In general, the mainstay in the diagnosis of FVII deficiency is FVII:C assay.

11.7 Differential Diagnosis

FVII deficiency can be readily differentiated from acquired FVII deficiency due to the absence of family history in patients with acquired FVII deficiency. Moreover, patients with acquired FVII deficiency may also exhibit a history of normal PT results. Acquired combined FVII is usually observed during excessive consumption and/or inadequate production of FVII, resulting in a concomitant decrease in other vitamin K-dependent clotting factors. In such cases, in addition to PT, APTT would also be increased. Assessment of other coagulation factors (FII, FIX, and FX) may be useful for excluding cases with acquired combined FVII deficiency [35].

Exclusion of vitamin K deficiency or other acquired causes of clotting factor deficiencies is useful, but it is not necessary because concomitant prolongation of APTT is observed [3].

In patients with isolated FVII deficiency, isolated prolongation of PT occurs, and APTT is normal. However, a prolonged APTT might occur in some patients with the presence of lupus anticoagulant (LA). In this situation, an isolated FVII deficiency should be diagnosed by assessing FVII: C levels. In addition, acquired isolated FVII deficiency, may develop in the presence of autoantibody against FVII, severe infections, or malignancy. In such cases, prolonged PT is observed to be the same as congenital FVII deficiency. In this regard, family history, the absence of bleeding episodes, FVII: C assessment post-treatment, and the presence of severe infections or malignancy can help in the differential diagnosis [48, 53].

Evaluation of other vitamin-K dependent clotting factors might be helpful to rule out other disorders. For further investigation, a mixing study should be performed. After the mixing study, if PT was prolonged, the presence of a specific FVII inhibitor is suspected, and the Bethesda assay could confirm the presence of specific FVII inhibitor [36]. In this way, the absence of bleeding history, the presence of malignancy (or other underlying conditions), and the absence of a family history of congenital FVII deficiency could all be useful during the process of diagnosis. In order to rule out transient FVII deficiency, the test should be repeated in a couple of weeks. On the whole, clinical manifestation, family history, and laboratory investigation constitute the main basis of FVII diagnosis [1].

11.8 Functional Assays of FVII

In order to confirm FVII deficiency, the FVII activity level must be determined. FVII activity assays can be measured through clotting- or chromogenic-based methods.

11.8.1 The One Stage PT-Based FVII Activity Assay

FVII:C usually determined by one-stage prothrombin time-based assay [54].

Box 11.1 The Principle of the One-Stage PT-Based FVII Activity Assay
The assay relies upon measuring the extent of PT correction in FVII deficient-plasma, following the addition of the test plasma. In this assay, a serial dilutions of standard and patients' plasma (1/10, 1/20, 1/40, 1/80, etc.) are prepared. Typically, the 1/10 dilution is considered as having 100% factor activity. Standard curves are usually prepared using seven or eight dilutions. The equal volume of each dilution is then mixed with substrate plasma (FVII deficient-plasma) and incubated at 37 °C. Following the addition of the pre-warmed thromboplastin containing calcium, the clotting time for each dilution is recorded. The clotting times of both standard and patients are plotted on logarithmic paper (on Y-axis, the clotting time (seconds). On the X-axis, the FVII activity level (IU/dL)) is drawn. All the points of standard and patient are connected by drawing a line (Fig. 11.3) [4].

Interfering variables: The accuracy and precision of the test depend on the FVII-deficient plasma, calibration materials, and type of thromboplastin. It is recommended that the mean FVII activity in FVII-deficient plasma should be less than 1%. FVII-deficient plasma with more than this threshold results in the overestimation of the FVII:C, especially in patients with low FVII level. Indeed, even slight contamination of thromboplastin with FVIIa can decrease sensitivity to a patient's plasma FVII:C, while increasing sensitivity to the patient's plasma level of FV, FX, and prothrombin [1, 55]. In spite of the presence of certified reference materials for the accuracy of calibrators, the different calibration materials may exhibit variable inter-laboratory precision, especially in cases with an FVII level below 20% [1, 56, 57].

Three types of thromboplastin reagents with different sensitivity including rabbit brain, ox brain or human recombinant thromboplastin are available [54, 58].

According to the type of thromboplastin, different results might be obtained, however, variability that caused by qualitative FVII defects such as FVII Padua (Arg304Gln), FVII Nagoya (Arg364Trp), and FVII Tondabayashi, or Shinjo (Arg79Gln) are more profound. For example, in Padua variant which usually associates with no bleeding history and the normal range of FVII:Ag, disparate results toward different thromboplastins could be obtained, so that normal results using ox brain thromboplastin and abnormal results by use of rabbit brain thromboplastin might be obtained [1, 59]. It should be noted that variable reactivity of different thromboplastins only occurs in type II deficiency such as Padua, not type I deficiency. In FVII Padua or Nagoya, the FVII:C, when measured by rabbit brain thromboplastin and ox brain thromboplastin, are around 5% and 100% of normal, respectively [1, 59]. In this framework, based on structural similarity of recombinant thromboplastins and human TF, use of this product is more reliable than other thromboplastins for FVII assay. Although this approach can be suitable in certain situations, such as control of coumarin treatment, it is not an absolute rule. In cases of FVII Padua and FVII Nagoya, the level of FVII: C with human-origin

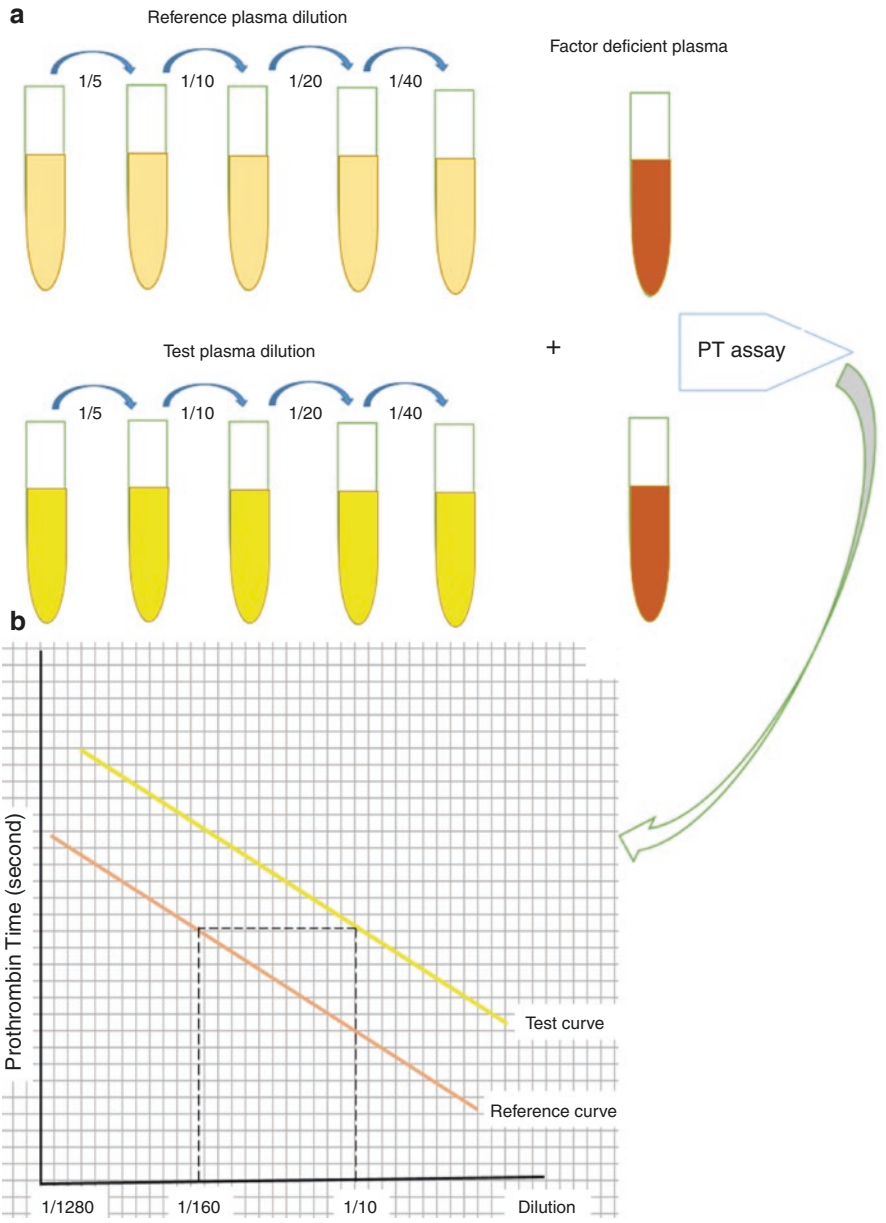

Fig. 11.3 One-stage PT-based factor VII activity assay. (**a**) Preparation of the serial dilutions of the standard and test plasma with factor VII deficient plasma. (**b**) Assessment of factor VII activity based on the reference curve

thromboplastin are about 35% of the normal levels which is different compared to other origins of thromboplastin [52, 60]. It seems that using a three-panel thromboplastin including rabbit brain, ox brain, and human origin thromboplastin is a robust approach to accurately identify all patients, especially those with qualitative FVII defect. Nevertheless, due to the high sensitivity of rabbit brain thromboplastin, it is recommended as a first-line screening test in cases with FVII defect [61]. More recently, a new variant of FVII (c.194C>G; p.Ala65Gly) has also found with discrepancies in its results when tested with different thromboplastin sources. In the corresponding study, FVII:C activity levels were found to be 4%, 28%, and 21%, respectively, using rabbit brain, recombinant human tissue factor, and human placental thromboplastin [62].

Finally, it should be noted that plasma FVII level is raised in some circumstances, such as female gender, increasing age and hyperlipidemia, especially hypertriglyceridemia [63].

Interpretation of the results: FVII deficiency is one of the RBDs with a poor correlation between FVII level and clinical picture. But on the whole, FVII activity less than 55% is considered a boundary for FVII deficiency. The relevant clinical picture usually develops in FVII:C <30% while FVII:C <2% is associated with an increased risk of severe hemorrhages. However, there are some cases with FVII<1% without spontaneous or provoked bleeding manifestations. In contrast, there are some patients with FVII:C >20% who experience severe bleeding [1, 64].

11.8.2 The Chromogenic Factor VII Assay

The chromogenic FVII assay is another approach for quantitatively measuring of FVII activity levels in citrated plasma.

Box 11.2 The Principle of Chromogenic Factor VII Assay
In this method, activated factor VII (FVIIa) triggers the activation of FX to FXa. A captured specific FVII antibody is coated onto wells. The test plasma is added to the microplate well, followed by an incubation period and subsequent washing. Captured FVII is activated to FVIIa upon the addition of a reagent containing TF (typically recombinant human TF), calcium, and FX. FX in the mixture is activated to FXa via TF/FVIIa complex during incubation. After the washing step, the specific FXa chromogenic substrate is added. The intensity of the FXa substrate, which is releases a yellow paranitroaniline (pNA) chromophore, is directly proportional to FVII activity.

Sample requirement: Blood samples should be collected into tube containing 3.8% citrate anticoagulant. The collected plasma could be stored in a plastic anticoagulant coated tube for up 4 h at room temperature and up to 24 h at 4 °C. The plasma sample can be kept at −20 °C for short and at −70 °C for long-term

storage. It should be considered that frozen plasma should be immediately thawed at 37 °C before testing.

11.9 Activated Factor VII Assay

In addition to the pivotal role of TF-FVIIa pathway in the initiation of the coagulation cascade, it has the important effect on the inflammatory pathway, regulation of inflammation and sepsis [31].

When recombinant activated FVII (rFVIIa) was introduced for the treatment of patients with inhibitor-complicated hemophilia, FVII deficiency, and also several other off-label indications such as retropubic prostatectomy bleeding, the interest for concentrates FVIIa assay was increased [1, 65, 66]. Although PT and FVII:C assay could also be used for monitoring of rFVIIa treatment, but as mentioned above, both of these tests have some limitations such as variability in results (mostly due to the source of thromboplastin), especially in a cross-reacting material-positive variants [67], even if different assays used thromboplastins with similar sensitivity, and high cost. Therefore, FVIIa assay may be more effective than PT and FVII:C for monitoring of these patients [1, 65]. FVIIa assay is not recommended for diagnosis of FVII deficiency [1, 65]. It should be noted that the level of FVIIa can be increased in some pathological conditions that are associated with coagulation activation, while it decreases during heparin therapy. The assessment of FVIIa could be performed by two methods including clotting-based and chromogenic-based assays.

11.9.1 The Clotting-Based Activated Factor VII Assay

The first method relies on PT-based clotting-based assay using recombinant soluble mutant TF molecule (sTF1–219), a TF without transmembrane and cytoplasmic domains. STF cannot activate FVII, but its FVIIa cofactor activity conserved [68, 69].

Box 11.3 The Principle of the Clotting-Based FVII Activity Assay
The truncated mutant TF molecule (sTF1–219) is the main material in the clotting-based FVIIa assay. The sTF1–219 is a recombinant TF without transmembrane and cytoplasmic domains, which is not able to activate FVII into FVIIa. However, it retains cofactor activity for FVIIa. Therefore, the presence of FVII in the plasma could not interfere with the assay. The citrated test plasma is diluted/mixed with FVII-deficient plasma. For the clotting assay, a reagent containing sTF, phospholipids, and CaCL2 is added, and the clotting time is recorded. The clotting time is converted to the FVIIa level using the standard curve. The clotting time is directly proportional to the FVIIa level. Recently, using highly purified and homogeneous sTF, a new modified method (HemoclotTM FVIIa), is developed. This method is more sensitive for FVIIa assay and can measure FVIIa in very low concentrations [1, 4].

Normal plasma FVIIa level using this technique is 0.5–8.4 ng/mL (mean 3.6 ng/mL), encompasses 1–3% of the total inactive zymogen form [3, 69], based on the specificity of the FVIIa assay [70].

Interfering variables: As mentioned above, in physiologic states, a small amounts of FVIIa are circulated in plasma. This quantity can increase in hypercoagulable states and certain pathological conditions associated with coagulation activation. However, during heparin therapy, FVIIa concentration can diminish [25, 53].

11.9.2 Chromogenic-Based Activated FVII Assays

The activated FVII on chromogenic-based assays is measured using enzyme-linked immunosorbent assay (ELISA) which is highly sensitive compared to clotting-based assays. When using this method, lower FVIIa concentration was reported compared to activity-based assay. However, a strong correlation is observed between the two methods. This method, use a high specific antibody against two-chain FVIIa that cannot reactive with FVII [69, 70]. The newly introduced chromogenic assay, BiophenTM FVIIa, utilizes sTF, which can attach specifically to FVIIa and then activate the FX to FXa. Following the addition of the chromogenic substrate releasing para-nitro-aniline (pNA), the FVIIa concentration is measured at 405 nM [53].

Reference range: approximately 0.0125 ng/mL (±0.01 ng/mL) [3].

11.10 Immunological Assay of Factor VII

The FVII:Ag can be determined by different methods including ELISA or immunoturbidimetric assays (IRMAs) using polyclonal or epitope-specific monoclonal antibodies against free-circulating FVII. Distinguishing between type I and II defects is feasible, using the FVII:Ag assay. The FVII:Ag level is not a good predictor of the severity of bleeding tendency, but it can aid in understanding of mutational mechanisms of FVII deficiency [1, 44].

Box 11.4 The Principle of Immunological Assay

Free-circulating FVII is sandwiched by the immobilized antibody specific for FVII. The biotinylated polyclonal antibody specific for another epitope of FVII is then added and followed by streptavidin-peroxidase conjugate that can be attached to the biotin antibody. Following the washing step, the OD is measured by a spectrophotometer after adding the peroxidase enzyme substrate.

11.11 Molecular Basis

Molecular studies have received noticeable attention in recent years and can reveal the disease's causative mutations. The poor correlation between the clinical picture and the FVII activity level makes molecular diagnosis useful for the exact diagnosis of disease as well as for prenatal diagnosis (PND) [71]. Moreover, molecular diagnosis can be used for the classification of FVII deficiency, especially in patients with qualitative defects. The *F7* gene spans 12.8 kb on chromosome 13q34 and contains nine exons and five short tandem repeats. These minisatellite DNA sequences cover more than a quarter of the introns sequence and more than one-third of 3′ untranslated region (UTR (of mRNA. The *F7* gene is located approximately 2.8 kb upstream of the *F10* gene and located near another vitamin K-dependent protein *Z* gene [5, 19, 30, 72]. The *F7* gene and protein are structurally homologous to other vitamin K-dependent coagulation factors, particularly FIX, FX, and protein C. The overall base compositions of the *F7* gene in exons and introns are similar (60% G-C and 40% A-T), which is similar to the protein C and the *F10* genes [19]. *F7* gene consists of nine exons. Exon 1 and 2 (classically exon 1b; an alternatively spliced target in 90% of factor VII mRNA transcripts) and a part of exon 3 encode 5′ UTR and a main part of the pre-pro leader. The Gla domain is encoded by exon 3. Exon 4 encodes the hydrophobic aromatic stack, exons 5 and 6 encode two epidermal-like growth factor (EGF) domains, exons 7 and 8 responsible for the encoding of the activation region, and finally, exon 9 encodes the catalytic domain as well as 3′ UTR including poly (A) tail (Fig. 11.4) [3].

A wide spectrum of mutations have been identified within the *F7* gene, and whole gene sequencing including exons, introns, boundaries, and promoter regions is recommended for mutation detection in patients with congenital FVII deficiency. This is mostly due to a large number of identified mutations within the *F7* gene, the short length of the gene and merely the possibility of the detection of a recurrence mutation [1]. In general, 90–92% of mutated alleles could be identified with the current routine direct sequencing methods, while ~10% of gene mutation could not be found. Although new techniques, such as next-generation sequencing (NGS) certainly can improve this situation, however, some of the cases with congenital FVII deficiency may result from mutations in other genes, which can make FVII deficiency still an open question [1, 22]. A wide spectrum of normal gene variations and disease-causing mutations, including missense, nonsense, splice site mutations, and insertions/deletions have been observed in the *F7* gene. Several functional and nonfunctional polymorphisms have also been observed (Table 11.3) [3, 22, 73]. For example, functional promoter polymorphism at position −402 (G > A) of the ATG codon leads to increased FVII:C, while promoter polymorphism at position −401 (G > T) decreases plasma FVII level [74]. Arg413Gln substitution in exon 8 (classically known as R353Q variant) arises from polymorphisms including G to A substitution at nucleotide 10,976 and is commonly found in association with the polymorphism of decanucleotide (10-bp sequence) insertion at position −323 in the 5′-flanking region of the *F7* gene. Arg403Gln, result in 30% and 23% of the variance in FVII:C and FVII:Ag, respectively, while the decanucleotide polymorphism

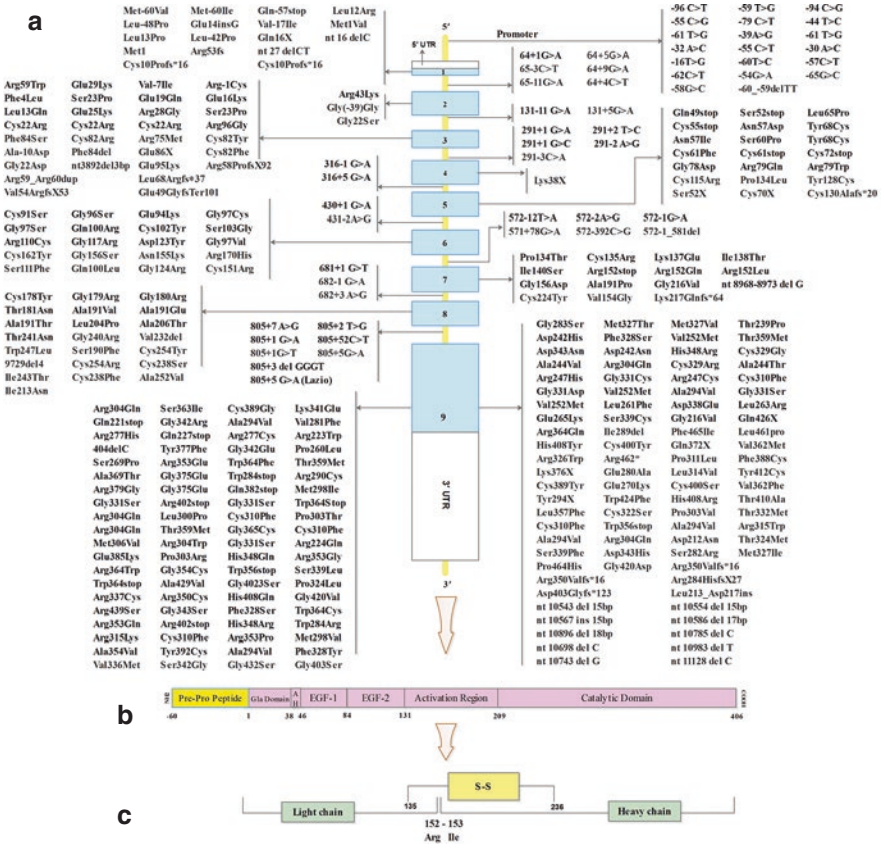

Fig. 11.4 *F7* gene and FVII protein structures and spectrum of gene mutations in *F7* gene. (**a**) *F7* gene contains nine exons that encode FVII protein. Exon 2 is usually alternatively spliced in 90% of the FVII mRNA transcript. (**b**) The FVII protein contains pre-pro sequence, Gla, EGF1, EGF2, and catalytic serine protease domains. (**c**) Cleavage at Arg152-Ile153 location leads to generation of a two-chain molecule that joins together by a disulfide bond between Cys135 and Cys236. Light chain contains residues +1 to 152 and heavy chain contains residues 153–406. *FVII* factor VII; *Gla* gamma-carboxyglutamate acid; *EGF* epidermal growth factor1

brings about 26% and 23% of the variance in FVII:C and FVII:Ag, respectively [3, 74, 75]. The *F7* gene has five short tandem repeats (STR) that span a quarter of the intron sequence and more than one-third of the 3′ untranslated region of mRNA. A small part of one of these STRs is located on the end of exon 8 (including 11 nucleotides "CGCGGTGCTGG"). This sequence in wild-type exon has 6 repeats with 26 spaces between them (795_805 + 26 [6]). Three polymorphisms with 6, 7, and 8 sequence repetition may be seen during direct sequencing in this location. The presence of STR, repeated regions, and polymorphism variation of repeats within the *F7* gene may be associated with misdiagnosis that requires special attention to design the PCR primers and the use of other ways than conventional polymerase chain reaction (PCR)-based techniques such as semi-quantitative multiplex

Table 11.3 Factor VII gene polymorphisms>

Polymorphism type	Location	MAF	Effect on FVII:C	Comment
Decanucleotide [CCTATATCCT] inser[a]	5′ region (−323)	0.23	Decrease	In linkage disequilibrium with p.Arg413Gln
G/T dimorphism	5′ region (−401)	0.2041	Decrease	In complete allelic association with c.-323ins10 and c.-122C
G/A dimorphism	5′ region (−402)	0.2326	Increase	–
c.64+9G>A (G73A)[a]	Intron 1	0.2096	Decrease	In linkage disequilibrium with p.Arg413Gln
c.525C>T (his 115=)	Exon 6	0.1419	–	–
VNTR repeat (37 bp monomer repeat, 9716ins)[b]	Intron 8	–	Decrease	VNTR[6] is the Wild Type
c.806-20G>A (G/A dimorphism)	Intron 8	0.1342	–	
c.1238G>A (Arg353Gln polymorphism)[a]	Exon 9	0.80 0.20	Decrease	In linkage disequilibrium with the c.-401 T/c.-325_-324ins10/c.-122C haplotype

MAF minor allele frequency; *NM* not mentioned. [a] The 10 bp insertion and the Arg353Gln polymorphism indicate a strong linkage disequilibrium, and therefore it is not clear whether the 73A allele or 10 bp insertion contributed per se to lowering FVII:C. [b] The high mRNA expression in quantitative mRNA analysis has shown that this polymorphism probably is associated with increased plasma FVII level, although there are contradictory results in this regard

fluorescent-PCR (SQF-PCR), multiplex ligation-dependent probe amplification or multiplex amplification and probe hybridization, especially in patients who have a discrepancy between genotype and FVII:C level or those that present the discrepancies in the inheritance pattern [76, 77]. For instance, in another study, two patients with the previously known homozygous mutation were further studied by SQF-PCR and were revealed that they carry a novel heterozygous large genomic rearrangement. As mentioned above, in cases with confounding results, these uncommon techniques may be useful [76].

According to databases available at https://f7-db.eahad.org/, and http://www.factorvii.org/index.php as well as our literature review [78], most of the mutations in the *F7* gene, similar to other congenital bleeding disorders are point mutation., however, in spite of the presence of some minor different in mutations number and percentage, in general, missense mutations are the most frequent while nonsense mutations are the rarest mutations. Exon 9 as the largest exon (1.6 kb) [19] in the *F7* gene that is responsible for encoding the catalytic domain has a considerable number of mutations (Fig. 11.5).

Prenatal diagnosis (PND) can be used in patients with congenital FVII deficiency, but it is more suitable for those families with severe factor deficiency and a history of life-threatening bleeding such as ICH [1, 79].

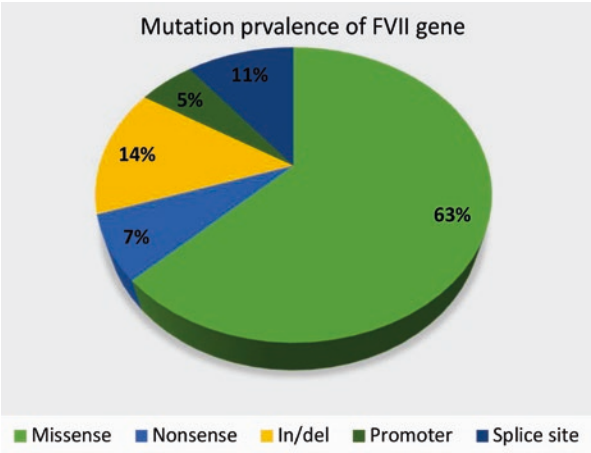

Fig. 11.5 Mutation prevalence of factor VII (FVII) gene in its exons. Most common mutations of FVII gene are missense mutations (79%), while small Ins/Del (9%), splice sites (8%) and nonsense (4%) mutations form others mutations in *F7* gene

11.12 Management

Due to highly variable clinical presentations and the low correlation between the severity of clinical presentations and FVII:C level, bleeding risk prediction and management of these patients remains controversial. The mainstay of treatment in patients with congenital FVII deficiency is on-demand replacement therapy which means the stop of bleeding as soon as possible after the occurrence of bleeding. It should be borne in mind that in the setting of high-risk bleeding conditions such as childbirth or surgery, this rule still holds, and thereby clinical history should be considered as an indicator of the need for prophylactic treatment. Nonetheless, there are very low reports about the use of rFVIIa in women with FVII deficiency who experienced vaginal delivery or cesarean and therefore don't exist definite guidelines to help caregivers to manage this process. However, in pregnant women with known severe FVII deficiency, rFVIIa administration is suggested during delivery (both vaginal and cesarean) and the decision about prophylaxis in cases with mild-moderate FVII deficiency is depending on bleeding tendency, multiple gestations, type of delivery, and PT or FVII levels in the third trimester. On the whole, management of delivery should be addressed on a case-by-case. In these situations, the efficient use of rFVIIa (with different treatment protocols) before delivery to prevent postpartum hemorrhage, has been shown. Due to the lack of specific guidelines to point to the specific dose and duration of treatment during delivery, the same dose of hemorrhage treatment is used for hemorrhagic events [80, 81].

In patients with a history of life-threatening bleeding such as ICH, secondary prophylaxis is recommended. Primary prophylaxis could be used for those patients

Table 11.4 Available therapeutic options for patients with factor VII deficiency

Factor	Advantage	Disadvantage
FFP	Easily available, cheap	Limited effectiveness, need to high volumes for treatment, fluid overload, risk of viral transmission
Pd-FVII	Effective; suitable for surgery	Unavailable in some countries, other vitamin K-dependent factors concentrations are higher than factor VII, risk of viral transmission, risk of TE
rFVIIa	Very effective Low dosage requirement for treatment No risk of viral transmission Not immunogenic in patients with hemophilia Not produces an anamnestic response in hemophilia patients with inhibitors Very low thrombogenicity	Risk of TE, expensive
PCC	Suitable for surgery	Risk of TE, concentration of other vitamin K-dependent factors is higher than factor VII and presence of activated factors. Variable amount of factor VII

Pd-FVII plasma-derived FVII; *FFP* fresh frozen plasma; *rFVIIa* recombinant FVIIa; *TE* thrombotic events; *PCC* prothrombin complex concentrates

with severe factor deficiency and risk of life-threatening bleeding. Different therapeutic choices including FFP, pd-FVII, PCC, aPCC, and rFVIIa are available for patients with FVII deficiency (Table 11.4) [1, 30]. In this setting, Pd-FVII and rFVIIa are the most commonly used treatments. However, in low-incoming countries, where access to these products is limited, FFP and PCC are also used, although they contain low levels of FVII [82].

The recommended dose and therapeutic target levels of FVII for on-demand, prophylaxis and surgeries are summarized in Table 11.5 [83].

rFVIIa (eptacog alfa) is a structurally similar product to plasma-derived coagulation factor VIIa but is manufactured using DNA biotechnology [84, 85]. The first report of successful treatment with rFVIIa was in 1988 with Novoseven® (rFVIIa; NovoSeven, Novo Nordisk, Copenhagen, Denmark) in a patient with severe haemophilia A during synovectomy [86]. Another generic rFVIIa, Aryoseven™, is claimed to have bio-similarity with Novoseven® as well as similar clinical safeness and effectiveness [87–89]. rFVIIa has been approved for the treatment of patients with congenital FVII deficiency, congenital hemophilia B with high-responding inhibitors, acquired hemophilia, and Glanzmann thrombasthenia with refractoriness to platelet transfusions, with or without antibodies to platelets and also recommended as the first-line therapeutic option for hemophilia A patients with high-responder inhibitors [84, 85]. rFVIIa has also been used in surgical bleeding caused by dilutional or consumptive coagulopathies or in patients with impaired liver function

Table 11.5 Recommended dose and therapeutic target levels for factor VII on on-demand and prophylaxis approaches in patients with factor VII deficiency

Recommended and Maintaining level	Plasma half-life	On-demand dosages	Major surgery	Minor surgery	Long-term prophylaxis dosages
>20%	2–4 h	Pd-FVII concentrate (30–40 U/kg)	rFVIIa Before surgery: 15–30 μg/kg After surgery: continue the same dose for first day with 4–6 h interval, and then change interval to 8–12 h or Pd-FVII concentrate: 8–40 IU/kg with similar intervals	Tranexamic acid 15–20 mg/kg or 1 g four times daily or Antifibrinolytics[a]	FFP: 10–15 mL/kg two times/week Pd-FVII: 30–40 U/kg three times/week rFVIIa: 20-30 μg/kg two or three times/week or a total weekly dose of 90 μg/kg with three times/week

Pd-FVII plasma-derived FVII; *FFP* fresh frozen plasma; *rFVIIa* recombinant FVIIa
[a] This recommendation need to further research because the quality of evidence is moderate

[90]. In addition, the rFVIIa can be used in various conditions, such as spontaneous bleeding, hemarthrosis, and major surgical procedures. However, it is also necessary to mention that one of the most important challenges of administering rFVIIa is inhibitor development [91]. According to FDA report, the risk of thrombotic events associated with rFVIIa is 2% of treated patients in rFVIIa clinical trials. More recently rFVIIa, eptacog beta (brand name SEVENFACT®, LFB Biotechnologies, Hema Biologics), has been approved by the FDA in 2020 for on-demand treating bleeding episodes in patients >12 to 75 years of age with hemophilia A or B complicated by inhibitors [92, 93]. Marzeptacog alfa (MarzAA), has passed phase 1/2/3, an open-label clinical trial study for the prophylaxis treatment of non-bleeding patients with congenital hemophilia A or B with or without inhibitors [67, 93]. However, the very low frequency of thrombotic events, no virus transmission and scarce production of inhibitory antibodies are advantages of rFVIIa, while expensiveness and short half-life of rFVIIa even than FVII and FVIIa are disadvantages of rFVIIa [1].

PCC is another therapeutic choice for patients with FVII deficiency. It usually includes FII (prothrombin), FIX, FX, and the varying amounts of FVII. In general, 2 commercially types of PCC are available, including 3-factor PCC (with absent or low levels of FVII) and 4-factor PCC (with high level of FVII) (Table 11.6), and also another form is activated PCC (factor eight inhibitor bypass activity; FEIBA), contains 4-factors which includes both inactive (FII, FIX, and FX) and active (FVII) forms [94–96]. The amount of FVII is variable in different manufactured PCC that

Table 11.6 Characteristics of some available four factor prothrombin complex concentrates (PCC)

PCC	Manufacturer/ country	Hemophilia B (factor IX)	Acquired deficiency (e.g., VKA)	Congenital deficiency (specific factor not available)	Other factor deficiency, such as factor II, VII, X	Reference
Beriplex P/N	CSL Behring GmbH/Germany		√	√		[105]
Kcentra[a]	CSL Behring GmbH/Germany		√			[106]
Cofact	Sanquin/ Netherlands		√	√		[107]
Kaskadil	LFB/France		√		√ (FII & FX)	[108]
Octaplex	Octapharma/ Vienna, Austria		√		√ (FII & FX)	[109]
Prothromplex Total 600 IU	Baxalta innovations GmbH/Vienna, Austria		√	√		[110]
Proplex T[b]	Baxter/ Glendale, USA	√			√ (FVII)	[111]

PCC prothrombin complex concentrates; *VKA* vitamin K antagonists; *FIX* factor IX; *FII* factor II; *FVII* factor VII; *FX* factor X

[a] Kcentra is indicated for the urgent reversal of acquired coagulation factor deficiency induced by vitamin K antagonists therapy in adults patients during acute major bleeding (It does not have indication in patients without acute major bleeding)

[b] Proplex T is indicated for treatment of bleeding episodes in patients with factor VIII deficiency with inhibitors

usually indicated by the manufacturer, thus after requirement calculation could be administered [3]. Some PCC may contain additional components such as anticoagulants, protein C, protein S, protein Z, and antithrombin III as well as heparin to mitigate thrombotic risk [95, 97, 98]. Overall clotting factors of these concentrates are approximately 25 times higher than normal plasma [99]. Some advantages of PCC over the FFP include a relatively constant high level of vitamin K-dependent coagulation factors (FII, FVII, FIX, and FX), a more rapid decrease in INR value, and no need for matching the blood groups or thaw the product [96, 100]. Several reports indicated both venous and arterial thrombosis associated with PCC, therefore, these concentrates should not be used in patients with liver disease, major trauma, or neonates (due to their relatively immature livers). The incidence of thrombotic events in patients treated with 4-factor and 3-factor PCC is 1.8% and 0.7%, respectively [96]. Another disadvantage of PCC is a high concentration of other vitamin K-dependent factors than FVII [1, 30, 101–103].

The pd-FVII is a useful product for prophylaxis in children with severe FVII deficiency and for long-term prophylaxis in the range of 30–40 U/Kg, three times a week. Various doses of Pd-FVII have successfully been used for surgery, ranging from 8 to 40 U/Kg every 4–6 h. For major surgeries, FVII level must be kept above

20 U/dL. Similar to PCC, pd-FVII has a lower FVII concentration than other vitamin K-dependent coagulation factors [3, 30, 104]. Acquired FVII deficiency is usually treated as same as inherited FVII deficiency by FFP, PCC, aPCC, and pd-FVII or rFVIIa. However, in these cases, the underlying diseases should be treated.

References

1. Sevenet P-O, Kaczor DA, Depasse F. Factor VII deficiency: from basics to clinical laboratory diagnosis and patient management. Clin Appl Thromb Hemost. 2016;23:1076029616670257.
2. Herrmann F, Wulff K, Auerswald G, Schulman S, Astermark J, Batorova A, et al. Factor VII deficiency: clinical manifestation of 717 subjects from Europe and Latin America with mutations in the factor 7 gene. Haemophilia. 2009;15(1):267–80.
3. Perry DJ. Factor VII deficiency. Br J Haematol. 2002;118(3):689–700.
4. Shams M, Dorgalaleh A. Congenital factor VII deficiency. Congenital bleeding disorders. Springer; 2018. p. 239–59.
5. Lapecorella M, Mariani G, Deficiency IRoCFV. Factor VII deficiency: defining the clinical picture and optimizing therapeutic options. Haemophilia. 2008;14(6):1170–5.
6. Peyvandi F, James P, Salomon O, Mikovic D. Rare bleeding disorders. Haemophilia. 2014;20(4):71.
7. Mariani G, Herrmann FH, Dolce A, Batorova A, Etro D, Peyvandi F, et al. Clinical phenotypes and factor VII genotype in congenital factor VII deficiency. Thromb Haemost. 2005;93(3):481.
8. Mackman N. The role of tissue factor and factor VIIa in hemostasis. Anesth Analg. 2009;108(5):1447.
9. Rosen ED, Chan JC, Idusogie E, Clotman F. Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. Nature. 1997;390(6657):290.
10. Peltier S, Kellum A, Brewer J, Duncan A, Cooper DL, Saad H. Psychosocial impact and disease management in patients with congenital factor VII deficiency. J Blood Med. 2020;11:297.
11. Moerloose P, Schved JF, Nugent D. Rare coagulation disorders: fibrinogen, factor VII and factor XIII. Haemophilia. 2016;22(S5):61–5.
12. Smith SA, Travers RJ, Morrissey JH. How it all starts: initiation of the clotting cascade. Crit Rev Biochem Mol Biol. 2015;50(4):326–36.
13. Hussain MA, Abogresha NM, Hassan R, Tamany DA, Lotfy M. Effect of feeding a high-fat diet independently of caloric intake on reproductive function in diet-induced obese female rats. Arch Med Sci. 2016;12(4):906.
14. Neuenschwander PF, Morrissey JH. Roles of the membrane-interactive regions of factor VIIa and tissue factor. The factor VIIa Gla domain is dispensable for binding to tissue factor but important for activation of factor X. J Biol Chem. 1994;269(11):8007–13.
15. Tavoosi N, Smith SA, Davis-Harrison RL, Morrissey JH. Factor VII and protein C are phosphatidic acid-binding proteins. Biochemistry. 2013;52(33):5545–52.
16. Fair DS. Quantitation of factor VII in the plasma of normal and warfarin-treated individuals by radioimmunoassay. Blood. 1983;62(4):784–91.
17. Vadivel K, Bajaj SP. Structural biology of factor VIIa/tissue factor initiated coagulation. Front Biosci. 2012;17:2476.
18. Eigenbrot C, Kirchhofer D, Dennis MS, Santell L, Lazarus RA, Stamos J, et al. The factor VII zymogen structure reveals reregistration of β strands during activation. Structure. 2001;9(7):627–36.
19. O'Hara PJ, Grant FJ, Haldeman BA, Gray CL, Insley MY, Hagen FS, et al. Nucleotide sequence of the gene coding for human factor VII, a vitamin K-dependent protein participating in blood coagulation. Proc Natl Acad Sci. 1987;84(15):5158–62.

20. Banner DW, D'Arcy A, Chène C, Winkler FK, Guha A, Konigsberg WH, et al. The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. Nature. 1996;380(6569):41–6.
21. Butenas S, Mann KG. Kinetics of human factor VII activation. Biochemistry. 1996;35(6):1904–10.
22. McVey JH, Boswell E, Mumford AD, Kemball-Cook G, Tuddenham EG. Factor VII deficiency and the FVII mutation database. Hum Mutat. 2001;17(1):3–17.
23. Spiezia L, Campello E, Dalla Valle F, Woodhams B, Simioni P. Factor VIIa-antithrombin complex: a possible new biomarker for activated coagulation. Clin Chem Lab Med. 2017;55(4):484–8.
24. Rao L, Nordfang O, Hoang A, Pendurthi U. Mechanism of antithrombin III inhibition of factor VIIa/tissue factor activity on cell surfaces. Comparison with tissue factor pathway inhibitor/factor Xa-induced inhibition of factor VIIa/tissue factor activity. Blood. 1995;85(1):121–9.
25. Monroe DM, Key N. The tissue factor–factor VIIa complex: procoagulant activity, regulation, and multitasking. J Thromb Haemost. 2007;5(6):1097–105.
26. Iakhiaev A, Pendurthi UR, Voigt J, Ezban M, Rao LVM. Catabolism of factor VIIa bound to tissue factor in fibroblasts in the presence and absence of tissue factor pathway inhibitor. J Biol Chem. 1999;274(52):36995–7003.
27. Lwaleed BA, Bass PS. Tissue factor pathway inhibitor: structure, biology and involvement in disease. J Pathol. 2006;208(3):327–39.
28. Rosen ED, Xu H, Liang Z, Martin JA, Suckow M, Castellino FJ. Generation of genetically-altered mice producing very low levels of coagulation factor VII. Thromb Haemost. 2005;94(3):493–7.
29. Tuddenham EGD, Pemberton S, Cooper DN. Inherited factor VII deficiency: genetics and molecular pathology article in thrombosis and haemostasis. Thromb Haemost. 1995;74(1):313–21.
30. Mariani G, Bernardi F. Factor VII deficiency. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2009.
31. Versteeg HH, Peppelenbosch MP, Spek CA. Tissue factor signal transduction in angiogenesis. Carcinogenesis. 2003;24(6):1009–13.
32. Krikun G, Schatz F, Taylor H, Lockwood CJ. Endometriosis and tissue factor. Ann N Y Acad Sci. 2008;1127(1):101–5.
33. Alexander B, Goldstein R, Landwehr G, Cook C, Addelson E, Wilson C. Congenital SPCA deficiency: a hitherto unrecognized coagulation defect with hemorrhage rectified by serum and serum fractions. J Clin Investig. 1951;30(6):596.
34. Triplett DA, Brandt JT, Batard M, Dixon J, Fair DS. Hereditary factor VII deficiency: heterogeneity defined by combined functional and immunochemical analysis. Blood. 1985;66(6):1284–7.
35. Girolami A, Santarossa C, Cosi E, Ferrari S, Lombardi AM. Acquired isolated FVII deficiency: an underestimated and potentially important laboratory finding. Clin Appl Thromb Hemost. 2016;22(8):705–11.
36. Mulliez SM, Devreese KM. Isolated acquired factor VII deficiency: review of the literature. Acta Clin Belg. 2016;71(2):63–70.
37. Moosavi L, Bowen J, Coleman J, Heidari A, Cobos E. Acute myelogenous leukemia with trisomy 8 and concomitant acquired factor VII deficiency. J Investig Med High Impact Case Rep. 2019;7:2324709619872657.
38. Toor A, Slungaard A, Hedner U, Weisdorf D, Key N. Acquired factor VII deficiency in hematopoietic stem cell transplant recipients. Bone Marrow Transplant. 2002;29(5):403.
39. Zaidi MH, Stanley A, Khan M. Acquired factor VII deficiency–a rare but important consideration. Scott Med J. 2019;64(3):119–22.
40. Biron C, Bengler C, Gris J, Schved J. Acquired isolated factor VII deficiency during sepsis. Pathophysiol Haemost Thromb. 1997;27(2):51–6.

41. Delmer A, Horellou M, Andreu G, Lecompte T, Rossi F, Kazatchkine M, et al. Life-threatening intracranial bleeding associated with the presence of an antifactor VII autoantibody. Blood. 1989;74(1):229–32.
42. Abu-Quider A, Asleh M, Fruchtman Y, Ben-Harosh M, Beck G, Abuhasira R, et al. Factor VII deficiency in patients receiving chronic packed cell transfusions. J Pediatr Hematol Oncol. 2021;43(2):e268–e71.
43. Napolitano M, Giansily-Blaizot M, Dolce A, Schved JF, Auerswald G, Ingerslev J, et al. Prophylaxis in congenital factor VII deficiency: indications, efficacy and safety. Results from the Seven Treatment Evaluation Registry (STER). Haematologica. 2013;98(4):538–44.
44. Napolitano M, Siragusa S, Mariani G. Factor VII deficiency: clinical phenotype, genotype and therapy. J Clin Med. 2017;6(4):38.
45. Mariani G, Mazzucconi M. Factor VII congenital deficiency. Pathophysiol Haemost Thromb. 1983;13(3):169–77.
46. Peyvandi F, Mannucci P, Asti D, Abdoullahi M, Di Rocco N, Sharifian R. Clinical manifestations in 28 Italian and Iranian patients with severe factor VII deficiency. Haemophilia. 1997;3(4):242–6.
47. Trillo A, Kronenfeld R, Simms-Cendan J, Davis JA, Corrales-Medina FF. High prevalence of congenital factor VII (FVII) Deficiency in adolescent females with heavy menstrual bleeding and iron Deficiency anemia. J Pediatr Adolesc Gynecol. 2022;35(6):647–52.
48. Giansily-Blaizot M, Verdier R, Biron-Adreani C, Schved J-F, Bertrand M, Borg J, et al. Analysis of biological phenotypes from 42 patients with inherited factor VII deficiency: can biological tests predict the bleeding risk? Haematologica. 2004;89(6):704–9.
49. Singh B, Modi V, Kaur P, Guron G, Maroules M. Unprovoked pulmonary embolism in factor VII deficiency. Acta Haematol. 2020;143(2):181–3.
50. Mariani G, Herrmann F, Schulman S, Batorova A, Wulff K, Etro D, et al. Thrombosis in inherited factor VII deficiency. J Thromb Haemost. 2003;1(10):2153–8.
51. Marty S, Barro C, Chatelain B, Fimbel B, Tribout B, Reynaud J, et al. The paradoxical association between inherited factor VII deficiency and venous thrombosis. Haemophilia. 2008;14(3):564–70.
52. Girolami A, Cosi E, Santarossa C, Ferrari S, Randi ML. The story of serum prothrombin conversion accelerator, proconvertin, stable factor, cothromboplastin, prothrombin accelerator or autoprothrombin I, and their subsequent merging into factor VII. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2015.
53. Amiral J, Dunois C, Amiral C, Seghatchian J. The various assays for measuring activity states of factor VIIa in plasma and therapeutic products: diagnostic value and analytical usefulness in various pathophysiological states. Transfus Apher Sci. 2017;56(1):91–7.
54. Poggio M, Tripodi A, Mariani G, Mannucci PM. Factor VII clotting assay: influence of different thromboplastins and factor VII-deficient plasmas. CISMEL Study Group. Thromb Haemost. 1991;65(2):160–4.
55. Smith S, Comp P, Morrissey J. Traces of factor VIIa modulate thromboplastin sensitivity to factors V, VII, X, and prothrombin. J Thromb Haemost. 2006;4(7):1553–8.
56. Zantek N, Hsu P, Refaai M, Ledford-Kraemer M, Meijer P, Van Cott E. Factor VII assay performance: an analysis of the North American specialized coagulation laboratory association proficiency testing results. Int J Lab Hematol. 2013;35(3):314–21.
57. Takamiya O, Ishikawa S, Ohnuma O, Suehisa H, Iijima K, Kayamori Y, et al. Japanese collaborative study to assess inter-laboratory variation in factor VII activity assays. J Thromb Haemost. 2007;5(8):1686–92.
58. Bleavins MR, Carini C, Jurima-Romet M, Rahbari R. Biomarkers in drug development: a handbook of practice, application, and strategy. Wiley; 2011.
59. Girolami A, Fabris F, Dal Bo ZR, Ghiotto G, Burul A. Factor VII Padua: a congenital coagulation disorder due to an abnormal factor VII with a peculiar activation pattern. J Lab Clin Med. 1978;91(3):387–95.

60. Girolami A, Treleani M, Scarparo P, Bonamigo E, Lombardi AM. Considerations on a tentative classification of FVII deficiency suited for practical clinical purposes. Clin Appl Thromb Hemost. 2012;18(6):654–7.
61. Kirkel D, Lin T-W, Fu SW, Dlott JS, Sahud MA, McCaffrey T, et al. Asymptomatic factor VII deficiency: gene analysis and structure–function relationships. Blood Coagul Fibrinolysis. 2010;21(1):91–4.
62. Gallardo CA, Wong LJL, Sum CLL, Goh LL, Ong KH. Compound heterozygous factor VII deficiency c.1025G>A p.(Arg342Gln) with novel missense variant c.194C>G p.(Ala65Gly). J Hematol. 2022;11(1):29.
63. Simpson H, Meade T, Stirling Y, Mann J, Chakrabarti R, Woolf L. Hypertriglyceridaemia and hypercoagulability. Lancet. 1983;321(8328):786–90.
64. Shams M, Dorgalaleh A, Safarian N, Emami AH, Zaker F, Tabibian S, et al. Inhibitor development in patients with congenital factor VII deficiency, a study on 50 Iranian patients. Blood Coagul Fibrinolysis. 2019;30(1):24–8.
65. Cid A, Lorenzo J, Haya S, Montoro J, Casana P, Aznar J. A comparison of FVII: C and FVIIa assays for the monitoring of recombinant factor VIIa treatment. Haemophilia. 2001;7(1):39–41.
66. Friederich PW, Henny CP, Messelink EJ, Geerdink MG, Keller T, Kurth K-H, et al. Effect of recombinant activated factor VII on perioperative blood loss in patients undergoing retropubic prostatectomy: a double-blind placebo-controlled randomised trial. Lancet. 2003;361(9353):201–5.
67. Gruppo R, Malan D, Kapocsi J, Nemes L, Hay C, Boggio L, et al. Phase 1, single-dose escalating study of marzeptacog alfa (activated), a recombinant factor VII a variant, in patients with severe hemophilia. J Thromb Haemost. 2018;16(10):1984–93.
68. Neuenschwander PF, Morrissey JH. Deletion of the membrane anchoring region of tissue factor abolishes autoactivation of factor VII but not cofactor function. Analysis of a mutant with a selective deficiency in activity. J Biol Chem. 1992;267(20):14477–82.
69. Morrissey JH, Macik BG, Neuenschwander PF. Quantitation of activated factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. Blood. 1993;81(3):734–44.
70. Philippou H, Adami A, Amersey RA, Stubbs PJ, Lane DA. A novel specific immunoassay for plasma two-chain factor VIIa: investigation of FVIIa levels in normal individuals and in patients with acute coronary syndromes. Blood. 1997;89(3):767–75.
71. Tabibian S, Shams M, Naderi M, Dorgalaleh A. Prenatal diagnosis in rare bleeding disorders—an unresolved issue? Int J Lab Hematol. 2018;40(3):241–50.
72. Colman RW. Hemostasis and thrombosis: basic principles and clinical practice. Lippincott Williams & Wilkins; 2006.
73. Peyvandi F, Garagiola I, Palla R, Marziliano N, Mannucci P. Role of the 2 adenine (g. 11293_11294insAA) insertion polymorphism in the 3′ untranslated region of the factor VII (FVII) gene: molecular characterization of a patient with severe FVII deficiency. Hum Mutat. 2005;26(5):455–61.
74. van't Hooft FM, Silveira A, Tornvall P, Iliadou A, Ehrenborg E, Eriksson P, et al. Two common functional polymorphisms in the promoter region of the coagulation factor VII gene determining plasma factor VII activity and mass concentration. Blood. 1999;93(10):3432–41.
75. Giansily-Blaizot M, Rallapalli PM, Perkins SJ, Kemball-Cook G, Hampshire DJ, Gomez K, Ludlam CA, McVey JH. The EAHAD blood coagulation factor VII variant database. Human Mutation. 2020;41(7):1209–19.
76. Giansily-Blaizot M, Thorel D, Khau Van Kien P, Behar C, Romey MC, Mugneret F, et al. Characterisation of a large complex intragenic re-arrangement in the FVII gene (F7) avoiding misdiagnosis in inherited factor VII deficiency. Br J Haematol. 2007;138(3):359–65.
77. Hewitt J, Ballard JN, Nelson TN, Smith VC, Griffiths TA, Pritchard S, et al. Severe FVII deficiency caused by a new point mutation combined with a previously undetected gene deletion. Br J Haematol. 2005;128(3):380–5.

78. Dorgalaleh A, Bahraini M, Shams M, Parhizkari F, Dabbagh A, Naderi T, et al. Molecular basis of rare congenital bleeding disorders. Blood Rev. 2022:101029.
79. Farah R, Al Danaf J, Braiteh N, Costa JM, Farhat H, Mariani G, et al. Life-threatening bleeding in factor VII deficiency: the role of prenatal diagnosis and primary prophylaxis. Br J Haematol. 2015;168(3):452–5.
80. Loddo A, Cornacchia S, Cane FL, Barcellona D, Marongiu F, Melis GB, et al. Prophylaxis of peripartum haemorrhage using recombinant factor VIIa (rfVIIa) in pregnant women with congenital factor VII deficiency: a case report and literature review. Eur J Obstet Gynecol Reprod Biol. 2019;235:77–80.
81. Lee EJ, Burey L, Abramovitz S, Desancho MT. Management of pregnancy in women with factor VII deficiency: a case series. Haemophilia. 2020;26(4):652–6.
82. Şenol BK, Zülfikar B. Clinical problems and surgical interventions in inherited factor VII deficiency. Turk Arch Pediatr. 2020;55(2):184.
83. Peyvandi F, Menegatti M. Treatment of rare factor deficiencies in 2016. ASH Educ Program Book. 2016;2016(1):663–9.
84. Asif M, Siddiqui A, Scott LJ. Recombinant factor VIIa (eptacog alfa): a review of its use in congenital or acquired haemophilia and other congenital bleeding disorders. Drugs. 2005;65(8):1161–78.
85. Croom KF, McCormack PL. Recombinant factor VIIa (eptacog alfa): a review of its use in congenital hemophilia with inhibitors, acquired hemophilia, and other congenital bleeding disorders. BioDrugs. 2008;22(2):121–36.
86. Hedner U, Glazer S, Pingel K, Alberts KA, Blonback M, Schulman S, et al. Successful use of recombinant factor VIIa in patient with severe haemophilia a during synovectomy. Lancet. 1988;332:1193.
87. Sadeghi N, Kahn D, Syed D, Iqbal O, Abro S, Eshraghi R, et al. Comparative biochemical and functional studies on a branded human recombinant factor VIIa and a biosimilar equivalent product. Clin Appl Thromb Hemost. 2014;20(6):565–72.
88. Faranoush M, Abolghasemi H, Toogeh G, Karimi M, Eshghi P, Managhchi M, et al. A comparison between recombinant activated factor VII (Aryoseven) and Novoseven in patients with congenital factor VII deficiency. Clin Appl Thromb Hemost. 2015;21(8):724–8.
89. Faranoush M, Abolghasemi H, Mahboudi F, Toogeh G, Karimi M, Eshghi P, et al. A comparison of efficacy between recombinant activated factor VII (Aryoseven) and Novoseven in patients with hereditary FVIII deficiency with inhibitor. Clin Appl Thromb Hemost. 2016;22(2):184–90.
90. Miller RD, Eriksson LI, Fleisher LA, Wiener-Kronish JP, Cohen NH, Young WL. Miller's anesthesia E-book. Elsevier Health Sciences; 2014.
91. Bauer KA. Treatment of factor VII deficiency with recombinant factor VIIa. Pathophysiol Haemost Thromb. 1996;26(Suppl. 1):155–8.
92. Chrisentery-Singleton T, Amos LE, Bonzo D, Escobar M, Giermasz A, Lagrue E, et al. A preliminary analysis of Athn 16: safety of coagulation factor VIIa (recombinant)-Jncw for the treatment of bleeding events in patients with congenital hemophilia a or B with inhibitors. Blood. 2022;140(Supplement 1):11360–1.
93. Meeks SL, Leissinger CA. The evolution of factor VIIa in the treatment of bleeding in haemophilia with inhibitors. Haemophilia. 2019;25(6):911–8.
94. Awad NI, Cocchio C. Activated prothrombin complex concentrates for the reversal of anticoagulant-associated coagulopathy. Pharm Ther. 2013;38(11):696.
95. Sørensen B, Spahn DR, Innerhofer P, Spannagl M, Rossaint R. Clinical review: prothrombin complex concentrates-evaluation of safety and thrombogenicity. Crit Care. 2011;15(1):201.
96. Dentali F, Marchesi C, Pierfranceschi MG, Crowther M, Garcia D, Hylek E, et al. Safety of prothrombin complex concentrates for rapid anticoagulation reversal of vitamin K antagonists. Thromb Haemost. 2011;106(3):429–38.
97. Grottke O, Rossaint R, Henskens Y, van Oerle R, ten Cate H, Spronk HM. Thrombin generation capacity of prothrombin complex concentrate in an in vitro dilutional model. PLoS One. 2013;8(5):e64100.

98. Pabinger I, Brenner B, Kalina U, Knaub S, Nagy A, Ostermann H. Prothrombin complex concentrate (Beriplex® P/N) for emergency anticoagulation reversal: a prospective multinational clinical trial. J Thromb Haemost. 2008;6(4):622–31.
99. Schulman S, Bijsterveld NR. Anticoagulants and their reversal. Transfus Med Rev. 2007;21(1):37–48.
100. Strengers P, Drenth J. PPSB as first choice treatment in the reversal of oral anticoagulant therapy. Acta Anaesthesiol Belg. 2001;53(3):183–6.
101. Lusher J, editor. Thrombogenicity associated with factor IX complex concentrates. Seminars in hematology. 1991.
102. Schulman S, Johnsson H, Lindmarker P. Thrombotic complications after substitution with a FVII concentrate. Thromb Haemost. 1991;66(5):619.
103. Escoffre M, Zini J, Schuamser L, Mazoyer E, Soria C, Tobelem G, et al. Severe arterial thrombosis in a congenitally factor VII deficient patient. Br J Haematol. 1995;91(3):739–41.
104. Ferster A, Capouet V, Deville A, Fondu P, Corazza F. Cardiac surgery with extracorporeal circulation in severe factor VII deficiency. Pathophysiol Haemost Thromb. 1993;23(1):65–8.
105. Behring CSL. Beriplex® P/N 250, 500 and 1000 IU [package leaflet]. Marburg, Germany: CSL Behring GmbH; 2014.
106. Behring CSL. KCENTRA (prothrombin complex concentrate (human)), [package insert]. Marburg Germany: CSL Behring GmbH; 2013.
107. Sanquin. Cofact 250, 500 IU [information for use]. Amsterdam, Netherlands: Sanquin Plasma Products B.V.; 2016.
108. LFB-BIOMEDICAMENTS. KASLADIL (human coagulation factor II) [Patient information leaflet]. FRANCE. 2010.
109. Octapharma. Octaplex 500, 1000. [package leaflet]. Vienna, Austria: Octapharma Pharmazeutika Produktionsges.m.b.H.; 2015.
110. Baxalta. Prothromplex TOTAL 600 IU [package leaflet]. Vienna, Austria: Baxalta Innovations GmbH; 2015.
111. Baxter. Proplex-T (factor IX complex). Glendale: Baxter Healthcare Corporation; 2000.

Congenital Factor X Deficiency, Diagnosis, and Management

12

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12.1 Introduction

Coagulation factor X (FX; synonyms: autoprothrombin III, Stuart–Prower factor) is a vitamin K-dependent glycoprotein that occupies a pivotal position in the coagulation cascade. After activation, activated FX (FXa) is the first enzyme in the common coagulation pathway and plays a key role in thrombin generation. Congenital FX deficiency is a very rare bleeding disorder that is inherited in an autosomal recessive manner estimated to occur in 1:1,000,000 individuals, although the prevalence is much greater in areas with a high rate of consanguineous marriage. Bleeding manifestations usually occur in homozygous and compound heterozygous cases, while heterozygous cases are generally asymptomatic. Regardless of the severity of FX deficiency, the most common bleeding symptoms are mucocutaneous bleedings including epistaxis, gum bleeding, and easy bruising. Patients with severe FX deficiency have a high incidence of spontaneous major bleeding. The bleeding may occur at any age; however, the cases with severe FX deficiency [FX:C) <1%] may be diagnosed early in life with an abnormal bleeding tendency. The affected women at reproductive age may present with menorrhagia. The diagnosis of FX deficiency is suspected following the finding of a prolonged prothrombin time (PT) and activated partial thromboplastin time (APTT) which corrects (unless an inhibitor is present) in a 50:50 mix with normal plasma and confirmed by measuring plasma FX levels either immunologically or functionally. Management includes on demand

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_12

and prophylactic therapy (if and when indicated). Current available therapies include antifibrinolytic agents; tranexamic acid and aminocaproic acid and blood-derived products; fresh frozen plasma (FFP), prothrombin complex concentrate (PCC), FIX products, and plasma-derived FX concentrate (pdFX).

Factor X is synthesized by the liver and as an inactive zymogen secreted into the plasma where the excision of a tribasic peptide Arg-Lys-Arg ("RKR": residues 180–182) occurs;. It circulates as a two-chain molecule with a concentration of 8–10 μg/mL (~135 nmol/L) and a half-life of 40 h in the plasma. FX belongs to the peptidase S1 family of proteins.

12.2 Factor X Structure and Function

FX, also known as autoprothrombin III and Stuart–Prower factor, is a serine protease mainly synthesized in the liver. Its zymogen form secretes to plasma and circulates as a two-chain molecule with a concentration of 8–10 μg/mL (~135 nmol/L) and a half-life of 40 h. FX belongs to the peptidase S1 family of proteins (Table 12.1) [1]. FX contains a 40-residue pre-propeptide and hydrophobic signal sequence (−37 to −22) in homology with the other vitamin K-dependent coagulation factors. The signal sequence is cleaved in a two-step process. The first cleavage occurs after Leu-29 and then the propeptide (−1 to −18) is removed from the N-terminus of the light chain by the second peptidase (between −1Arg and + 1Ala). The propeptide is essential for intracellular post-translational modifications and the proper function of FX. The light chain of FX contains 11 glutamic acid residues which are modified to γ-carboxyglutamic acid (Gla) domains. Ten residues are encoded by exon II and the last one is encoded by exon III. The Gla domain has a key role in the binding of Ca^{2+} ions and anchoring to negatively charged phospholipid membranes.

Table 12.1 Characteristics of *F10* gene

Exon	Size (bp)	Domain
1	70	Pre-pro-leader sequence (signal peptide) (aa-40 to −17)
2	161	Propeptide region containing Gla domain (aa-17 to +37)
3	25	Linking segment of the aromatic amino acid (aromatic stack) (aa38 to 46)
4	114	EGF-1 (aa46 to 84)
5	132	EGF-2 (aa84 to 128)
6	245	Connecting region and activation peptide (aa128 to 209)
7*	118	Catalytic domain (aa210 to 249)
8*	599	Catalytic domain (aa249 to 448)

bp base pair; *aa* amino acid; *Gla* γ-carboxyglutamic acid-rich; *EGF* epidermal growth factor; *Exons VII and VIII encode two active serine protease domains containing the residues for the catalytic triad (His236, Asp282, and Ser379)

Mature FX consists of two light (17 kDa) and heavy chains (45 kDa), covalently linked by a tripeptide residue (Arg-Lys-Arg) and a disulfide bond between Cys 89 and Cys124. The light chain consists of 139 amino acids organizing the Gla-rich domain (which contains 11 Gla residues from Ala1-Gla39, three α-helices A1–3 and one π-helix P1), a high proportion of aromatic amino acids segment (hydrophobic aromatic stack, residues Phe40-Lys45) and two epidermal growth factor (EGF)-like domains: EGF1 (Asp46-Phe84 forming two β-strands A-B) and EGF2 (thr85-Gly128 forming four β-strands A-D). Each EGF-like domain contains three conserved Gly and six Cys residues forming three unique disulfide bonds; these domains are important to maintain the correct conformation of FXa. The heavy chain consists of 346 residues forming a catalytic serine protease domain (254 residues forming 13 β-strands A-M, three minor α-helices, three 310-helices, and one π-helix) and a 52-amino acid activation peptide. In homology with other vitamin K-dependent factors and trypsin-like enzymes, the catalytic domain contains the highly conserved catalytic site—His236, Asp282, and Ser379. When FX is activated, the activation peptide cleaves (Fig. 12.1) [2–7]. A number of crystal structures for FX exist and have been used to model a number of the naturally occurring FX mutations [8].

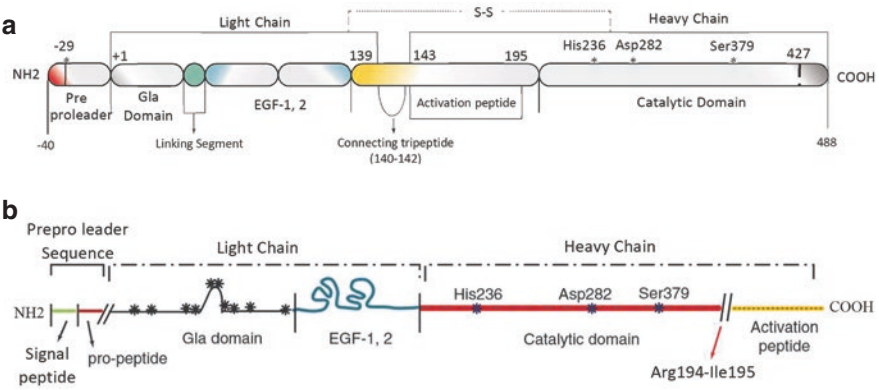

Fig. 12.1 (**a**) Factor (F) X polypeptide structure and its functional domains. The signal peptidase probably cleavages FX protein at residue −29 and the mature protein initiates from residue +1. The light chain consists of 139 amino acids (residues +1 to 139) containing Gla domain and EGF-1, 2 domains, while the heavy chain consists of 364 residues (143–448) containing activation peptide and catalytic domain in which activation peptide is located at residues 143–195. The position of connecting tripeptide (Arg-Lys-Arg) is between residues 140 and 142. Clotting FX is activated by cleavage at Arg194-Ile195. The second cleavage at residue 427 generates FXaβ. The catalytic site within the catalytic domain is formed by His236, Asp282, and Ser379. (**b**) Schematic of FX structure. The separated part indicates cleavage of the pre-pro-leader sequence. The Gla domain is shown with Gla residues and the active sites His, Asp, and Ser are shown in the catalytic domain. Two light and heavy chains are linked by a disulfide bond. The cleavage site of the activation peptide (yellow part) is indicated by the red arrow. *EGF* epidermal growth factor

12.3 Factor X Activation

Normally, there is a mixture of both zymogen and activated forms of FX in plasma with the zymogen form predominating. This balance shifts in favor of FXa concentration once the scissile peptide bond is cleaved between the light and heavy chains. The zymogen form of coagulation FX is cleaved at the Arg194-Ile195 peptide bond in the heavy chain releasing the 52-amino acid activation peptide and activated FXa. When activated, the N-terminus of the serine protease domain is remarkably redirected (a major difference between the zymogen and activated form of FX) and the new N-terminus of Ile195 inserts into the hydrophobic substrate-binding pocket within the catalytic domain by forming a strong salt bridge with Asp378. It is a required orientation to trigger the events promoting the FXa catalytic activity. The second cleavage of FX often occurs at the C-terminus (Lys427-Ser428) to produce FXaβ that has no significant functional hints [2, 4, 9, 10].

FX may be activated either in vivo or in vitro. Physiologically, FX is activated by FIXa (FIXa)/FVIIIa through the "intrinsic pathway" or by TF-FVIIa through the "extrinsic pathway." In-vitro FX can also be activated by Russell viper venom (RVV), a metalloproteinase isolated from the venom of the snake *Vipera russelli*. A complex of tissue factor (TF), FVIIa, Ca^{2+} ion, and appropriate phospholipid membrane is required for in vivo activation of FX and FIX (extrinsic pathway of coagulation cascade) (please refer to Chap. 1). Physiologically activation of FIX by TF/FVIIa complex is more important than direct activation of FX. Generation of FVIIa is amplified via a positive feedback loop by production of FXa. The GLa domain evokes the negative charge essential for Ca^{2+} binding and constructs the required conformation for binding to an anionic phospholipid surface. A variety of cell types such as fibroblast, monocyte-macrophage, and endothelial cells as well as tumor cells provide this anionic phospholipid membrane for activation. Naturally, the membrane of these cells is anticoagulant since phosphatidylserine (PS) and phosphatidylethanolamine (PE) are abundant in the inner leaflet while sphingomyelin and phosphatidylcholine (PC) are sequestered to the outer surface. Disruption and externalization of PS and PE following cellular injuries and activation of platelets provide the suitable binging surface for coagulation factors. Given that mammalian cells' membrane contains about 10% PS, recent studies displayed that in the presence of excess PE, only one molecule of PS is required for binding of FX Gla domain to the negatively charged membrane, indicating the significance of PS/PE synergy to develop membrane binding sites for clotting factors assembly. Conversion of FX to FXa also occurs through coagulation intrinsic pathway by the interaction of FIXa, FVIIIa, Ca^{2+} ion, and phospholipid membrane (platelet and/or endothelial cells) (Fig. 12.2). In vitro activation of FX may occur by Russell viper venom of the snake *V. russelli.* It contains a metalloproteinase which can directly activate FX without Ca^{2+} ion or phospholipid membrane [2, 3, 6, 10, 11]. The presence of all the constituents results in a tenfold decrease in Km for the reaction and a 1000-fold increase in kcat accelerating the generation of FXa by at least 10,000-fold relative to rates observed for FVIIa and Ca2+ alone.

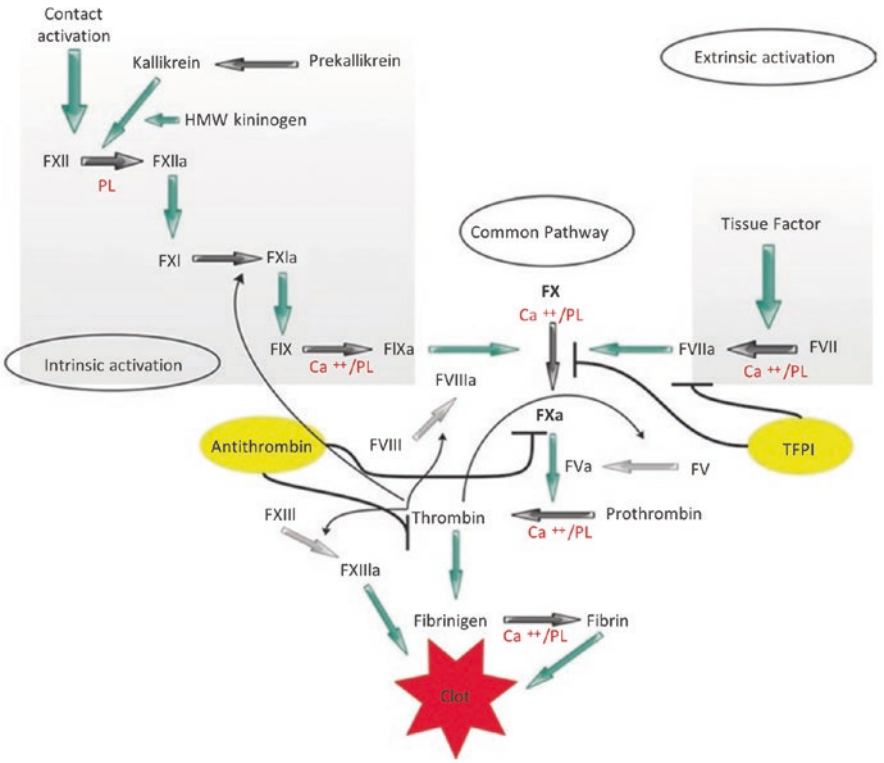

Fig. 12.2 Coagulation cascade. This illustration represents the role and activation of clotting factor (F) X in the coagulation cascade. FX is the first enzyme of the common pathway activated by a complex of TF/FVIIa/Ca^{2+}/PL through the extrinsic pathway and FIXa/FVIIIa/Ca^{2+}/PL via the intrinsic route. Then, a complex of FXa/FVa/Ca^{2+}/PL (prothrombinase complex) cleavages prothrombin to the thrombin subsequently leading to the cleavage of fibrinogen to fibrin. Finally, FXIIIa stabilizes the generated clot. FV, FVIII, FIX, and FXIII also are activated by thrombin. Activation of FX may be inhibited by tissue factor pathway inhibitor (TFPI) and antithrombin. TFPI is a pivotal inhibitor of the extrinsic pathway that in complex with FXa/TF/FVIIa inhibits their catalytic activity. Antithrombin also affects FXa and generates a stable inactive complex (please refer to Chap. 1). *TF* tissue Factor; *FVII* factor VII; *FVIIa* activated factor VII; *FX* factor X; *FXa* activated factor X; *FXII* factor XII; *FXIIa* activated factor XII; *FXI* factor IX; *FIXa* activated factor IX; *FXa* activated factor IX; *FVIII* factor VIII; *FVIIIa* activated factor VIII; *FV* factor V; *FVa* activated factor V; *FXIII* factor XIII; *FXIIIa* activated factor XIII; *TFPI* tissue factor pathway inhibitor; *PL* phospholipid

FXa could be generated independently of either TF/FVIIa or FIXa/FVIIIa, Ca^{2+} ion, and phospholipid membrane. For instance, in some malignant cells, cysteine proteinase may be involved in FX activation [3, 6].

FXa exerts a central role as a key thrombin activator in a way that one molecule of FXa can produce more than 1000 molecules of thrombin. A complex of FXa, FVa, and Ca^{2+} ion at a suitable phospholipid membrane (prothrombinase complex) can 280,000-fold accelerate prothrombin transition. This complex assembles on the

surface of platelets, lymphocytes, monocytes, neutrophils, and endothelial cells. The suggested model for the prothrombinase complex shows that, initially, FVa binds to the negatively charged phospholipid membrane through its light chain and provides a suitable receptor for FXa. In turn, FXa anchors to the membrane surface via Gla domains of the light chain on one side and interacts with FVa by the heavy chain on the other side. FVa then interacts with prothrombin through the heavy chain, independent of Ca^{2+} ions. Subsequently, prothrombin binds to the membrane surface by the formation of Ca^{2+} bridges (Gla residues) and later, following membrane assembly, the prothrombinase complex converts prothrombin to the active form, thrombin which promotes clot formation. FVa is a cofactor of FXa which enhances the catalytic efficacy of FXa, also the negatively charged phospholipid membrane is an appropriate surface that increases the local concentration of FXa, FVa, and prothrombin to accelerate the conversion of prothrombin to the active serine protease, thrombin. In addition to prothrombin, FXa has the potential of converting FV, FVII, and FVIII to their active form. Moreover, activation of FVII and FX is a reciprocal pathway, so that TF/FVIIa complex produces FXa, FXa next activates FVII and increases the level of FVIIa in a positive feedback loop [3], therefore more FXa brings more FVIIa which in turn enhances FXa.

12.4 Regulation of Factor X Activity

The principal regulators of FX activity (FXa) are antithrombin, TF pathway inhibitor (TFPI), and protein Z-dependent protease inhibitor (ZPI).

Antithrombin (III) FXa is inhibited by antithrombin. The latter forms a stable inactive complex that inhibits FXa. Inactive complexes of FX are then removed from the circulation by the liver. Low molecular weight heparin (LMWH) enhances the anti-Xa activity of antithrombin. Various sulfated glycosaminoglycans may accelerate the antithrombin activity.

Tissue Factor Pathway Inhibitor (TFPI) TFPI is the pivotal inhibitor of the coagulation extrinsic pathway. It binds to FXa in a 1:1 ratio and forms a quaternary complex with TF-FVIIa. The complex of TFPI/FXa/TF/FVIIa lacks TF/FVIIa catalytic activity thereby blocking the extrinsic pathway to thrombin generation (please refer to Chap. 10).

Factor VIII and Activated Factor X FXa is involved in the inactivation of FVIIIa. Therefore FXa is inhibited via a negative feedback loop [6].

Protein Z (PZ) A plasma vitamin K-dependent protein functions as a cofactor to enhance the inhibition of FXa by the serpin ZPI.

Pathophysiological conditions such as inflammation may reduce the levels of FXa inhibitors owing to degradation or devastated synthesis leading to an impaired anticoagulant pathway and unconstrained formation of FXa [12].

12.4.1 Factor X Gene

The human FX gene (*F10*) is 22 kb, mapping on the long arm of chromosome 13 at 13q34, 2.8 kb downstream of *F7*gene (Fig. 12.3) [13]. For the first time, the Davie laboratory isolated the *F10* gene in 1986 [14]. As there is significant homology in the *F10* gene and the other vitamin K-dependent clotting factors (*F9*, *F7,* and protein C), both in structure and organization, an evaluation from a common ancestral gene is suggested. The *F10* gene spread approximately 27 kb of genomic sequence. It contains eight exons and seven introns; each exon encodes a specific part of FX protein. The introns vary in size from 950 bp to 7.4 kb (Fig. 12.6) [5, 15, 16]. Exon I encodes the signal peptide; exon II codes the propeptide and γ-carboxyglutamic acid-rich (Gla) domain. Exon III codes a short linking segment of the aromatic amino acid (aromatic stack), exons IV and V encode two regions homologous to EGF, and exon VI codes activation peptide at the amino terminus of the heavy chain. Exons VII and VIII encode two active serine protease domains containing the residues for the catalytic triad (His236, Asp282, and Ser379) [5] (Fig. 12.4).

The *F10* cDNA consists of 120 bp encoding 40 amino acids pre-pro-leader sequence and 1344 bp coding 488 amino acids of the mature protein. There is a short 3′ untranslated region of 10 bp preceding the poly (A) tail. An obvious TATA box has not been identified at the 5′ end of the *F10* gene, but almost six different transcription initiation sites have been detected at the 5′ region like other TATA-less promoter genes. Analysis of the *F10* gene promoter shows that a 200-bp region upstream of the coding region is vital for its function. *F10* gene has been cloned by different groups. Isolated *F10* colons with a lack of 9-bp sequence encoding a tripeptide (Lys-Val-Arg) have been detected by different groups. At first, it was considered an artifact but later Tuddenham and Copper suggested that it is a result of the formation of a "semi-cryptic" splice site that led to alternative processing. Several polymorphisms also have been detected within the *F10* gene [3, 5].

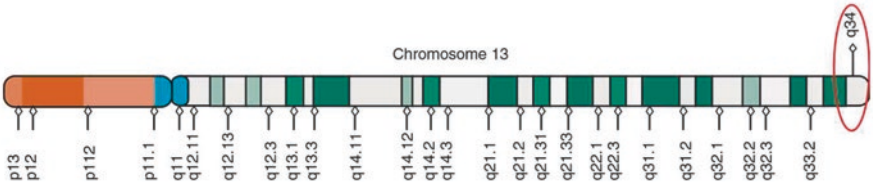

Fig. 12.3 Chromosomal location of *F10* gene. The *F10* gene is located in long arm of chromosome 13 at 13q34

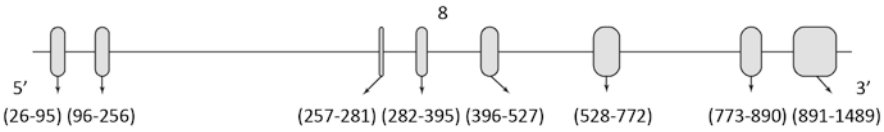

Fig. 12.4 *F10* gene organization. The *F10* gene consists of eight exons. The diagram shows the location of the first and last nucleotides of each exon

12.5 Congenital Factor X Deficiency (FXD)

Historical background of FXD is shown in Fig. 12.5.

The first clues of the FX function date back to **1904** when **Paul Morawitz** brought some evidence of the entity of a physiologic central activator of prothrombin in the coagulation system; it was termed **thrombokinase**. During the following 50 years, the first attempts to isolate and purify thrombokinase were carried out by **Haskell Milstone** in **1947–48**. Later congenital FX deficiency was identified by two independent groups in the 1950s [6, 15, 17]. First**, Telfer et al**. **1956** described menorrhagia and bleeding after dental extraction and tonsillectomy associated with an abnormal thromboplastin generation test and prolonged prothrombin time (PT) in a 22-year-old patient **(Power)**; then 1 year later, **Hougie et al., 1957** reported a 36-year-old man **(Stuart)** with prolongation of the activated partial thromboplastin time (APTT), abnormal thromboplastin generation test (TGT), and a prolonged Russel viper venom time (RVVT). At first, they thought that bleeding tendency in these patients was caused by FVII deficiency, but later they found that direct mixing of the patients' plasma with FVII-deficient plasma led to correction of prolonged prothrombin time (PT) yet aPTT, RVVT, and TGT are prolonged in the patient, while both aPTT and TGT are normal in patients with FVII deficiency highlighting the fact that there was an insufficiency in a clotting factor other than FVII. Hence, **Telfer and Hougie** named it the **Stuart-Prower** factor. Furthermore, **Duckert et al. in 1954** described a type of factor deficiency that was distinct from FVII and FIX shortage in a patient who received coumarin anticoagulants. In 1962 the new characterized factor was officially named **FX** (Fig. 12.5) [3, 6, 13, 17–19].

12.5.1 Prevalence/Inheritance

FX deficiency is inherited in an autosomal recessive manner equally affecting both genders. The estimated incidence of FX deficiency in homozygous form is 1:1,000,000. It is more frequent in areas with a high rate of consanguineous

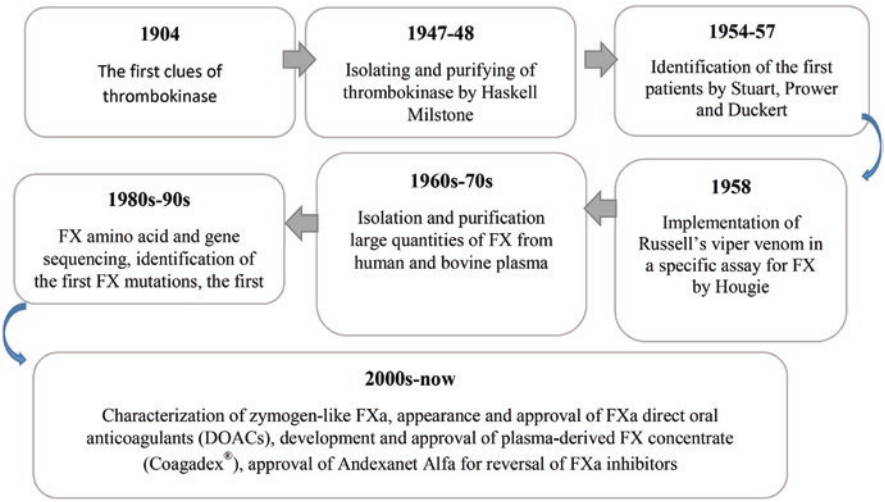

Fig. 12.5 Chronological blocks (timeline) of significant events in the history of FX discovery and development from the beginning until now (adopted from Rodney M.Camire [15] with some modifications)

marriages such as Iran where the frequency is reporetd to be 1:200,000~. The prevalence of heterozygotes is estimated as high as ~ 1:500 [18, 20–23]. FX deficiency accounts for 1.3% of patients with inherited coagulation deficiencies in Iran, 0.4% in Italy, and 0.5% in the UK. Heterozygous FX deficiency (carrier state) is often clinically asymptomatic [24] but can be symptomatic. Although compound heterozygotes have been reported, pedigree analysis frequently identifies consanguineous parents, supporting the presence of a homozygous defect. Indeed in the first case of factor X deficiency reported by Hougie et al., the father and mother of the index case were aunt and nephew. FX deficiency encompassed 8% of all rare bleeding disorders (Fig. 12.6). FX is activated through extrinsic and intrinsic coagulation pathways and has a pivotal role in the common pathway. In view that it is the key part of the prothrombinase complex, FX deficiency causes the impaired formation of the complex which leads to bleeding. Congenital FX deficiency presents with a wide variable bleeding tendency from asymptomatic in heterozygotes to life-threatening manifestation in some homozygous. Bleeding symptoms may occur as a result of insufficient enzymatic activity or interference of mutant gene product with the normal reaction of the coagulation pathway [6, 19].

12.6 Classification

FX deficiency could be simply classified based on the results of both immunological and functional (PT, APTT, and time-based RVV) laboratory assays [19] and highlights the interesting observation that a number of dysfunctional variants of FX show differing FX activities depending upon the method employed in their assay. Type 1 is described by a concomitant reduction in antigenic and functional levels of FX, whereas type 2 is demonstrated with a decrease in FX:C and normal or nearly normal antigenic level of FX (FX:Ag) [3]. The first classification of FX deficiency was established in 1969–1970 including type I or cross-reacting maternal negative (CRM-) (Stuart family), type II or CRM+ (Prower family), and CRM Friuli form. CRM-type is described by simultaneous reduction of both FX:Ag and FX:C levels because of abnormal synthesis or secretion of the protein, while CRM+ form is characterized by normal or nearly normal FX:Ag and reduction of FX:C. For years a wide range of *F10* gene defects has been identified that affect only extrinsic or intrinsic coagulation pathways. So, it became clear that type II or CRM+ is a heterogeneous type with various presentations in different families. Therefore, this classification was insufficient to describe all types of FX deficiency. Establishing molecular biology techniques studying the *F10* gene in the last two decades leads to the identification of several mutations in association with different types of FX

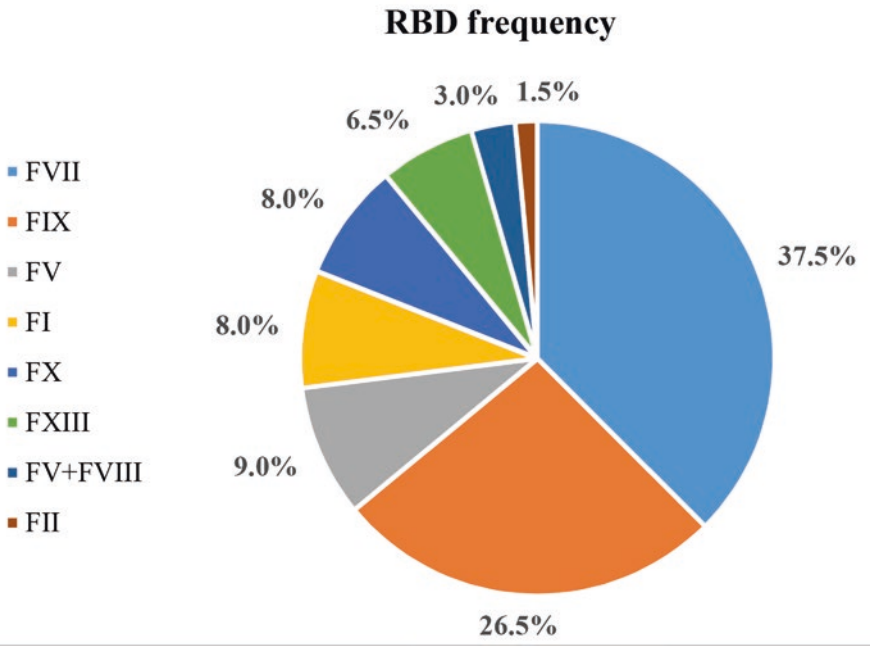

Fig. 12.6 Relative percentage of rare bleeding disorders. (**Worldwide distribution of RBDs derived from the WFH and EN-RBD**)

Table 12.2 Classification of factor X deficiency

Type		Clotting assay			Chromogenic assay	Antigenic assay (FX:Ag)
		PT	aPTT	RVVT		
I	CRM negative	↓	↓	↓	↓	↓
II	CRM positive	↓	↓	↓	↓	N or nearly N
III	Friuli like	↓	↓	N	↓	N or nearly N
	Padua like	↓	N	N	↓	N or nearly N
	Melbourne like	N	↓	N	↓	N or nearly N
	–	↑	↑	↑	H	N or nearly N
IV[a]	–	↓	↓	↓	↓	↓

CRM cross-reacting material; *PT* prothrombin time; *aPTT* activated partial thromboplastin time; *RVVT* Russell viper venom time; *FX* factor X; *FX:Ag* factor X Antigen; *N* normal, *H* high
[a] FX deficiency concomitant deficiency of other clotting factors especially factor VII

deficiency. According to molecular studies, FX deficiency could be classified based on the type of mutations, e.g., nonsense, missense, insertion, deletion, etc. There is also a classification based on of the sites where mutations occur including promoter, Gla domain, EGF domain, activation peptide, and the catalytic domain. Despite these investigations, no obvious correlation has been found between genotype and phenotype in FX deficiency. Mutations in the same area may accompany different phenotypes, whereas the same phenotypes may be due to different mutations. The only exception is variants in the Gla domain of the light chain that present similar phenotypes in different cases.

In conclusion, because of the great heterogeneity in genotype and phenotype of FX deficiency, it seems that a more suitable classification could be achieved by using immunological, functional, and clotting assays as well as molecular biology studies. According to these considerations, the following classification has been proposed by Denson, Fair, Edgington, and then Girolami et al., 2011 [19] that is more acceptable and practical. It is shown in Table 12.2.

- *Type I*: CRM negative or Stuart-like (the classic form of congenital FX deficiency)
- *Type II*: CRM positive or Prower-like, inert protein
- *Type III*: CRM positive with distractive protein that is categorized in four levels:
  - CRM positive *Friuli-like*: Defects in all clotting assays except for RVVT
  - CRM positive *Padua-like*: Defects only or mainly in the extrinsic pathway
  - CRM positive *Melbourne-like*: Defects only or mainly in the intrinsic pathway
  - *Defects with contradiction in the chromogenic assay*
- *Type IV*: concurrent deficiency in FX and other coagulation factors mostly FVII in the effect of abnormality in chromosome 13 (Table 12.2) [19, 25].

It is worth noting that the pattern of clotting assays is variable among different patients based on the involved area of the *F10* gene and protein. For instance, in 2008 Girolami et al. described some cases of mutations in the catalytic domain and light chain C-terminal of FX protein which affect mainly the intrinsic pathway.

They also reported some variants in the Gla domain of the light chain, EGF domain, and catalytic domain that predominantly affect the extrinsic pathway [3, 6, 25].

12.7 Clinical Manifestations

Although FX deficiency produces a variable bleeding tendency, patients affected by severe FX defects tend to be the most seriously affected among those with rare bleeding disorders (RBDs). Clinical features of the patients with FX deficiency are associated with the plasma level of FX protein, but the correlation between the plasma level of FX and bleeding tendency is not as strong as in hemophilia A and B [13, 26]. On the basis of a one-stage prothrombin-based FX assay using rabbit thromboplastin, patients with FX deficiency have been classified into three grades of severity and correlated with the bleeding phenotype (Table 12.3) [13, 14, 18, 27].

Generally, regardless of the severity of the disease, mucosal bleeding, particularly epistaxis, and easy bruising are the most common symptoms of congenital FX deficiency. Common symptoms reported at all severity levels include epistaxis and menorrhagia. Symptomatic patients with clotting factor deficiencies are usually homozygous or compound heterozygous; however, there are some reports of significant post-dental extraction and postpartum bleeding in about one third of heterozygotes of FX deficiency who did not receive prophylactic replacement therapy [26].

Less severely affected patient may bleed only after some challenge to the hemostatic system, e.g., trauma, surgery. Some cases are identified incidentally during routine screening or family studies (Table 12.3).

Herrmann et al. analyzed clinical manifestations of 102 patients with FX deficiency from Europe and Latin America with *F10* gene mutations in 2006. The most severe bleeding symptoms among 42 symptomatic patients (homozygous, heterozygous, and compound heterozygous) were ICH, GI bleeding, and hemarthrosis which were observed in cases with FX:C <2%. However, there were differences among the bleeding patterns of the patients due to corresponding genotypes and the type of mutation. Among clinically symptomatic subjects, 13% were heterozygous, mainly

Table 12.3 Classification and clinical presentations of patients with congenital factor X deficiency

Severity	Factor X level	Clinical features (predominantly)
Mild	6–10 U/dL	Usually asymptomatic and identified based on family studies or occasionally on routine laboratory evaluations but can present with easy bruising, epistaxis, and menorrhagia [3, 13, 14]
Moderate	1–5 U/dL	Mucosal bleeding and post traumatic or post-surgical bleeding [3, 13, 14]
Severe	<1 U/dL	Mucosal bleeding, CNS, umbilical cord bleeding during the neonatal period, hemarthrosis, hematoma, hematuria, and GI bleeding [1, 5, 13, 14, 26]

CNS central nervous system; *GI* gastrointestinal

Table 12.4 Prevalence of bleeding symptoms in patients with congenital factor X deficiency

Symptom	Herrmann et al. (n = 42) (%)	Peyvandi et al. (n = 32) (%)	Acharya et al.		Anwar et al. (n = 20) (%)
			Homo (n = 19) (%)	S Hetero (n = 15) (%)	
Epistaxis	36	72	45[a]	75[a]	35
Gum bleeding	31	–	–	–	35
Easy bruising	55	–	–	–	45
Hematoma	43	66	27	–	–
Hematuria	7	25	–	–	5
Hemarthrosis	33	69	–	–	5
GI bleeding	12	38	4–9	12	10
CNS bleeding	21	9	15	–	–
Umbilical cord bleeding	–	28	–	–	15
Post-circumcision bleeding	–	–	–	–	30
Menorrhagia [b]	71	505	–	–	10
Post-operative hemorrhage	–	–	4	13	–

Homo homozygous; *S Hetero* symptomatic heterozygous; *GI* gastrointestinal; *CNS* central nerves system

[a] Skin and mucus membrane bleeding

[b] Women in reproductive age

with mucocutaneous hemorrhage. It could occur either due to insufficient enzymatic activity of the FX wild type or inhibition of one reaction in the clotting cascade by FX mutant protein [28].

The most common bleeding symptom among 32 Iranian patients, regardless of the disorder severity, was epistaxis (72%). Other clinical manifestations including GI bleeding and hematuria were mainly observed in the cases with an undetectable level of FX. In this study, umbilical stump bleeding was found unexpectedly in nine patients occurring at the time of stump detachment (7–10 days after birth). A direct correlation between FX level and bleeding diathesis was observed in this study (Table 12.4) [18, 28–30].

The biggest four studies with a total of 128 patients show the wide bleeding phenotype of FXD as shown in Table 12.4 with epistaxis being the commonest noted bleeding symptom.

The thrombogram has been used to investigate the relation between FX levels in an attempt to establish at what level of FX normal thrombin generation is achieved. The thrombogram in rare inherited coagulation disorders: its relation to clinical bleeding. In FX deficiency, half-normal endogenous thrombin potential (ETP) is seen at an FX:C of 5 U/dL. Ten individuals with FX deficiency (FX levels 1–50% of normal) were studied and when functional FX activity was <10 the parameters of the thrombogram (lag time and peak height) were markedly abnormal. The ETP was

similar irrespective of the method of activation of FX (intrinsic or extrinsic pathway). In patients with FX activity between 10 and 50 U/dL only the lag time and peak height were abnormal and the ETP remained within normal limits. These patients had no bleeding even after trauma and this suggests the threshold range of FX required to obtain normal thrombin generation time is approximately10% of normal.

12.8 Diagnosis

Similar to other rare bleeding disorders the diagnosis of FX deficiency includes taking detailed history containing family history, assessment of the patient's bleeding phenotype and clinical evaluation as well as first-line screening tests, and specific coagulation tests.

12.8.1 History Taking and Clinical Evaluation

A detailed history taking including family history and pedigree is mandatory. Bleeding phenotype and severity are assessed using the ISTH-BAT and clinical bleeding severity score. Clinical assessment should include full clinical examination including determination of site, type, and duration of bleeding, treatment received as well as other co-morbidities [31] (Table 12.5).

12.8.2 First-Line Screening Tests (Primary Assays)

Following clinical assessment, laboratory screening should be performed. Complete blood count (CBC) and peripheral blood smear examination are necessary to determine any abnormality in the number and morphology of platelets in a bleeding episode in an undiagnosed patient. PT, aPTT, mixing study, and thrombin time (TT), as well as RVVT, should be established in a case suspected of having FX deficiency. FX has a pivotal role in both intrinsic and extrinsic coagulation pathways so prolonged prothrombin time (PT) and activated partial thromboplastin time (APTT)

Table 12.5 Categories of clinical bleeding severity

Clinical bleeding severity	Definition
Asymptomatic	No documented bleeding episodes
Grade I bleeding	Bleeding that occurred after trauma or drug ingestion
Grade II bleeding	Spontaneous minor bleeding*:* bruising, ecchymosis, minor wounds, oral cavity bleeding, epistaxis, and menorrhagia
Grade III bleeding	Spontaneous major bleeding: hematomas, hemarthrosis, CNS, GI, and umbilical cord bleeding

which corrects (unless an inhibitor is present) in a 50:50 mix with normal plasma [3, 18, 31–33].

It is noteworthy to mention that, a number of patients with FX deficiency harbor mutants that affect just extrinsic coagulation pathway exhibiting prolonged PT and normal aPTT (type III, Padua-like).On the contrary, other patients harbor mutations in which only intrinsic pathway is affected and they have a prolonged aPTT and normal PT (type III, Melbourne-like). There are also reports of some variants in the *F10* gene that represent a normal RVVT (type III, Friuli-like).

12.8.2.1 Russell Viper Venom FX Assay

The Russell viper venom (RVV) FX assay is designed based on a special component of some snakes' venoms. The family of *Viperidae* snakes produces a specific venom with the ability to influence the hemostatic system by boosting or impeding platelet aggregation, blood coagulation, and fibrinolysis. The venom's protein content includes two types of proteases, snake venom serine protease (SVSPs) and snake venom metalloprotease (SVMPs) that can promote coagulation cascade by converting some of the clotting factors to their active form. SVSPs exclusively activate FV, while either SVSPs or SVMPs can activate FX and prothrombin. The SVMPs in *Daboia russelii* venom cleaves a specific peptide in the N-terminal domain of the FX heavy chain and activate it directly. Therefore, RVV starts up the common pathway of the coagulation cascade. Prolonged RVVT may be associated with a deficiency in FX, FV, prothrombin, and fibrinogen [18, 32], although, by using FX-deficient plasma as substrate, this assay would be specific for FX deficiency [13, 18, 32, 34].

12.8.2.2 The Principle of the Russell Viper Venom FX Assay

After preparing serial dilutions of standard and patients plasma, an equal volume of each dilution is mixed with FX-deficient plasma and incubated at 37 °C. The RVV-platelet substitute is next added and incubated at 37 °C again. Then the clotting time is recorded following the addition of the pre-warmed Cacl2 (Fig. 12.9). At last, the results of clotting time are plotted on logarithmic paper [2, 3, 35–38].

Interpretation RVVT results are interpreted similar to the PT-based FX assay. Note that RVVT is normal in patients with type III (Friuli-like) FX deficiency (Fig. 12.7).

12.8.3 Specific Coagulation Assays

If a patient is suspected of having FX deficiency based on first-line screening tests, more specific analyses should be considered in the next step. There are various methods for the measurement of both antigenic and functional levels of FX. Plasma levels of FX:Ag may be determined by several immunological assays such as

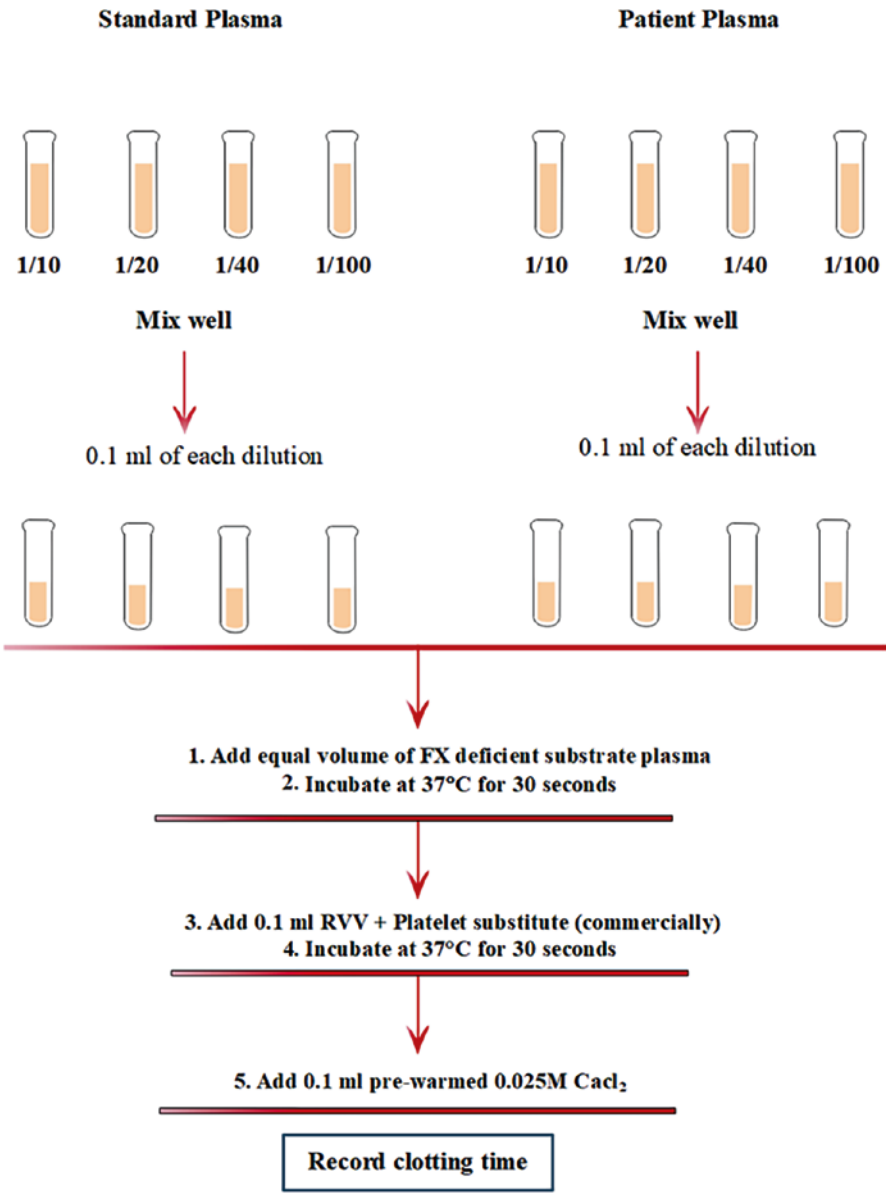

Fig. 12.7 The principle of Russell viper venom (RVV) FX assay

electro-immunoassay, immunodiffusion, radioimmunoassay, antibody neutralization, and laser nephelometry as well as enzyme-linked immunosorbent assay (ELISA) (Figs. 12.8, 12.9, and 12.10).

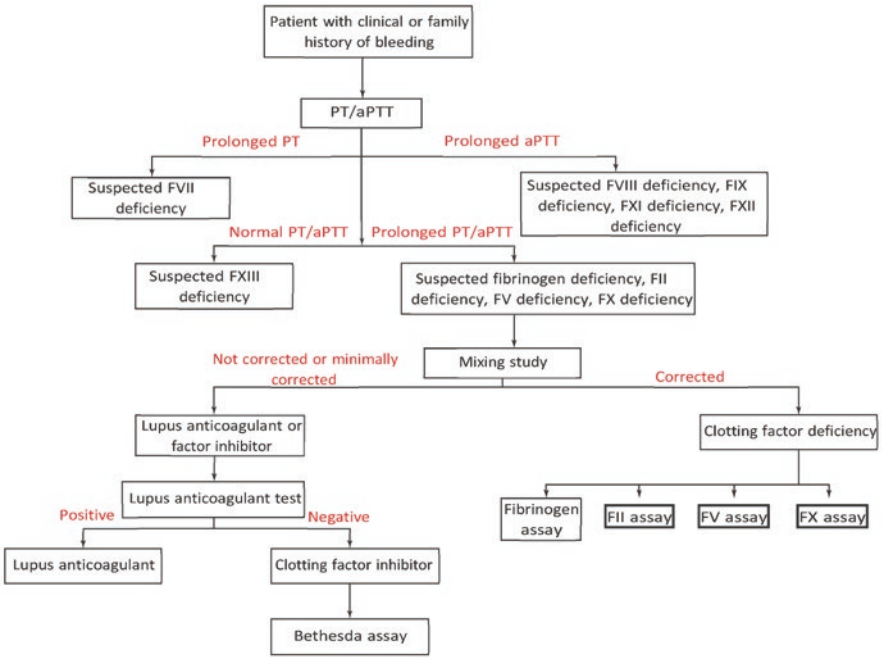

Fig. 12.8 An algorithm approach to diagnose congenital factor (FX) deficiency. To evaluate a patient presenting clinical or family history of bleeding, screening tests such as PT and aPTT are the first step. Prolonged PT with normal aPTT is suspected to FVII deficiency (extrinsic pathway), while normal PT with prolonged aPTT may be due to FVIII deficiency, FIX deficiency, FXI deficiency, or FXII deficiency (intrinsic pathway). Normal PT and aPTT along with bleeding symptom may be suspected to FXIII deficiency or other bleeding disorders that further specific assays are required. In presence of prolonged PT and aPTT, deficiency in fibrinogen, FII, FV, or FX is suspected (common pathway). Then the mixing study by using equal volume of normal and patient plasma could be performed to determine presence of an inhibitor or deficiency in the coagulation factors. Uncorrected or minimally corrected mixing test should be checked for the presence of lupus anticoagulant or coagulation factor inhibitors, while corrected mixing study is an evidence for one or more than one factor deficiency. In the next step, specific assays for the coagulation factors in common pathway are required. *PT* prothrombin time; *aPTT* activated partial thromboplastin time; *FII* factor II; *FV* factor V; *FVII* factor VII; *FVIII* factor VIII; *FIX* factor IX; *FX* factor X; *FXI* factor XI; *FXII* factor XII; *FXIII* factor XIII

12.8.3.1 Functional Assays

- One-stage FX assay (PT-based).
- One-stage FX assay is one of the most common methods to determine precise FX:C level in plasma. It is based on using FX-deficient plasma as substrate in mixing with normal or patient plasma. PT is performed before and after diluting of FX-deficient plasma with patient plasma and the correlated ratio of the clotting time is calculated [13, 18].

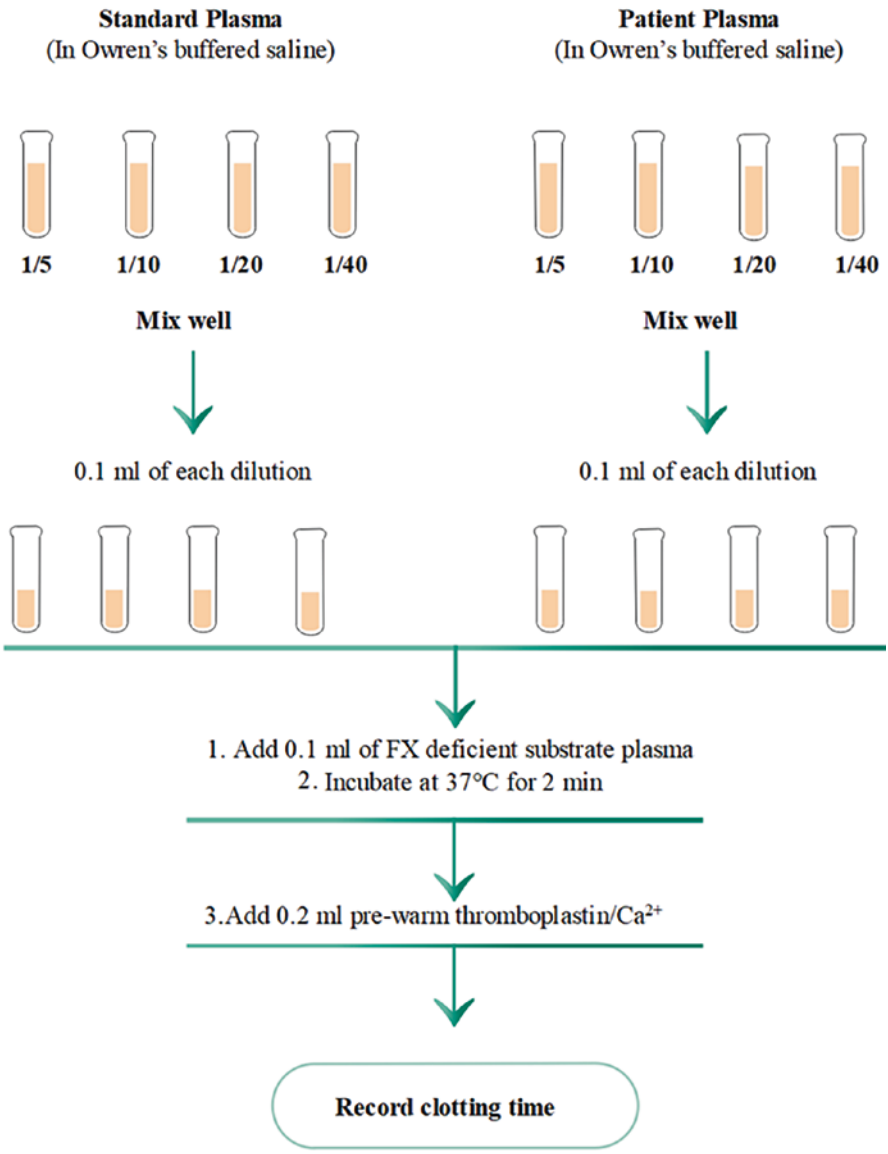

Fig. 12.9 The principle of one-stage FX assay (PT based). Serial dilutions of the standard and test plasma are prepared. The equal volume of each dilution is mixed with substrate plasma. Following the addition of the activating agent, the clotting time is recorded. The FX activity level is determined based on the prepared standard curve

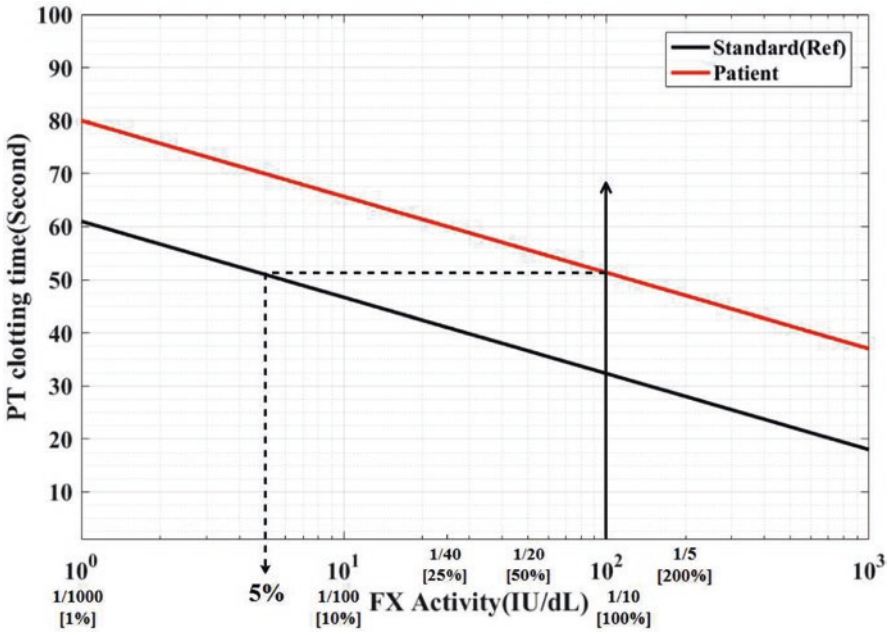

Fig. 12.10 The standard and patient curve of one-stage FX assay (PT based). For determining FX activity level in a patient's specimen, from 1/10 standard point (FX:C 100%), a vertical line is drawn to discontinue the patient's curve. Then, the horizontal line is drawn to intersect the standard curve. This new point shows the FX activity level of the patient specimen (FX:C 5%)

12.8.3.2 The Principle of the One Stage PT-Based FX Activity Assay

First, four dilutions of the patient and standard plasma in Owren's buffered saline at 25 °C (1/5, 1/10, 1/20, and 1/40) are prepared and an equal volume of each dilution is mixed with FX-deficient substrate plasma and incubated at 37 °C. Then, the pre-warmed thromboplastin containing calcium is added to each dilution and immediately the clotting time is recorded. Each standard and patient dilutions should be performed in duplicate and included the blank (Fig. 12.11). Finally, the clotting times of the standard and patient are plotted on logarithmic paper. The Y-axis represents the clotting time and the X-axis represents the FX activity level (U/dL) on the curve. All points of standard and patient are connected by drawing a line with a downward slope. The 1/10 standard dilution is arbitrary considered as the value of 100%. Based on 1/10 standard point, FX activity level in the patient's specimen is determined (Fig. 12.12) [14, 39, 40].

Interfering variables Disproportionate amount of anticoagulant to blood, not reaching the temperature of the reagents to 37 °C, and presence of inhibitors may interfere with the test.

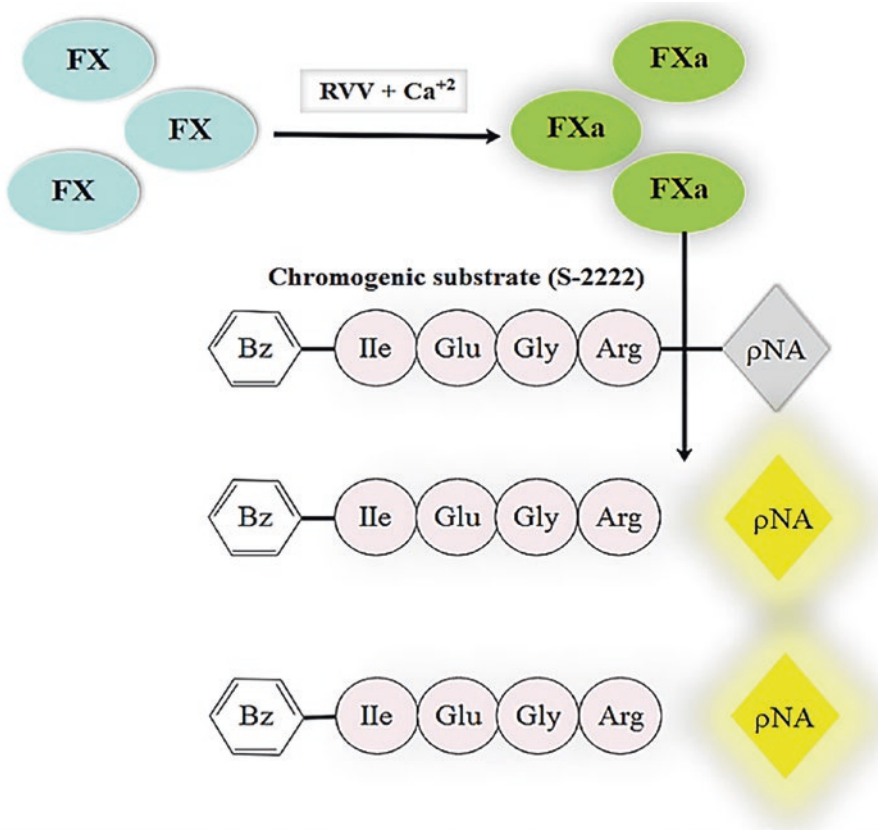

Fig. 12.11 The two-stage mechanism of the chromogenic assays of FX. First, activation of plasma FX to FXa by Russell viper venom and calcium. Second, the generated FXa proteolysis the chromogenic substrate (S-2222) and releases a yellow para-nitroaniline (pNA) chromophore

12.8.3.3 Chromogenic Assays

Chromogenic assay of FX (CFX) is another trustable quantitative method to assess plasma FX:C level in citrated plasma samples using a specific chromogenic substrate. The method is performed based on the two-stage assay. First, CFX is initiated by activation of FX by RVV and calcium, and then aFX proteolyzes a FXa-specific chromogenic substrate (S-2222 or spectrozyme FXa) resulting in the release of a yellow para-nitroaniline (pNA) chromophore (Fig. 12.13). The maximum absorbance of the generated color is next detected by spectrophotometer at 405 nm wavelength, the color intensity is directly proportional to the plasma FXa enzymatic activity. More activity levels of FX result in more FXa, so the color will be more intense [2, 3, 35]. It is important to know that because of nonspecific nature of the chromogenic substrate, the FX level may be estimated higher in some cases [13].

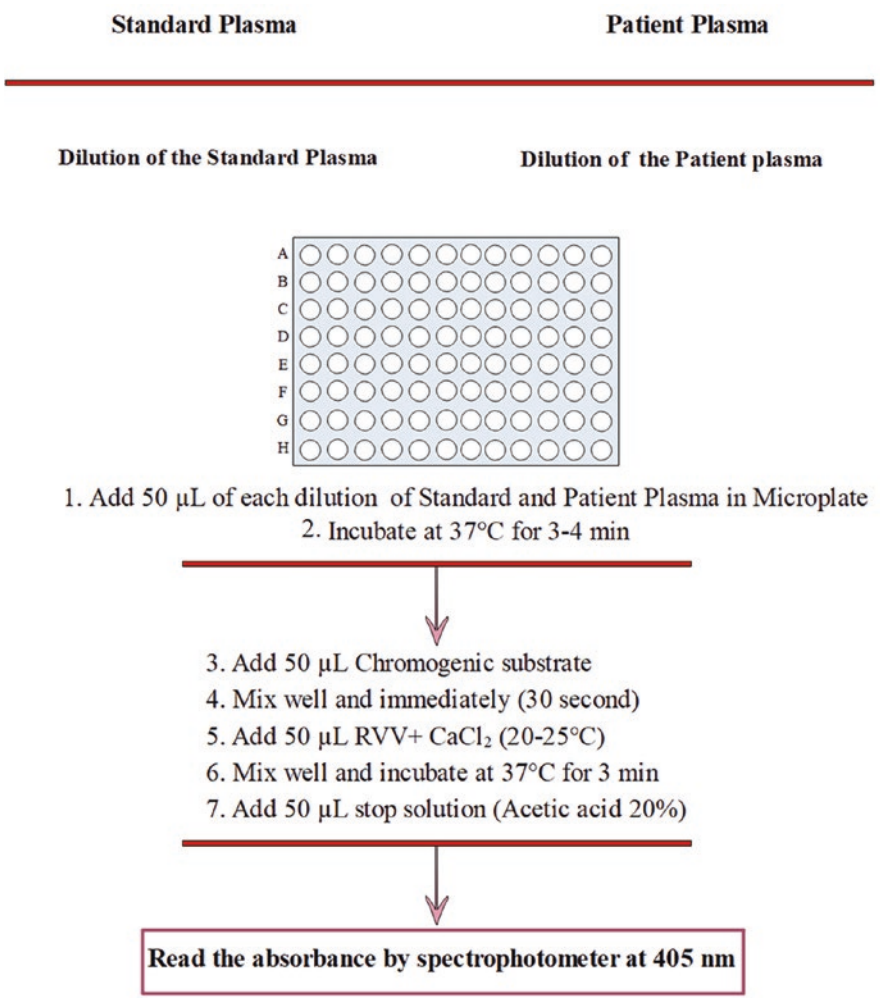

Fig. 12.12 The principle of the chromogenic assay of FX. Each standard and patient dilutions should be performed in duplicate and averaged

12.8.3.4 The Principle of the Chromogenic FX Assay

At the onset, the dilutions of the standard plasma are prepared. Afterward, each volume of prepared dilutions of the standard plasma or patient plasma is added to the microplate wells to start the assay. After incubation at 37 °C, the chromogenic substrate is added and mixed well. The RVT+ Cacl2 is then added and incubated at 37 °C. Subsequently, the reaction is stopped by using the acidic solution and the maximum absorbance of the standard and patient plasma is measured by spectrophotometer at 405 nm (Fig. 12.14). Finally, the standard curve is drawn by using the standards absorbance values against the FX concentrations. Based on the absorbance of the patient plasma, the FX concentration of the patient is determined on the

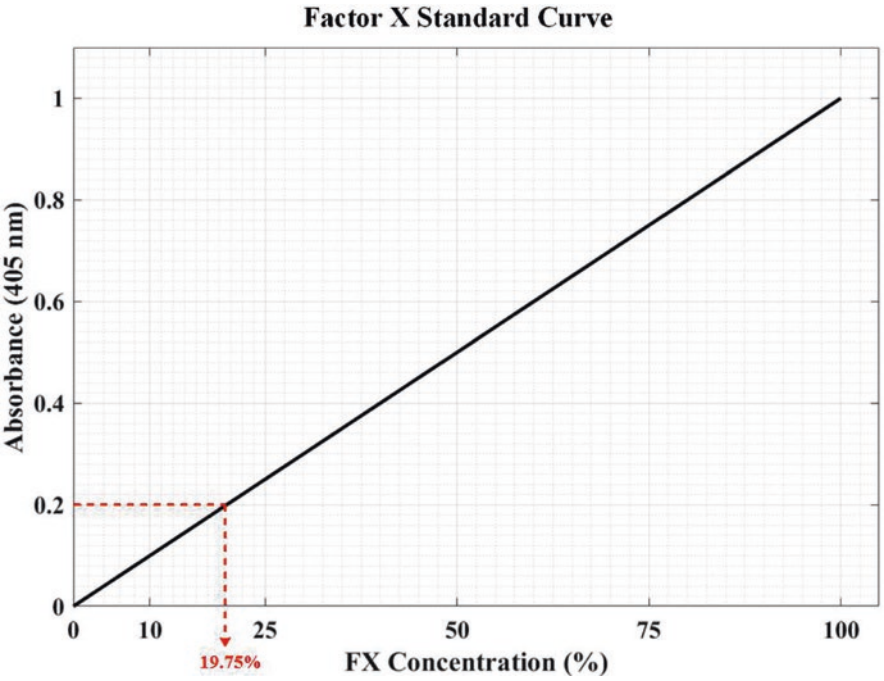

Fig. 12.13 The FX standard curve of the chromogenic assay. The standard curve is drawn using the standard absorbance values against the FX concentrations and based on the absorbance of the patient (OD = 0.2), the FX concentration of the patient is determined (FX:C 19.75%). Each standard and patient dilutions should be performed in duplicate and averaged

standard curve (Fig. 12.15). Each standard and patient dilutions should be performed in duplicate.

Interfering variables All samples should be mixed well to form a homogeneous reaction mixture. Extreme shaking of the specimens causes the deposition of protein in the foam. Observing the time and temperature of incubation is very important. The icterus, hemolysis, and lipemic plasma may interfere with absorbance readings. In these cases, the same patient's plasma blank should be used.

12.8.3.5 Immunological Assays

Various immunological assays including radioimmunoassay, laser nephelometer, immunodiffusion, antibody neutralization, and electro immunoassay as well as enzyme-linked immunosorbent assay (ELISA) have been used for the detection of congenital FX Deficiency. *Radioimmunoassay* is a more objective method that measures very low plasma levels of FX antigen. It is noticeable that other plasma proteins do not interfere with this technique. *The laser nephelometer* is a rapid method in comparison to other immunological assays and may be used for the rapid evaluation of FX antigen. The possibility of several plasma testing in a batch is another

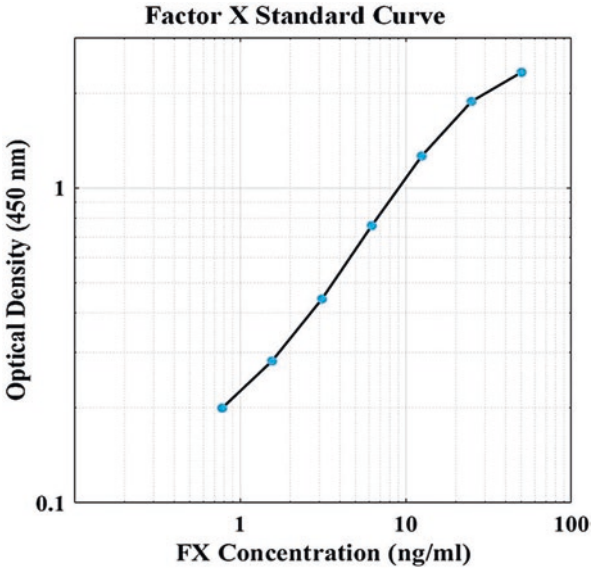

Fig. 12.14 The standard curve of FX antigen assay. The standard curve is plotted based on the optical density (OD) of the standards against FX antigen. The patient concentration of FX antigen is determined by the OD of patient plasma on the standard curve

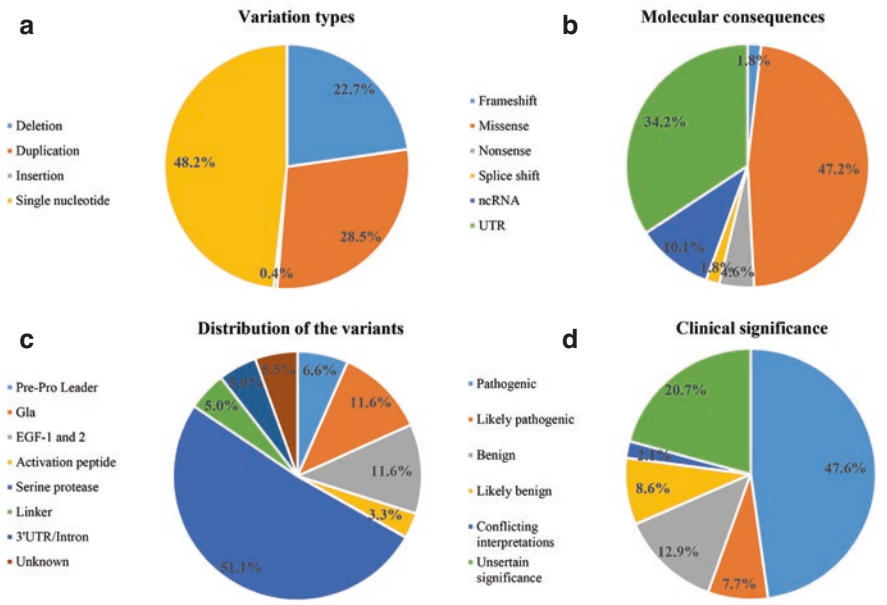

Fig. 12.15 Distribution and clinical significance of the recognized 180 mutations within the *F10* gene. The (**a**–**d**) charts represent relative frequency of the variations types, molecular consequences, and relative distribution of the variants across the protein domains as well as the clinical significance of the variants identified within the *F10* gene, respectively

advantage of this assay, though it needs a relatively large amount of antiserum. *ELISA* is one of the most commonly used immunological assays.

12.8.3.6 The Principle of the Enzyme-Linked Immunosorbent Assay (ELISA)

In ELISA assay, a polyclonal antibody against FX is coated at the bottom of the microplate wells. The standard and patient's plasma are applied and FX binds to the coated antibodies. After washing the microplate, unbound antibodies and materials are removed, a peroxidase conjugated antibody is added to bind to the captured antigen. After washing, a TMB substrate is added and the generated color is measured at 450 nm. Finally, the standard curve is plotted based on optical density (OD) of standards against standards concentrations of FX antigen (commercial standards). The patient concentration of FX antigen is determined by OD of the patient plasma on the standard curve (Fig. 12.14).

Interpretation The FX:Ag is reduced in *Type I* and *IV* of FX deficiency but type *II* and *III* with dysfunctional FX protein (CRM positive) have normal or nearly normal FX: Ag levels [3, 37, 38, 41].

FX antigen levels are reduced to approximately 50% of normal in patients being treated with vitamin K antagonists, e.g. warfarin although FX activity is lower [42]. The reduction in FX antigenic levels in such patients may reflect an increased catabolism of the acarboxylated form or to a reduced secretion by the hepatocyte. In addition to routine coagulation assessments, using other processes including *thrombin generation* and *fibrin polymerization* as well as *thromboelastography* to evaluate the total blood coagulation pathway dynamically is useful in the diagnosis of FX deficiency.

It is noteworthy that the reliability of results of FX assays depends on both preanalytical and analytical variables for example amount and type of anticoagulant, sampling, centrifuging of the sample, preservation of reagents and instruments as well as using suitable quality controls in analytical phase [14].

Anti-FXa assay is a chromogenic method monitoring the level of low molecular weight heparin (LMWH). LMWH binds to antithrombin specifically and more effectively inactivates FXa than thrombin. The remaining FXa in the patient's plasma cleaves a chromogenic substrate and produces color. The intensity of the produced color is measured by a spectrophotometer. The intensity of color is inversely associated with the amount of LMWH. It should be noticed that in the case of renal failure or due to contamination of the sample with heparin, the level of anti-Xa may be overestimated. This method is also appropriate to monitor direct oral FXa inhibitors if a suitable standard curve is available [43].

12.9 Important Points

1. Immunological assays measure only antigenic levels of FX, so cases with dysfunctional FX protein without any reduction of antigenic level may be missed. During warfarin therapy, both antigenic and activity levels of FX are decreased to about 50%, although the functional levels may be lower [13].
2. It is noticeable that the FX level in newborns and infants under 6 months is lower than in adults. Therefore, FX assay results should be calculated according to age- and gestational age-matched reference ranges. The FX level in healthy term newborns ranges from 0.12 to 0.68 IU/mL and increases gradually during the first 6 months.
3. FX level can be affected by liver disease and vitamin K deficiency so it is necessary to rule out acquired causes of FX deficiency. On the other hand, vitamin K deficiency in preterm or term neonates may complicate the diagnosis of FX deficiency, especially in cases with mild deficiency. Therefore, a re-assessment should be done after replacement therapy with vitamin K and also at 6 months of age [13, 18].
4. Human (15/102) is a new reference reagent for FXa which has established by WHO Expert Committee on Biological Standardization in October 2017. Each vial has a potency of 6.7 units. Currently, several laboratories use an existing non-WHO Reference Material (75/597) for the measurement of FXa and calibration of local standards. It is a bovine FXa reference material provided by the National Institute for Biological Standards and Control (NIBSC. Since it was not formulated by NIBSC and there was no information about the uniformity or stability of the content of the vials using it can be inaccurate. On the other hand, due to noticeable differences between the coagulation and chromogenic activity of bovine and human FXa, using a bovine standard to assess human FXa can cause discrepancies in the assay [44].

12.9.1 Molecular Diagnosis

12.9.1.1 F10 Gene Mutations and Its Clinical Significance/ Genotype Phenotype Correlation

During the last two decades, several *F10* gene mutations have been identified and the majority were unique and restricted to special families. DNA amplification and direct sequencing mostly are used to find underlying mutations. Therefore, establishing a genetic diagnosis method is challenging and costly, identification of recurrent variants of each population is more practical and also is helpful in reducing the use of direct sequencing, but it is not applicable because in most families the mutations are unique.

FX has a leading role in the coagulation cascade common pathway as absolute lack of FX seems to be incompatible with life. Some surveys on ***F10* gene** knockout mice have proven that the complete absence of FX is lethal for mice, but it is different in humans. Patients with the undetectable activity of FX as a result of

null mutations can survive, although they are dependent on constant replacement therapy. However, mice with FX activity levels of 1–3% of normal show a complete rescue of lethality. In **2019**, **Ferrarese et al.** investigated the structure and function of FX in a neonate who had almost complete FX deficiency presenting with life-threatening ICH due to underlying molecular defects of *Trp421Ter (missense, c.1382G>A) and Leu211Pro (nonsense, c.752 T>C)*. Interestingly, the trace level of FX:C was sufficient to arrest a lethal phenotype, though the patients needed regular prophylaxis because of life-threatening breakthrough bleeds. Moreover, maternal transferring of a trace amount of FX might be effective for embryonic survival. The variants of *Glu29Lys and Phe31Ser in the Gla domain* are other examples. They are not potentially severe defects, but some situations such as the deficiency of vitamin K and required cofactors during pregnancy causing imperfect carboxylation of the Gla domain and FX binding defect can compromise the patient's condition. This concomitant presence of congenital and acquired deficiency of FX can be prenatally lethal [3, 5, 45]. There is no hot spot region in the *F10* gene, and the number of mutations occurring in each exon relatively is proportional to its length; however, most mutations happen in exon 8 [3, 14, 16]. Almost all types of mutations such as deletion, nonsense, missense, splice site, and frameshift have been reported in the ***F10* gene** in which *missense single-point mutations, mostly localized in Gla and catalytic domains (exons 2, 7, 8)*, are the most reported *F10* gene lesions (Fig. 12.15).

According to the reports, more than half of the identified variants within the F10 gene are pathogenic or likely pathogenic that can be along with clinical manifestations (Fig. 12.8d) [46]. Generally, *missense mutations* are the most frequent variants that account for most congenital bleeding disorders. Based on a published report from **Harris et al. in 2021**, approximately 46% and 16% of pathogenic mutations cause phenotypically type I and type II of FX deficiency, respectively whilst 38% of the variants have unknown phenotypes. Most **type II-causing mutations are distributed** in the catalytic (~64%) and Gla (~14%) domains, while the **type I-causing mutations** are more frequently distributed in the EGF-1 and EGF-2 domains. The way the variants are distributed within the FX protein domains highlights the point that catalytic and Gla domains are involved in the functional activity, while EGF-1 and EGF-2 domains have a central role in the term of correctly-FX protein folding. Accordingly both FX:C and FX:Ag levels are reduced in type I FXD, but the level of FX:C is predominantly lower than FX:Ag in type II FXD.

There are also some reports of FX deficiency as a part of vitamin K-dependent clotting factors deficiency due to some defects in either γ-glutamyl carboxylase (*GGCX*) or subunit 1 of vitamin K epoxide reductase complex (*VKORC1*) (please refer to Chap. 9). Among different reported *F10* gene mutations, some mutations are in association with severe and life-endangering manifestations. *Gly380Arg, IVS7-1G>A,* and *Tyr163delAT* mutations are associated with a *high incidence of ICH*, and *Gly-20Arg mutation* is associated with *severe hemarthrosis*. Also, some mutations such as *Leu32pro, Glu102Lys, and Gly114Arg* are more common in patients with *mild deficiency* of FX [3, 4, 6, 13, 14, 18, 26, 28, 47].

1. **Chromosomal abnormalities/gene deletions**

   The first molecular abnormality affecting the *F10* gene was 13q34 monosomy reported by **Scambler and Williamson**, **1985** who described a patient having a concomitant deficiency of FX and FVII, while her brother was trisomic for 13q34 and had increased levels of FX and FVII. Interestingly *FX* chromosomal deletions affecting both the F10 and the F7 genes which are positioned close to each other form part of the 13qsyndrome. *San Antonio* arises from an 838-T single nucleotide deletion that leads to frameshift mutation and production of a stop codon at residue 232. *San Giovanni Rotondo FX* is also due to a single nucleotide deletion (556-C) that leads to the creation of a stop codon in residue 226.

2. **Point mutations**

   The following ten mutations are the most frequent variants occurring in the *F10* gene, the first four mutations are associated with *type I* FXD whilst the rest are associated with *type II* FXD:

   Factor X Voralberg (type I): two variants Glu14Lys (c.160G>A) and Glu102Lys (c.424G>A), Known as *FX Voralberg* were reported by Watzke in 1990 for the first time. Glu14Lys occurs in the Gla domain (Exon 2) and interferes with FX activation by FVIIa/TF and FIXa/FVIIIa complexes, while Glu102Lys involves the EGF-2 domain (exon 5). In homozygous cases of *FX Voralberg*, *FX activity is <10% (PT based) and the antigen level is about 20%.*

   FX Santo Domingo (type I): It was reported by Watzke in 1991. The variant results from c.61G>A substitution leading to the replacement of glycine at position −20 to arginine in the Pre-Pro leader domain (Gly-20Arg, exon 1), and interferes with FX cleavage signal peptidase impairing FX secretion into the endoplasmic reticulum. *FX activity is <1% and the antigenic level is <5% in the homozygous stat*e [3].

   Gly222Asp (type I): This mutation, reported by **Peyvandi et al. in 2002**, occurs in c.785G>A substitution and involves the serine protease domain (exon 7). It disrupts the protein folding and catalytic activity due to its vicinity to the catalytic triad.

   FX Friuli (type II): FX Friuli was the first dysfunctional mutant FX and the best well-characterized mutation in the *F10* gene reported by **Girolami et al. in 1970**. It came from a northeastern region, named Friuli in Italy and all the recognized patients originated from this area, never reported in other areas of Italy or other countries. Pro343Ser (c.1147C>T) causes new hydrogen bridge formation between Ser343 and Thr318 in the catalytic domain. So the tertiary structure of the domain and its catalytic potential are affected. FX Friuli is characterized by FX normal antigen level and near-normal Stypven time, but prolonged PT and aPTT-based clotting assays. It occurs in both heterozygous and homozygous states. The *FX:C level is about 3–6%* in Friuli cases which seems adequate to arrest hemarthrosis and brain hemorrhage, usual symptoms in the classical form of FX deficiency. Another difference between these two conditions is the heterozygous cases where heterozygote cases diagnosed with Friuli type and have

FX:C level of 40–60% sometimes experience mild bleeding tendency while the heterozygote cases of the classic disease are commonly asymptomatic.

FX Riyadh (type II): Glu51Lys substitution, was reported by **Al. Hilali et al. 2007**, involves the EGF-1 domain (exon 4) disturbing the structure folding and activity of FX.

FX Stockton (type II): Asp282Asn (c.964G>A) was reported by **Messier et al. in 1996**. The substitution effects residue 282 in the catalytic triad and FX catalytic activity.

FX Marseille (type II): Ser334Pro (c.1120C>T) was reported by **Bernardi et al. 1994** affects the serine protease domain (exon 8) causing the reduction of FX activation by FIX and RVV. It hinders the cognition of FX by its activators.

FX Nagoya II (type II): Gly366Ser (c.1216G>A) that was identified by **Miyata et al. in 1998** is due to the substitution of Gly366 in the serine protease domain (exon 8) by a larger hydrophilic residue, serine, possibly affecting the substrate-binding and losing FX activity.

Gly381Asp (type II): The substitution of Gly381 residue by Asp in the serine protease (exon 8) disturbs key active site interactions causing the loss of FX activity and function. Because the side chain of Asp has a bulky charged nature. The variants were recognized by **Camire et al. in 2001** [3, 4, 6, 48–51].

Leu211Pro (type I): It is reported by **Ferrarese et al. in 2019**. The residue of Lue 251 is located in a highly conserved hydrophobic region in the serine protease domain (exon 7). Its substitution by proline would change the residue hydrophobicity of the region and affect the native structure of FX protein. FX folding, secretion, and activity properties are disrupted by this alteration, though they are not fully suppressed. The variants of Gly204Arg, Gly380Arg, and Cys364Arg also involve the catalytic domain in a manner similar to Leu211Pro, resulting in a very low FX level in plasma. Gly204Arg or FX Debrecen (c.730G>A, exon 6) was identified first by **Bereczky et al. in 2008**. The change of Gly204 which is located in a small disulfide-bridge loop induces structural changes in terms of default secretion. Gly380 is a conserved residue in the catalytic domain among the serine protease family including FVII, FIX, FX, and protein C, even among different species. In the alteration of Gly380Arg or FX Padua III (**reported by Vianello et al. in 2001**), the side chain of Arg may interact with the catalytic triad and change its function. Cys364Arg **(reported in 2000 by Millar et al.)** interferes with a highly conserved intra-chain disulfide bond in the serine protease domain (exon 8) disturbing the native FX folding and stability [4, 45, 52].

FX Nice I (type I): It arises from Met-40Val (c.1A>G) substitution in the pre-pro leader (exon 1). FX Nice I usually occurs in concomitant with *FX Nice II* (Pro304Ser, c.1030C>T), a substitution in the serine protease domain (exon 8).

The reported levels *The FX:C and FX:Ag were about 4% (based on PT assay) and 7%,* respectively, in the patients. The replacement of methionine with valine results in the elimination of the ATG start codon and the complete loss of translation of FX, while the Pro304Ser mutation destabilizes the FX hydrophobic core interfering with the interactions between EGF-2 and protease domain. It results in a defective FX secretion. Both mutations were recognized by **Miyata et al. in 1998** [3, 4, 6, 52].

FX St. Louis II: The variant of Glu7Gly (c.140A>G) in the Gla domain (exon 2) was reported by **Rudolph et al. in 1996**. The replacement of a Gla by glycine causes the loss of one Gla residue affecting the Ca^{2+} binding interactions in the molecule. It does not affect the synthesis or secretion of FX protein. FX St. Louis II presents with a *normal level of FX:Ag and FX:C less than 1%.*

FX Frankfurt I (type I): Glu25Lys (c.193G>A), reported by **Huhmann et al. in 1998**, occurs in the Gla domain (exon 2). It was identified in a patient with *FX:C and FX:Ag levels of 56% and 55%,* respectively [3, 6, 48].

Ser322Asn (type II): it is a novel homozygous missense mutation in the serine protease domain (exon 8), reported by **Lu et al.** in a Chinese family, **in 2020**. The substitution of highly conserved Ser322 by Asn (c.1085G>A) probably destabilizes the catalytic structure leading to the *reduction of FX:C level and secretion* [4, 52, 53].

Leu487Phe (type II): It is a novel homozygous variant in the catalytic domain (exon 8), reported by **Fallah et al. in 2022** in a male Iranian patient presenting with *FX:C level of 13%*. The mutation probably reduces the flexibility and also increases the number of H-bond in the catalytic domain [54].

Cys17Arg and Gln135:* These two new variants were reported by **Borhany et al.** in two Pakistani patients in **2018**. Cys17Arg (c.169T>C), identified at a compound heterozygous state with Gly380Arg, occurs in the Gla domain (exon 2) and apparently disrupts the disulfide bond formation (Cys57-Cys62). The homozygous nonsense variant of Gln135* (c.523T>C) in the linker segment (exon 6) was recognized in an infant with *delayed ICH, at 10 days of birth*. This mutation results in premature chain termination and a *lack of functional FX in plasma* [52, 55].

Ser322Asn and Tyr344Ter (type II): It is a compound heterozygote of two missense and termination mutations, reported **by Lu et al.** in a Chinese pedigree in **2020**. The proband presented with moderate bleeding, prolonged PT and aPTT, and *FX:C level of 1.7%*. They occur in the serine protease domain (exon 8) causing destabilization of the catalytic site structure that leads to reduced activity and secretion of FX protein [52, 56].

Characterizations of some of the FX gene mutations are listed in Table 12.6.

Table 12.6 Characterizations of some FX gene mutations

Name	Mutation	Involved exon and domain	Type of variant	Type of FXD/no of patients	Clotting assay			FX:C	FX:Ag	Comments
					PT	aPTT	RVVT			
Nice I	Met-40Val (c.1A>G)	Exon 1/ preproregion	Missense	I/3	↓	ND	ND	ND	↓	Start codon is destroyed by loss of Met, first report by Miyata et al. 1998
Nice II	Pro304Ser (c.1030C>T)	Exon 8/ catalytic domain	Missense	I/2	↓	ND	ND	ND	↓	Hydrophobic core is destabilized, problem in secretion, first report by Miyata et al. 1998
Stuart	Val246Met (c.856G>A)	Exon 7/ catalytic domain	Missense	I/1	↓	↓	↓	↓	↓	First report by Perry 1997
Santo Domingo	Gly-20Arg (c.61G>A)	Exon 1/ pre-propeptide	Missense	I/21	↓	↓	↓	↓	↓	Disturbing the FX cleavage, preventing of the secretion, first report by Watzke et al. 1991
St Louis II	Glu7Gly (c.140A>G)	Exon 2/Gla domain	Missense	II/2	↓	↓	ND	ND	N	Disturbing Gla domain interactions, Affects Ca^{2+} binding, first report by Rudolph et al. 1996
Ketchikan	Glu14Gly (c.161A>G)	Exon 2/Gla domain	Missense	I/1	↓	↓	↓	ND	↓	Changing of FX affinity for Ca^{2+} binding, first reported by Kim, Thompson and James 1995
Vienna	Gly204Glu (c.731G>A)	Exon 6/ activation domain	Missense	I/1	↓	↓	ND	↓	↓	First reported by Watzke et al. 1993
Voralberg	Glu14Lys (c.160G>A)	Exon 2/Gla domain	Missense	I/16	↓	↓	↓	↓	↓	Interfering with FX activation by FIXa/FVIIIa and FVIIa/TF complexes, first reported by Watzke et al. 1990
Voralberg	Glu102Lys (c.424G>A)	Exon 5/ EGF-2	Missense	I/12	↓	↓	↓	↓	↓	First reported by Watzke et al. 1990
Malmo 4	Glu26Asp (c.198G>C)	Exon 2/Gla domain	Missense	U/U	↓	↓	ND	↓	ND	First reported by Wallmark et al. 1991

Tokyo	Glu32Gln (c.214G>C)	Exon 2/Gla domain	Missense	II/U	↓	NN	↓	ND	N	Affects Ca^{2+} binding Preventing of standard carboxylation of FX, interfering with processing, first report by Zama et al. 1999
Wenatchee I	Arg139Cys (c.535C>T)	Exon 6/ activation domain	Missense	II/U	↓	↓	↓ND	↓	↓	Preventing FX proteolytic cleavage and standard processing, first reported by Kim et al. 1995
Wenatchee II	Asn57Thr (c.290A>C)	Exon 4/EGF	Missense	II/U	↓	↓	↓ND	↓	↓	First reported by Kim et al. 1995
Kurayoshi	Arg139Ser (c.535C>A)	Exon 6/ activation domain	Missense	II/2	↓	↓	↓	N	N	Preventing FX proteolytic cleavage and standard processing, disturbing FX interactions with FVa and FVIIIa, first reported by Iijima et al. 2001
Nagoya I	Arg306Cys (c.1036C>T)	Exon 8/ catalytic domain	Missense	I/U	↓	↓	ND	ND	↓	Alpha-helix is destroyed, first reported by Miyata et al. 1998
Nottingham	Ala404Thr (c.1332G>A)	Exon 8/ catalytic domain	Missense	II/3	↓	↓	↓	ND	N	Inducing conformational alteration, interfering with the nearby disulfide bond, probably causing complete unfolding of the protein, first reported by Deam et al. 2001
Taunton	Arg405Gly (c.1335C>G)	Exon 8/ catalytic domain	Missense	II/5	BL	BL	BL	ND	N	Disturbing catalytic activity and local conformation, first reported by Deam et al. 2001
Marseille	Ser334Pro (c.1120C>T)	Exon 8/ catalytic domain	Missense	II/14	↓	↓	↓	↓	N	Reduction of FX activation by FIXa and RVV, first reported by Bernardi et al. 1994
Stockton	Asp282Asn (c.964G>A)	Exon 8/ catalytic domain	Missense	II/15	↓	↓	ND	↓	N	Affecting catalytic activity of FX, first reported by Messier et al. 1996

(continued)

Table 12.6 (continued)

Name	Mutation	Involved exon and domain	Type of variant	Type of FXD/no of patients	Clotting assay			FX:C	FX:Ag	Comments
					PT	aPTT	RVVT			
Friuli	Pro343Ser (c.1147C>T)	Exon 8/ catalytic domain	Missense	II/25	↓	↓	NN	↓	N	Changing in FX catalytic domain structure, hindering the activation, first reported by Girolami et al. 1970
Roma	Thr318Met (c.1073C>T)	Exon 8/ catalytic domain	Missense	II/7	↓	↓	N	↓	N	Interfering with catalytic activity without changing in the structure, first reported by De Stefano et al. 1988
Frankfurt1	Gla25Lys (c.193G>A)	Exon 2/Gla domain	Missense	I/U	↓	N	↓	ND	↓	First reported by Nöbauer-Huhmann et al. 1998
Unnamed	Asp63His (c.307G>C)	Exon 4/ EGF-1	Missense	U/3	ND	ND	ND	↓	ND	Disruption with β-hydroxylation at residue 63, loss FX function, first reported by Karimi et al. 2008
Unnamed	Leu-34Ile (c.19C>A)	Exon 1/ preproregion	Missense	U/U	↓	↓	ND	ND	↓	First reported by Camire et al. 2001
Unnamed	Gly323ser (c.1087G>A)	Exon8/serine protease	Missense	I/3	↓	↓	ND	ND	↓	Conformational changes and misfolding of the protein, first reported by Millar et al. 2000
Unnamed	Asp368Asn (c.1222G>A)	Exon8/serine protease	Missense	U/U	↓	↓	ND	ND	↓	First reported by Camire et al. 2001
Unnamed	Gly381Asp (c.1262G>A)	Exon8/serine protease	Missense	II/13	↓	↓	ND	ND	↓	Interfering with active site interactions, FX activity, and function, First reported by Camire et al. 2001
Unnamed	Arg-1Thr (c.119G>C)	Exon 2/ preproregion	Missense	II/6	↓	↓	↓	N	N	Disrupting FX cleavage by signal peptidase, production of dysfunctional FX, first reported by Peyvandi et al. 2002

Unnamed	Cys81Tyr (c.362G>A)	Exon 4/ EGF-1	Missense	I/2	↓	↓	↓	↓	↓	Disulfide bond formation, EGF-1 domain folding, and overall structure of FX are disrupted, first reported by Peyvandi et al. 2002
Unnamed	Gly94Arg (c.400G>A)	Exon 5/ EGF-2	Missense	I/5	↓	↓	↓	↓	↓	Interfering with FX structure and function, first reported by Peyvandi et al. 2002
	Asp95Glu (c.405C>A)	Exon 5/ EGF-2	Missense	I/2	↓	↓	↓	↓	↓	First reported by Peyvandi et al. 2002
Unnamed	Cys109Tyr (c.446G>A)	Exon 5/ EGF-2	Missense	I/2	↓	ND	ND	ND	↓	Disulfide bond is disrupted, interfering with folding and overall structure of FX, first reported by Millar et al. 2000
Unnamed	Thr-2Met (c.116C>T)	Exon 2/ pre-proleader	Missense	I/1	↓	ND	ND	ND	↓	First reported by Millar et al. 2000
Unnamed	Cys111Tyr (c.452G>A)	Exon5/EGF-2	Missense	I/1	↓	ND	ND	ND	↓	Disulfide bond is disrupted, interfering with folding and overall structure of FX, first reported by Millar et al. 2000
FX Debrecen	Gly204Arg (c.730G>A)	Exon 6/serine protease	Missense	I/10	↓	↓	ND	ND	↓	Changing the structure and interfering with the secretion of FX, first reported by Bereczky et al. 2008
Unnamed	Glu264Lys (c.910G>A)	Exon 8/serine protease	Missense	I/3	NN	ND	ND	ND	NN	First reported by Millar et al. 2000
Unnamed	Arg287Trp (c.979C>T)	Exon 8/ catalytic domain	Missense	U/1	↓	ND	ND	ND	ND	Changing overall FX electrostatic potential, affects FVa binding, first reported by Cooper et al. 1997

(continued)

Table 12.6 (continued)

Name	Mutation	Involved exon and domain	Type of variant	Type of FXD/no of patients	Clotting assay			FX:C	FX:Ag	Comments
					PT	aPTT	RVVT			
Unnamed	Glu310Lys (c.1048G>A)	Exon 8/ catalytic domain	Missense	I/3	↓	ND	ND	ND	↓	First reported by Millar et al. 2000
Unnamed	Gly323Ser (c.1087G>A)	Exon 8/ catalytic domain	Missense	I/3	↓	ND	ND	ND	N	Conformational changing and the protein mis-folding, first reported by Millar et al. 2000
Unnamed	Cys364Arg (c.1210T>C)	Exon 8/ catalytic domain	Missense	I/5	↓	ND	ND	ND	↓	Disulfide bond formation is disrupted, disturbing folding and stability of FX, first reported by Millar et al. 2000
Unnamed	His383Gln (c.1269C>G)	Exon 8/ catalytic domain	Missense	U/1	ND	ND	ND	↓	ND	Substitution of His is near to catalytic domain, affecting catalytic triad interactions, disrupting FX catalytic activity, first reported by Odom et al. 1994
Unnamed	Trp421Arg (c.1381T>C)	Exon 8/ catalytic domain	Missense	U/1	ND	ND	ND	↓	ND	First reported by Odom et al. 1994
Unnamed	Gly380Arg (c.1258G>A)	Exon 8/serine protease	Missense	I/10	ND	ND	ND	↓	ND	Immediately after the active site, causes formation of a new hydrogen bond with Ala234, causes ICH, first reported by Vianello et al. 2001
Unnamed	Tyr163delAT	Exon 6/ activation peptide	Frameshift	I/U	↓	↓	↓	↓	↓	Interruption with reading frame leading to a stop codon 163 in exon 6, premature chain termination, degradation of the truncated protein, causes ICH, first reported by Herrmann et al. 2005

Unnamed	Glu32Asp	Exon 2/Gla domain	Missense	U/U	ND	ND	ND	ND	ND	First reported by Girolami et al. 2019
Unnamed	Cys17Arg (c.169T>C)	Exon 2/Gla domain	Missense	U/1	ND	ND	ND	ND	ND	Disrupting the formation of the disulfide bond and FX structure, first reported by Borhany et al. 2018
Unnamed	Cys17Phe (c.170G>T)	Exon 2/Gla domain	Missense	I/1	ND	ND	ND	ND	ND	Disrupting the formation of the disulfide bond and FX structure, first reported by Mitchell et al. 2019
Unnamed	p.Glu77* (c.349G>T)	Exon 4/ EGF-1	Nonsense	I/2	ND	ND	ND	ND	ND	Premature chain termination, first reported by Arita et al. 2018
Unnamed	Cys81Arg (c.361T>C)	Exon 4/ EGF-1	Missense	I/3	ND	ND	ND	ND	ND	Interfering with disulfide bond formation, EGF-1 folding, and FX structure, first reported by Jin et al. 2018
Unnamed	Cys206Arg (c.736T>C)	Exon 6/serine protease	Missense	I/6	ND	ND	ND	ND	ND	Interfering with disulfide bond formation and FX structure, first reported by Mitchell et al. 2019
Unnamed	Leu211Pro (c.752T>C)	Exon 7/serine protease	Missense	I/1	ND	ND	ND	ND	ND	The native FX structure is disrupted, but the secretion or activity of FX is not completely suppressed, first reported by Ferrarese et al. 2019
Unnamed	p.Gly298= (c.894C>T)	Exon 8/serine protease	Silent	None/U	N	N	N	N	N	Polymorphism, first reported by Borhany et al. 2018

(continued)

Table 12.6 (continued)

Name	Mutation	Involved exon and domain	Type of variant	Type of FXD/no of patients	Clotting assay			FX:C	FX:Ag	Comments
					PT	aPTT	RVVT			
Unnamed	Tyr279His (c.955T>C)	Exon 8/serine protease	Missense	II/1	ND	ND	ND	ND	ND	Localization of the residue in the domain interferes with catalytic activity of FX, first reported by Mitchell et al. 2019
Unnamed	Ser322Asn (c.1085G>A)	Exon 8/serine protease	Missense	II/5	ND	ND	ND	ND	ND	Destabilization structure of FX, reduction in activity and secretion, first reported by Lu et al. 2020
Unnamed	P.Tyr344* (c.1152C>A)	Exon 8/serine protease	Nonsense	II/4	ND	ND	ND	ND	ND	Destabilization structure of FX, reduction in activity and secretion, first reported by Lu et al. 2020
Unnamed	p.Gln371* (c.1231C>T)	Exon 8/serine protease	Nonsense	I/1	ND	ND	ND	ND	ND	Premature chain termination, first reported by Mitchell et al. 2019
Unnamed	Gly410Arg (c.1348G>A)	Exon 8/serine protease	Missense	II/1	ND	ND	ND	ND	ND	Catalytic activity is affected without interfering with the native structure, first reported by Mitchell et al. 2019
Unnamed	p.Trp421* (c.1382G>A)	Exon 8/serine protease	Nonsense	I/1	ND	ND	ND	ND	ND	Premature chain termination, first reported by Mitchell et al. 2019
Unnamed	Cys50Arg (c.268T>C)	Exon 4/ EGF-1	Missense	I/1	ND	ND	ND	ND	ND	Disturbing FX structure, first reported by Mitchell et al. 2019
Unnamed	c.70+4A>G	Splice site	Splice site	U/1	ND	ND	ND	ND	ND	Formation of a new splice site, first reported by Ferrarese et al. 2019
Unnamed	c.71-1G>C	Splice site	Splice site	U/1	ND	ND	ND	ND	ND	First reported by Mitchell et al. 2019
Unnamed	c.257-1G>C	Splice site	Splice site	U/1	ND	ND	ND	ND	ND	Abolishing of donor/acceptor splice site, first reported by Mitchell et al. 2019

Unnamed	Lue487Phe	Exon 8/ catalytic domain	Missense	U/1	ND	ND	ND	↓	ND	Reduction in flexibility and increasing H-bond numbers, negative effects of the function of FX, first reported by Fallah et al. 2022
Deletion	Del (exon 2)	Exon 2/Gla domain	Deletion	U/1	ND	ND	ND	ND	ND	First reported by Mitchell et al. 2019
Deletion	Complete deletion of gene and partial deletion including exon 7–8	–	–	–	↓	↓	↓	ND	↓	Probable germline mosaicism in the father
San Antonio	c.813delC	Exon 7/serine protease	Frameshift	I/1	↓	N	ND	↓	↓	leading to stop codon, truncated protein is not functional, first reported by Reddy et al. 1989
San Giovanni Rotondo	c.556delC	Exon 6/ activation peptide	Frameshift	I/7	↓	↓	↓	↓	N	leading to stop codon at residue 226, limited synthesis or secretion of truncated protein, first reported by Simioni et al. 2001
Deletion	17 bp deletion in exon 8	Exon 8/ catalytic domain	–	–	↓	ND	ND	ND	↓	Additional mutation at GTG > ATG Val298Met

PT prothrombin time; *aPTT* activated partial thromboplastin time; *RVVT* Russell viper venom rime; *FVa* activated factor V; *FVIIIa* activated factor VIII; *FX:Ag* factor X antigenic assay; *FX:C* factor X activity by chromogenic assay; *FX* factor X; *N* normal; *ND* no data; *BL* borderline; *ICH* intracranial hemorrhage; *NN* near normal; *U* unknown

12.10 Prenatal Diagnosis (PND)

Prenatal diagnosis (PND) can be used for families at risk of having a severely affected infant in cases when both parents are carriers or have at least one affected child. To diagnose an affected fetus, chorionic villous sampling (CVS) is performed at weeks 10–12 of gestation, and then extracted DNA is analyzed to determine the parents' underlying mutations. In special geographic areas with recurrent mutations, molecular characterization, carrier detection, and prenatal diagnosis remain key steps for genetic counseling and education. So establishing a mutation-screening method may be helpful to reduce the rate of affected offspring [14].

12.11 Diagnosis of Acquired Factor X Deficiency

In the case of dealing with a patient suffering from plasma cell dyscrasias and/or Al amyloidosis concomitant with bleeding, acquired FX deficiency should be considered, and investigating the patient's coagulation profile is essential. On the other side, when a patient refers with known or new-onset coagulopathy without any personal or family history, the related underlying diseases to acquired FX deficiency should be concerned as a differential diagnosis. Prolonged PT and aPTT with normal bleeding time are suspicious and more study is required; the level of FX:C and FX:Ag should also be measured along with hematological assessment. Besides, correction or not of PT and aPTT with normal plasma, in the mixing study, can differentiate the acquired FX deficiency with inhibitor presence (e.g. lupus anticoagulant or AiFXD with neutralizing specific FX antibody) from the other acquired types.

It is worth noting in this regard, that the levels of FDPs and D-dimer are increased moderately in Al-amyloidosis, while they are normal or slightly elevated in AiFXD; therefore these parameters are valuable in the differential diagnosis. Plasmin-α_2-plasmin inhibitor complex (PIC) in a hyperfibrinolytic state and thrombin-anti-thrombin complex (TAT) in a hypercoagulation state can be mentioned as other valuable parameters in the differential diagnosis between Al amyloidosis and AiFXD [57, 58].

12.12 Treatment

The proportion of patients with FX deficiency that require treatment is higher than most other rare bleeding disorders according to UK Hemophilia Centre Doctors' Organization (UKHCDO) registry data. Management of FXD can be difficult as evidence-based guidelines are lacking. The UK Haemophilia Centre Doctors' Organisation (UKHCDO) has published guidelines on the management of FX deficiency (and other rare inherited bleeding disorders) based upon a literature review and personal experience. The main mode of therapy in patients with congenital FX deficiency is on-demand therapy (stopping hemorrhage as soon as possible) whilst regular prophylaxis is used in patients with the risk of severe hemorrhagic manifestations. Replacement therapy in congenital FX deficiency is guided by the particular hemorrhagic episode. Current therapeutic options for the management of FXD

patients include antifibrinolytic agents; tranexamic acid and ε-aminocaproic acid (EACA) and blood-derived products; solvent detergent treated fresh frozen plasma (FFP), prothrombin complex concentrate (PCC), FIX products, and plasma-derived FX concentrate (pdFX) [13, 18, 21, 59, 60]. The biological half-life of FX is 20–40 h so an adequate level can be achieved with repeated infusions. Factor levels of 10–20 U/dL are generally sufficient for hemostasis, even in the immediate postoperative period although some data suggest that levels of 5 IU/dL may be sufficient for adequate hemostasis and the thrombogram would suggest that levels of >10 U/dL are sufficient to restore thrombin generation to normal.

12.12.1 Antifibrinolytic Agents

They are indicated in mucous membrane bleeding including epistaxis, oral bleeding, and menorrhagia and can be quite effective either alone or as adjunctive therapy to blood products or FX concentrates.

Aminocaproic acid is usually administrated either as mouthwash (15 mL every 6 h) or orally taken (50–100 mg/kg every 6 h) to control nosebleeds and bleeding from the oral cavity.

Tranexamic acid can be quite effective in managing menorrhagia. It is prescribed orally in dosage of 20–25 mg/kg/6–8 h or IV in a dose of 10–15 mg/kg/6–8 h or as local TAMW (tranexamic acid mouthwash).

Nosebleeds Quick Release™ powder (Biolife; LLC, Sarasota, FL, USA) is a hydrophilic polymer that is administrated for the management of epistaxis [14, 18].

12.12.2 Blood-Derived Products

12.12.2.1 Nonspecific FX Replacement Therapy

Fresh Frozen Plasma (FFP) Although virus-inactivated FFP is an option to control both traumatic and spontaneous bleeding, it is not universally available. A level of 10–20% of FX:C is sufficient for normal hemostasis, so given that the half-life of FX is 20–40 h in plasma, doses of 3–6 mL/kg twice a day increase FX:C level to 10–20%. In cases of surgery and active or severe bleeding, a dosage of 15–20 mL/kg of FFP is required [3, 14, 18, 20]. Due to the low or actually unknown concentration of other clotting factors in FFP, a large volume is required to achieve the hemostatic level of FX; therefore fluid overload is a challenging issue, especially in children and elderly patients suffering from cardiovascular disease. Allergic reaction, elevated risk of transfusion-associated lung injury (TRALI), and delayed efficacy are other concerns. Antihistamines can be given in patients with a history of allergy and using plasma only from men and/or non-multiparous women, as well as using diuretics and slower infusion rate can help in managing these complications [13, 14, 18, 61, 62].

Prothrombin Complex Concentrates (PCC) Virally inactivated PCC is plasma-derived concentrates in which there are three (FII, FIX, and FX) or four (FII, FIX, FVII, and FX) clotting factors and also some anticoagulants such as heparin, anti-

thrombin and protein C, S, and/or Z. It is noticeable that different products contain different amounts of each clotting factor, or even more, product batches may be variable in the contents. This variability can be challenging for severe deficient patients who need frequent infusions.

PCC contains a 1:1 ratio of FX/FIX that is administrated to manage bleeding diathesis in patients with FX deficiency. By a dose of 1 IU/kg, PCC increases the level of FX:C to 1.5%, and due to FX's long half-life (about 30 h), a daily infusion is not necessary. In case of severe deficiency, a daily dosage of 20–30 IU/kg PCC is expected to elevate the FX activity level by 40–60 IU/dL; however, the optimal dose should be determined with regard to the type of bleeding and residual level of FX:C [3, 14, 18]. The efficacy of PCC has been shown in various bleeding scenarios including control of minor bleeding diathesis, hemostatic maintenance in operations, and prophylactic treatment in severe FX deficient patients. It is important to note that PCC also contains an unknown amount of other activated clotting factors (FVIIa, FIIa, and FIXa), so it may be associated with a high risk of thrombotic complications. As FII and FX (60 and 30 h respectively) have long half-lives recurrent infusion of PCC may result in a cumulative effect Therefore, during long-term treatment or in case of regular prophylaxis, monitoring of FIX and FX and D-dimer should be monitored. Several factors can predispose to thrombosis related to PCC including the quality of products, patients' risk profile, and infusion dose and rate. Thrombotic complications have been reported more in acquired coagulation factor deficiencies rather than congenital ones. PCC also should be administrated with caution in cases of liver disease, major trauma, large hematoma, and antithrombin deficiency as well as in neonates [62, 63]. It is noticeable that the concomitant use of antifibrinolytic as tranexamic acid and PCC is contraindicated due to the high risk of thromboembolism (Table 12.7) [13, 14, 18].

Table 12.7 PCC concentrates available in the United State, Canada, or Europe and their FX content

Name of the product	The content of FX (IU)
Profilnine®, three-factor	100
Prothrombinex® VF, three-factor	100
Prothromplex TIM3, three factor	100
Prothromplex total, four-factor	100
Proplex-T, four-factor	100
Confidex, four-factor	100–200
Beriplex® P/N, four-factor	110–190
Octaplex®/Ocplex®, four-factor	72–120
Cofact, four-factor	56–140
Kcentra®, four-factor	125–165
Kanokad, four-factor	56–140
Kaskadil, four-factor	160
Uman complex DI, three-factor	80
FEIBA NF or VH	Not available

12.12.3 Factor IX/X Products

In view of several complications related to PCCs and FFP administration, freeze-dried concentrates containing specific quantities of human FIX and FX were developed to retrieve these factors' deficiencies. Several products have been introduced to the market including:

- **Factor X P Behring (CSL Behring AG, Bern, Switzerland):** a dual-factor powder containing 800–1200 IU and 600 IU human FX and FIX respectively, with heparin and antithrombin III. The efficacy of the product has been approved by a recent trial involving 10 FX-deficient patients who were on prophylactic treatment for 1 year (a dosage of 20 IU/kg once a week). *It is the only approved dual-factor product for FX deficiency treatment and is licensed just in Switzerland for now.*
- **Factor IX HS® (ZLB Behring [now CSL Behring])** another two-factor product, pasteurized and derived from plasma containing 800 IU and 1200 IU human FX and FIX respectively. According to Greifswald Registry, the efficacy of this product on seven FX-deficient patients taking regular prophylactic treatment to control the bleeding episodes was satisfactory.
- **Immune VH (Baxter AG, Vienna, Austria):** a lyophilized human single-FIX powder which is highly purified and virus-inactivated. It also contains a smidgen amount of other clotting factors including FII, FVII, and FX (<0.02 IU per IU of FIX).
- **AlphaNine® SD (Grifols Biologicals, Inc, Los Angeles, CA, USA):** another purified freeze-dried human single-FIX concentrate, virus-inactivated and derived from plasma, also containing trace amounts of FII, FVII, and FX (<0.05, <0.04, and <0.05 U/IU FIX respectively).

When using these products, associated complications such as thrombosis, nephrotic syndrome, and hypersensitivity reactions as well as the development of inhibitors should be considered [14, 60, 62].

Fibrin Glue Fibrin glue is generally used to facilitate local hemostasis, particularly in sites of surgery [18, 20].

12.12.3.1 Specific Factor X Products

High-Purity Human Plasma-Derived Factor X Concentrate (pdFX) The first high-purity and high-potency single-factor FX concentrate (pdFX) with the commercial name of Coagadex (Bioproducts Laboratory, Elstree, UK) was approved by the Food and Drug Administration (FDA) in 2015. Coagadex is manufactured from plasma of healthy and virally negative (hepatitis A, B, and C and HIV-1 and HIV-2 viruses) donors. Virus-inactivated pdFX is produced as a lyophilized powder that is injected after reconstitution with sterile water. It contains 100 IU/mL FX, less than 1 IU/mL of FII and FIX, and no added proteins. pdFX activity level is more than 100 IU/mg protein by using the chromogenic FX activity assay.

Indication of Coagadex It is indicated in the United States (in adults and children) and Europe (in all age groups) for on-demand and prophylactic treatments of bleeding episodes, perioperative management of bleeding and in order to achieve sufficient hemostasis in subjects with congenital FX deficiency. Coagadex could be administrated as pre-operative management in patients with mild FX deficiency although its efficacy for major surgery in patients with moderate and severe deficiency has not been investigated. It should be avoided in patients with allergies to any components of the product.

The safety, clinical efficacy, and pharmacokinetics of pdFX were assessed in two open-label, multicenter, and nonrandomized phase III trials, Ten01 and Ten03. Moreover, the efficacy of pdFX as prophylaxis was evaluated in the phase III TEN02 study and the TEN05 retrospective study (Table 12.8) [60, 62, 64].

12.12.3.2 TEN01

The first study (Ten01) was conducted on a population of 16 patients (208 bleeding episodes) aged >12 years with moderate to severe FX deficiency (<5 IU/dL) that experienced ≥1 bleed in the last year. pdFX was administrated at a dose of 25 IU/kg for the baseline pharmacokinetics (PK1) measurement including recovery rate and half-life. Then the patients are treated on-demand for spontaneous, traumatic, and heavy menorrhagia bleeding diathesis for more than 6 months and till >1 bleeding episode had been treated. After which, the secondary endpoints including PK2, and the number of required infusions, were evaluated. The mean recovery rate was 2.0 IU/dL per IU/kg, and the terminal half-life ($t_{½}$) of pdFX was approximately 30 h suggesting that a prophylactic regime once or twice infused weekly is adequate (depending on the required activity level of FX). *The results of pdFX efficacy were good or excellent in treating 98.4% of the 187 bleeding episodes.* The score of 'excellent' was described as stopping obvious bleeds or menorrhagia with one dose within 12 h or with ≤2 doses within 48 h, while the 'good' score meant overt bleeding and menorrhagia stopping with ≤2 doses within 24 h with 3 or 2 doses within 48 h. Clearance (CL) of pdFX through the hepatic route is slow so it remains in plasma for a long time. It is noteworthy that the PK of pdFX depends on the plasma volume as well as the body weight of patients. Therefore, it varies from younger children to older children and adults.

12.12.3.3 TEN02

This study was designed with the aim of assessing the potency of pdFX in reducing/preventing and/or treating the bleeds in nine children aged <12 years with FX:C <5 IU/dL and a history of severe bleeding. All patients received beginning, routine prophylaxis and end doses of 50, 40–50 and 50 IU/kg respectively, for ≥26 weeks. Totally, 537 prophylactic infusions with the mean dose of 38.8 IU/k every 3.1 days were taken by all patients. The mean IR was 1.74 IU/dL per IU/kg and all patients maintained levels >5 IU/dL after the fourth visit. *The pdFX preventing efficacy was 'excellent' meaning that the patients indicated no minor or major bleeding or that it was lower than expected based on the patient's history and condition.*

Table 12.8 Summary of data of clinical studies of Coagadex

Name of the study	Ten01 (NCT00930176)	Ten03 (NCT01086852)	Ten02 (NCT01721681)	Ten05
Design	Multicenter, nonrandomized, open-label, and prospective phase III trial	Multicenter, nonrandomized, open-label, and prospective phase III trial	Multicenter, nonrandomized, open-label, and prospective phase III trial	Retrospective, multicenter, open-label, international
Patients	– 16 patients aged ≥12 years with severe or moderate congenital FXD (FX:C <5 IU/dL) – Required replacement therapy for ≥1 spontaneous bleed	Two patients aged ≥12 years with plasma FX:C <20 IU/dL	– Nine patients aged <12 years with moderate or severe congenital FXD (FX:C <5 IU/dL) – History of severe bleeding – Or an *F10* mutation causing a severe bleeding phenotype	15 patients (13 aged ≥12 years and two patients aged <12 years) with moderate or severe congenital FXD
Aim of the study	– Overall efficacy assessment and safety in the management of bleeding diathesis up to 2 years – On-demand treatment for ≥6 months – Short-term preventing therapy – PK after a single dose of 25 IU/kg – Perioperative management for the surgical patients	Perioperative management for the surgical patients	Assessment of pdFX in preventing/treating bleeding episodes over ≥26 weeks	Assessment of pdFX based on compassionate use between March 30, 2011, and December 31, 2015, for: – Routine prophylaxis – On-demand treatment – Short-term preventing – perioperative hemostatic coverage

(continued)

Table 12.8 (continued)

Name of the study	Ten01 (NCT00930176)	Ten03 (NCT01086852)	Ten02 (NCT01721681)	Ten05
Dosing	– The mean dose per injection: 25.3 IU/kg – The mean total dose: 30.4 IU/kg – Supporting dose of 25 IU/kg twice a week for prophylaxis in adolescents and children	–	– A beginning and last dose of 50 IU/kg on day one and end of the study – Routine prophylaxis with 40–50 IU/kg twice weekly with adjustment of dose and frequency through week 6 to maintain a trough level FX:C >5 IU/dL	– The specific dosing was tailored to each patient and the dosing regime was decided by the investigator – Mean dose per infusion 32.5 IU/kg for routine prophylaxis
Findings	– Mean half-life: 29.4 h – Mean IR: 2.00 IU/dL per IU/kg – Excellent or good efficacy for on-demand management of episodes: 98% of cases	– Excellent efficacy for the management of blood loss before surgery: 100% – No bleeding after surgery – No need for blood transfusion – Expected or less than expected blood loss	– Overall mean IR: 1.74 IU/dL per IU/kg – Excellent efficacy in all the patients – No patient experienced minor or major bleeding or the frequency of bleeding was lower than expected based on the patient's medical history	– The investigators rated the efficacy of pdFX as 'excellent' in all patients – The overall median bleeding rate per patient per month was 0.04 in the prophylaxis group compared with 0.8 in the on-demand group
Complications	– Mild headache – One subject with pain in the injection site – One subject with erythema in the injection site, fatigue and back pain	No possibly complications	No serious adverse reaction was reported	Not mentioned

FX factor X; *FX:C* factor X functional activity; *PK* pharmacokinetic; *IR* incremental recovery

12.12.3.4 TEN03

The second study investigated the pre-operatively treatment of Coagadex in two surgical patients aged >12 years with mild to severe FX deficiency (FX:C <20 IU/dL).

12.12.3.5 TEN05

TEN05 was a multicenter data-collection study on 15 patients with moderate or severe congenital FX deficiency to assess the efficacy of pdFX for on-demand and routine prophylactic treatment, short-term prevention and perioperative management for 5 years. Thirteen patients aged ≥12 and two patients were <12 years from whom seven patients were on prophylaxis, seven patients took on-demand treatment and one patient was between these two conditions. The dosing regime was decided by the researchers and designed for each patient. A total of 1239 infusions were taken as prophylactic treatment with a mean dose of 32.5 IU/kg per infusion. Four out of eight patients who were on prophylaxis experienced 17 bleeds totally. *The effectiveness of pdFX was rated as 'excellent' in all patients meaning that pdFX regularly met or overstepped expectations.*

- The most commonly reported adverse event was a mild headache in the TEN01 study, while erythema and pain in the site of fusion, back pain, and fatigue were observed in both TEN01 and TEN03 studies. No associated cases of thrombosis, inhibitor development, safety concerns, and serious side reaction were reported in clinical studies.
- In brief, according to the evidence, the effectiveness and tolerability of pdFX were promising for children<12 years, adults, and adolescents. *The recommended doses for prophylaxis are 25 IU/kg twice per week for adolescents and adults and 40 IU twice a week in children <12 years.*
- As Coagadex is manufactured from human plasma, there may be contamination with infectious agents such as viruses or the agent of Creutzfeldt-Jakob disease (theoretically). Also, because possibly the formation of FX inhibitors and other complications by using Coagadex, closely monitoring and dosages adjustment of clinical responses and FX levels, via suitable laboratory, assays are necessary [60, 62, 64–27].

12.13 Management of Factor X Deficiency in Surgery

The target level of FX in surgery is variable in different patients according to the residual FX level in the plasma and based on the type and duration of surgery. A satisfactory surgery could be achieved in severely affected patients with FX deficiency (FX <1%) by carefully using FFP, PCC, or other products. A level of 20 IU/dL FX in plasma seems to be adequate for hemostasis and controlling of bleeding, so a loading dose of 15–20 IU/kg PCC followed by 10–15 IU/kg daily or every other day for minor surgery is recommended (Table 12.9) [13, 14, 63].

Table 12.9 Recommended dose of different products for the management of bleeding episodes or prophylaxis patients with factor X deficiency

	Products	Recommended dose
Mucosal bleeding	TA	10 mL of a 5% solution as mouthwash every 8 h
	FFP	A loading dose of 10–20 mL/kg followed by 3–6 mL/kg twice a day to keep FX:C level above 10–20 IU/dL[a]
	PCC	1 IU/kg, PCC raises FX:C level by 1.5%[b]
Acute bleeding in severe factor X deficiency	FFP	A loading dose of 10–20 mL/kg followed by 3–6 mL/kg twice a day to keep FX:C level above 10–20 IU/dL
	PCC	20–30 IU/kg once a day
	pdFX	25 IU/kg (patients aged ≥12 years)
Surgery in severe factor X deficiency[c]	FFP	15–25 mL/kg, an alternative if PCC is unavailable
	PCC	A loading dose of 15–20 IU/kg followed by 10–15 IU/kg after surgery
Prophylaxis	PCC (FIX)	15–40 IU/mL two or three times per week 20–70 IU/mL weekly
	FX	15–20 IU/kg weekly

TA tranexamic acid; *FFP* fresh frozen plasma; *PCC* prothrombin complex concentrate; *pdFX* plasma-derived factor X; *FX* factor X

[a] Level of 10–20 IU/dL FX seems to be sufficient for hemostasis; however, some reports suggest that 5 IU/dL FX may also be adequate

[b] As biological half-life of FX is 20–40 h daily infusion is not usually required

[c] Replacement therapy is not required in patients with factor IX level >10% without significant bleeding history

[d] Generally a daily dose is sufficient; however, in minor surgery, a dose every other day may be adequate

12.14 Treatment in Women: Pregnancy, Delivery, and Menorrhagia

Menorrhagia occurs in more than 50% of women with FX deficiency. Therapeutic options for the control of menorrhagia include medical treatments (such as antifibrinolytics, oral contraceptives, levonorgestrel intrauterine device, and clotting factor replacement), and surgical treatments (such as endometrial ablation and hysterectomy). In particular, tranexamic acid was reported to be useful (tranexamic acid 15 mg/kg every 8 h, in practice 1 g every 6–8 h, may be effective when taken for the duration of the menstrual period).

Affected women are also at risk of gynecologic problems including hemoperitoneum and corpus luteum bleeding in relation to ovulation.

12.14.1 Factor X Deficiency in Pregnancy

Pregnancy is accompanied by increased concentrations of FX. However, there are some concerns regarding FX-deficient women during their childbearing period. Recurrent abortion and placental abruption appear only in cases with severe

deficiency. Their opportunity for fertilization is lower than the normal population because of either higher frequency of bleeding in some organs such as the ovary or prolonged menstruation, especially in women with FX levels less than 1%. Also, they are at high risk of severe hemorrhagic complications following some necessary invasive procedures, e.g., chorionic villus sampling (CVS), amniocentesis, or axillary reproductive techniques. A sampling of CVS and amniocentesis are required for prenatal diagnosis (PND). Another concern is the mode of inheritance of FX deficiency so in countries with a high rate of consanguinity, either affected or mandatory carrier offspring would be expected more than in other areas [67].

Therefore, for FX-deficient pregnant women or who want to try pregnancy, a team of experts and an adequately equipped centre preferably a hemophilia treating center or a tertiary hospital are required for appropriate management of patients during pregnancy and labor. In such conditions, more affected women can experience low-risk pregnancy and delivery of healthy infants [3, 67].

Women with severe FX deficiency and a history of adverse outcome such as abortions, placental abruption, or premature births may benefit from replacement therapy throughout pregnancy. However, the associated risk of thrombosis must be carefully evaluated, particularly with the use of PCCs that contain appreciable quantities of coagulation factors other than FX. It is of relevant importance to note also that heterozygote subjects were reported to have bleeding after delivery without prophylactic replacement therapy, which required treatment with FFP. However, management of women with FX deficiency requires additional monitoring of the hemostatic parameters and awareness of the increased risk of bleeding with any surgical intervention.

During pregnancy, sufficient replacement therapy is required to prevent adverse outcomes such as miscarriage, preterm birth, and placental abruption. In severely affected patients (FX:C <2 IU/dL), who are in the third trimester of pregnancy and require a cesarean section, a loading dose of 20–40 IU/kg, PCC is required to achieve FX:C >4 IU/dL and then followed by 10–20 IU/kg once a day to keep FX:C >3 IU/dL for at least 3 days [68].

Among patients participating in the TEN01 study, ten women and girls were registered that received a total of 267 pdFX infusions, 178 for on-demand (a mean dose of 30.5 IU/kg per bleed) and 89 for prophylactic treatment. A total of 149 bleeding episodes were treated in which the pdFX efficacy score was “excellent,” “good,” “poor,” and “un-assessable” in 116, 13, 2, and 1 episodes, respectively. The overall efficacy score was 97.7% for the women and girls. Generally, in this study, women and girls took more infusions than men and boys (average 2.48 vs. 1.62 per month).

FX evaluation is required either before delivery or at birth (cord blood sample) to reduce the risk of hemorrhage in neonates. Further, severely affected neonates should carefully be evaluated by cranial ultrasound due to the high risk of ICH and then re-evaluated and screened at 6 months of age. Prophylactic care also must be considered a necessity during the neonatal period.

No trauma during expected vaginal delivery or using ventouse, or forceps during delivery.

Circumcision and vaccination should be postponed till the baby is screened.

Replacement therapy is not usually required to manage moderately factor X deficiency (FX:C >2 IU/dL) without significant bleeding history (despite hemostatic challenges). However, any prior hemostatic challenges related to bleeding history and the kind of surgery must be considered.

12.14.2 Recommendation for Management of Women with FX Deficiency

- Women suffering from severe deficiency and a history of unsuccessful delivery or unfortunate outcome should be treated with aggressive FX replacement.
- Pregnant women with heterozygous mutations should be evaluated for the risk of bleeding before delivery.
- Women with severe deficiency who experienced recurrent bleeding or unfavorable outcomes during pregnancy should receive prophylactic therapy before delivery.
- Based on the recommendations from UK guidelines, a level of FX:C >30 IU/dL should be achieved and maintained following delivery in women with a history of bleeds and low FX:C level, and also for women who need a cesarean operation [13, 40, 62].

12.15 Prophylaxis

As FX deficiency is one of the most severe rare bleeding disorders, prophylaxis is recommended for those patients at risk of severe bleeding including CNS, GI bleeding, hematoma, and hemarthrosis particularly in patients with FX <0.05 IU/mL. Previous data on thrombin generation in patients with FX deficiency showed that roughly 5% of residual FX in plasma might be enough to obtain at least 50% of thrombin generation and to prevent severe bleeding. Based on these data, an individual prophylaxis could be planned targeting this level. According to a case series data from the Greifswald registry, nine FX-deficient patients took a dose of 15–20 IU/kg Factor IX HS concentrate (once a week in seven patients and every other in two patients) leading to a significant reduction of hemorrhage, especially in children. Another case series showed that the bleeding episodes were significantly reduced in 10 patients with severe FX deficiency who received 20 IU/kg Behring Factor X P concentrate per week as prophylaxis [13, 14, 62, 69]. If PCC is used, doses of 15–40 IU/mL twice or three times a week are shown as more effective than 20–70 IU/mL weekly. Also, four neonates and three children with severe FX deficiency presenting with umbilical cord bleeding and ICH achieved hemostasis by receiving a dose of 50–70 IU/kg PCC once or twice a week [62, 69]. As described before, the efficacy of pdFX for prophylactic treatment was evaluated in two TEN02 and TEN05 studies (please refer to Sect. 12.12.3.3 and 12.12.3.5). Similar to on-demand treatment, no serious complications and inhibitor development were

reported in patients receiving prophylaxis [62]. The different prophylactic strategies described in these studies demonstrate clearly that the clinical levels of FX required to achieve adequate hemostasis, and the dose and frequency of factor administration, remain to be determined.

A prophylactic approach could also be crucial to prevent CNS bleeding at birth in families already with one severely affected child.

12.16 Intracranial Hemorrhage (ICH)

It is one of the most challenging bleeding sites in patients (generally neonates) diagnosed with severe congenital FX deficiency **(53% had FX activity <0.01 U/mL)**. 9–26% of cases, especially with a Gly380Arg mutation developed cerebral bleeding or ICH. Some cases received prophylactic therapy in the form of FFP and PCC. Dosages of 40–70 IU/kg FIX once or twice per week in some patients and 50 IU/kg activated PCC twice a week in another report have been shown promising, though the infusion of FFP has been insufficient. There is a report of successful treatment with pdFX in a young male patient with severe FX deficiency presenting with subdural hematoma [62].

12.17 Prognosis

By early identification and diagnosis of patients, bleeding complications of FX deficiency would be effectively managed. Special consideration is also required for affected women and children. On the other hand, in heterozygous cases, genetic counseling may be greatly helpful to prevent the birth of affected offspring [18].

12.18 Acquired Factor X Deficiency

In addition to an inherited form of the disease, FX deficiency may occur secondary to other disorders such as AL amyloidosis, myeloma, tumors, infections, and drug consumption as well as liver diseases [3]. Acquired deficiency of FX may present either as an isolated deficiency or along with other factors deficiencies, as FV deficiency [13, 20].

1. **Liver disease or vitamin K deficiency**

   The deficiency of vitamin K due to its malabsorption or oral anticoagulants may result in FX deficiency. On the other hand, affected patients with dysfunctional liver or hepatocellular damages such as hepatocellular carcinoma (HCC) are also at risk of acquired FX deficiency. However, in such disorders, there is a concomitant decrease of other hepatic-derived or vitamin K-dependent coagulation factors [13, 26].

2. **AL amyloidosis**

For the first time, Korsan-Bengsten et al. reported a case of amyloidosis with concomitant FX deficiency. They described an association between amyloidosis and FX deficiency. Since then, more similar cases were reported in several studies, and the correlation between systemic amyloidosis and FX deficiency became clearer [3].

Amyloidosis is a general name to define some clinical disorders in which insoluble abnormal fibril proteins are aggregated extra- or intracellularly on the affected tissues leading to functional defects of involved organs. Primary or systemic light-chain amyloidosis that is also called AL amyloidosis is a myeloma-associated disorder. It is characterized by the deposition of immunoglobulins' abnormal light chains that involves variable organs and may lead to thrombotic and hemorrhagic complications [70]. Isolated FX deficiency is the most clinically significant coagulopathy in association with primary amyloidosis that occurs in 6.3–14% of cases [59]. Several mechanisms are accounted for the underlying pathophysiology of acquired FX deficiency including the irreversible binding of FX to the abnormal paraproteins or adsorption by amyloid fibrils with subsequent sequestration in the liver, spleen, and vasculature leading to rapid clearance of both endogenous and exogenous FX from plasma. FX activation may also be interrupted by a specific FX inhibitor (IgG4-mediated). Therefore in these situations, either antigenic or functional levels of FX are reduced, although the reduction in the level of FX:C is more profound than FX:Ag [13, 20, 57]. The most common clinical manifestations in acquired isolated FX deficiency similar to the congenital form are epistaxis, easy bruising, and menorrhagia. Amyloidosis is usually accompanied by other hemostatic disorders such as other clotting factor deficiencies due to liver damage, vascular fragility because of amyloid infiltration, and defects in platelet aggregation as well as fibrin polymerization. Therefore, it is not practically possible to determine an obvious correlation between the severity of the disorder and the FX:C level. Acquired FX deficiency in relation to secondary or AA amyloidosis is very rare [3, 13, 59].

3. **Miscellaneous**

Besides acquired FX deficiency due to AL amyloidosis, there are few reports of non-amyloid deficiency of FX [20]. Some malignancies such as myeloma without amyloidosis, acute myeloid leukemia, thymoma, as well as renal, adrenal, and gastric carcinoma may lead to a reduction in FX level. However, the exact mechanism is not clear. In this scenario, **Reynolds et al.** reported a rarely acquired FX deficiency in a patient with multiple myeloma in **2019.** The coagulation profile of the case revealed prolonged PT and aPTT that were corrected in the mixing study with normal plasma. There were reductions in both FX:Ag and FX:C levels, <2% and <10% respectively. This survey highlighted several significant points in these specific situations, for instance, the consideration of acquired coagulopathy in patients with multiple myeloma with or without amyloidosis, or conversely, attention to underlying multiple myeloma and/or amyloidosis in a patient with known or new-onset coagulopathy. It should be noted that the presence of amyloidosis is related to serious prognostic and therapeutic con-

sequences; so thorough evaluations for possibly underlying amyloidosis are valuable.

In addition to malignancies, a transient FX deficiency has been reported about viral or bacterial upper respiratory tract infections, especially due to mycoplasma pneumonia. It is probably due to similar antigenic determinants of FX and infectious agents. Therefore, circulatory FX is cleared by the patient's immune system [3, 13, 20, 57, 59]. Some medicines may also be in association with isolated reduction of FX as sodium valproate or treatment with fungicides and amsacrine [59]. Warfarin and other oral anticoagulants could be a cause of acquired FX deficiency along with the reduction of other coagulation factors [13].

12.19 Management of Acquired Factor X Deficiency

Usually acquired FX deficiency is a transient and short-lived condition, so that coagulation test may turn to normal without any special treatment. Treatment of the underlying cause of acquired FX deficiency is the first step in the management of acquired FX deficiency. Then based on the severity of the deficiency, a suitable therapeutic choice should be selected. However, due to shortened half-life of infused FX, as it is adsorbed by amyloid fibrils, administration of FFP, PCC, or other products increases FX level transiently and so improves the bleeding tendency just for short time; therefore, they are not actually useful. Nevertheless, infusion of human FX concentrate is preferred to FFP and PCC owing to possible side effects (thrombosis, hepatitis, allergic reactions, fluid overload, and TRALI). Administration of vitamin K is recommended in case of severe liver dysfunction. Antifibrinolytic agents may also be useful to improve the hemostatic system in some cases [40, 57, 59, 62]. Recombinant FVII (rFVII) is another useful product for primary amyloid-associated FX deficiency [26]. It is suggested that the level of FX:C be maintained at $\geq$10% to limit the risk of bleeding. For patients with life-threatening hemorrhage, the source of bleeding should be controlled immediately, and then, in the following step, the FX:C level must be kept above 50% by administration of FX concentrates twice weekly. Given that, a large amount of amyloid deposition fibrils aggregate in the spleen, splenectomy could be a highly beneficial therapeutic choice for AL amyloid-related FX deficiency that provides a long-term recovery of FX deficiency by restricting the FX rupture in the RES [40, 57, 59]. In cases of Al amyloidosis and plasma cell dyscrasias, a combination of induction chemotherapy (melphalan), followed by autologous stem cell transplant has been reported highly promising for long-lasting improvement of FX deficiency without the need for FX infusion.

Hemostatic coverage by using FFP, PCC, and vitamin K administration for AiFXD patients has been reported in the literature, though FFP and vitamin K had no true effect to arrest the bleeding episodes; it must have been due to the small amount of FX in FFP and also that the deficiency in vitamin K is not the cause of symptoms. As there is no hyper-fibrinolytic state in AiFXD subjects, opposing Al amyloidosis, antifibrinolytic agents do not have therapeutic potency for these patients. There are some reports of successful management of acquired FX

deficiency associated with inhibitors by using plasmapheresis and steroid therapy as well as intravenous immunoglobulins [3, 10, 58, 71]. It must be implied that there is no standardized therapy for acquired FX deficiency in general and infusion of pdFX is not yet licensed in these conditions [62].

12.20 Autoimmune Factor X Deficiency and Factor X Inhibitors

Although rare, coagulation factors may turn into targets for the immune system with the formation of autoantibodies resulting in bleeding; an extremely rare condition referred to as autoimmune coagulation factor deficiency (AiCFD). Noteworthy that, autoimmune FX deficiency (AiFXD) is less frequent than the others (AiFVIIID>AiFVD>AiFXIIID>AiVWFD). Some patients may be diagnosed with no underlying condition, whereas anti-FX autoantibodies in others might be either specific or non-specific due to the presence of lupus anticoagulant or may occur as a result of burns, leprosy, and infection disease mainly involving the respiratory tract. In the case of infectious diseases, treatment with antibiotics could be accounted for anti-FX auto-antibodies development. According to a report by **Akitada Ichinose et al. in 2021**, both neutralizing and non-neutralizing anti-FX antibodies may be detected in the patients. Neutralizing antibodies or FX-inhibitors bind to the FX in a Ca2+-dependent manner and inhibit the functional activity of FX leading to an extreme reduction in the specific activity of FX (FX:C <1%). Patients with non-neutralizing anti-FX antibodies also have extremely low FX:C levels though in the absence of FX inhibitors (presenting with an undetectable or invaluable titer of FX inhibitor). Reported patients in this study demonstrated soft tissues and intramuscular bleeding, and as many as 70% and 22% of the patients indicated Grade III and Grade II bleeding, respectively, emphasizing that AiFXD could be even more severe than congenital FX deficiency.

When a symptomatic patient presents with prolonged PT and aPTT as well as low FX activity regardless of the correction of mixing tests based on PT or aPTT, without a personal or family history of bleeding and taking any anticoagulant, AiFXD should be considered by the clinicians. Of great concern, since both neutralizing and/or non-neutralizing autoantibodies can cause AiFXD, a functional FX inhibitor assay is not sufficient to certainly diagnose antibodies and immunological examination should be performed. Moreover, AL-amyloidosis is the most important condition that should be concerned for differential diagnosis [3, 11, 14, 58, 59].

References

1. Mannucci PM, Duga S, Peyvandi F. Recessively inherited coagulation disorders. Blood. 2004;104(5):1243–52.
2. Roshanzamir F, Dorgalaleh A. Congenital factor X deficiency. Congenital bleeding disorders. Springer; 2018. p. 261–89.
3. Uprichard J, Perry DJ. Factor X deficiency. Blood Rev. 2002;16(2):97–110.

4. Harris VA, Lin W, Perkins SJ. Analysis of 180 genetic variants in a new interactive FX variant database reveals novel insights into FX deficiency. TH Open. 2021;5(04):e557–e69.
5. Karimi M, Menegatti M, Afrasiabi A, Sarikhani S, Peyvandi F. Phenotype and genotype report on homozygous and heterozygous patients with congenital factor X deficiency. Haematologica. 2008;93(6):934–8.
6. Perry D. Factor X and its deficiency states. Haemophilia. 1997;3(3):159–72.
7. Shen Q, Tang P, Ma M, Bao B. Insights into the structure of human blood coagulation Factor X. Med Res. 2019;3(3):190012.
8. Peyvandi F, Menegatti M, Santagostino E, et al. Gene mutations and three-dimensional structural analysis in 13 families with severe factor X deficiency. Br J Haematol. 2002;117(3):685–92.
9. Li F, Chen C, Qu S-Y, Zhao M-Z, Xie X, Wu X, et al. The disulfide bond between Cys22 and Cys27 in the protease domain modulate clotting activity of coagulation factor X. Thromb Haemost. 2019;119(06):871–81.
10. Versteeg HH, Spek CA, Peppelenbosch MP, Richel DJ. Tissue factor and cancer metastasis: the role of intracellular and extracellular signaling pathways. Mol Med. 2004;10(1–6):6.
11. Osaki T, Souri M, Ogawa Y, Sato H, Mitsui T, Ichinose A. Retrospective examination of coagulation parameters in 33 patients with autoimmune coagulation factor deficiencies in Japan: a single-center analysis. Thromb Res. 2022;213:154–62.
12. Papadaki S, Tselepis AD. Nonhemostatic activities of factor Xa: are there pleiotropic effects of anti-FXa direct oral anticoagulants? Angiology. 2019;70(10):896–907.
13. Christine A, Lee EEB, Hoots WK. Textbook of hemophilia. Blackwell Publishing Ltd; 2010.
14. Menegatti M, Peyvandi F, editors. Factor X deficiency. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2009.
15. Camire RM. Blood coagulation factor X: molecular biology, inherited disease, and engineered therapeutics. J Thromb Thrombolysis. 2021;52(2):383–90.
16. Mariani G, Herrmann F, Schulman S, Batorova A, Wulff K, Etro D, et al. Thrombosis in inherited factor VII deficiency. J Thromb Haemost. 2003;1(10):2153–8.
17. Jackson CM. Structure and function of factor X: properties, activation, and activity in prothrombinase. A retrospective in a historical context. J Thromb Thrombolysis. 2021;52(2):371–8.
18. Brown D, Kouides P. Diagnosis and treatment of inherited factor X deficiency. Haemophilia. 2008;14(6):1176–82.
19. Girolami A, Vettore S, Scarparo P, Lombardi A. Persistent validity of a classification of congenital factor X defects based on clotting, chromogenic and immunological assays even in the molecular biology era. Haemophilia. 2011;17(1):17–20.
20. Lee G, Duan-Porter W, Metjian A. Acquired, non-amyloid related factor X deficiency: review of the literature. Haemophilia. 2012;18(5):655–63.
21. Palla R, Peyvandi F, Shapiro AD. Rare bleeding disorders: diagnosis and treatment. Blood. 2015;125:2052.
22. Peyvandi F, Duga S, Akhavan S, Mannucci PM. Rare coagulation deficiencies. Haemophilia. 2002;8(308–21):6.
23. Karimi M, Yarmohammadi H, Ardeshiri R, Yarmohammadi H. Inherited coagulation disorders in southern Iran. Haemophilia. 2002;8(740–4):7.
24. Graham JB, Barrow EM, Hougie C, Stuart clotting defect. II. Genetic aspects of a "new" hemorrhagic state. J Clin Invest. 1956;36:497–503.
25. Girolami A, Scarparo P, Scandellari R, Allemand E. Congenital factor X deficiencies with a defect only or predominantly in the extrinsic or in the intrinsic system: a critical evaluation. Am J Hematol. 2008;83(8):668–71.
26. Menegatti FPM. Inherited deficiencies of coagulation factors II, V, V+VIII, VII, X, XI, and XIII. Williams hematology. 9th ed. Cenveo Publisher Services; 2016. p. 40.
27. Austin S, Brindley C, Kavakli K, Norton M, Shapiro A. Pharmacokinetics of a high-purity plasma-derived factor X concentrate in subjects with moderate or severe hereditary factor X deficiency. Haemophilia. 2016;22(3):426–32.

28. Herrmann F, Auerswald G, Ruiz-Saez A, Navarrete M, Pollmann H, Lopaciuk S, et al. Factor X deficiency: clinical manifestation of 102 subjects from Europe and Latin America with mutations in the factor 10 gene. Haemophilia. 2006;12(5):479–89.
29. Acharya S, Coughlin A, Dimichele DM. Rare bleeding disorder registry: deficiencies of factors II, V, VII, X, XIII, fibrinogen and dysfibrinogenemias. J Thromb Haemost. 2004;2(2):248–56.
30. Peyvandi F, Mannucci P, Lak M, Abdoullahi M, Zeinali S, Sharifian R, et al. Congenital factor X deficiency: spectrum of bleeding symptoms in 32 Iranian patients. Br J Haematol. 1998;102(2):626–8.
31. Borhany M, Shamsi T, Moiz B, Hasan K, Hashmi KZ, Ayyub M, et al. Guidelines on the laboratory diagnosis of congenital bleeding disorders in Pakistan. J Pakistan Med Assoc. 2012;62(5):477.
32. Markland FS. Snake venoms and the hemostatic system. Toxicon. 1998;36(12):1749–800.
33. Kaushansky K, Williams WJ, Joseph W. Williams hematology. McGraw-Hill Medical; 2010.
34. Latinović Z, Leonardi A, Koh CY, Kini RM, Trampuš Bakija A, Pungerčar J, et al. The procoagulant snake venom serine protease potentially having a dual, blood coagulation factor V and X-activating activity. Toxins. 2020;12(6):358.
35. Budzynski AZ. Chromogenic substrates in coagulation and fibrinolytic assays. Lab Med. 2001;32(7):365–8.
36. Kessler CM. A systematic approach to the bleeding patient: correlation of clinical symptoms and signs with laboratory testing. In: Kitchens CS, Kessler CM, Konkle BA, editors. Consultative hemostasis and thrombosis. 3rd ed. Philadelphia: Saunders, Elsevier; 2013. p. 16–32.
37. Kitchen S, McCraw A, Echenagucia M. Diagnosis of hemophilia and other bleeding disorders. World Federation of Hemophilia; 2010.
38. Perry DJ. Factor X and Factor X deficiency. Textbook of Hemophilia; 2014. p. 421–7.
39. Chatterjee T, Philip J, Nair V, Mallhi R, Sharma H, Ganguly P, et al. Inherited factor X (Stuart–Prower factor) deficiency and its management. Med J Armed Forces India. 2015;71(Suppl 1):S184.
40. Tabibian S, Motlagh H, Naderi M, Dorgalaleh A. Intracranial hemorrhage in congenital bleeding disorders. Blood Coagul Fibrinolysis. 2018;29(1):1–11.
41. Nagaya S, Akiyama M, Murakami M, Sekiya A, Asakura H, Morishita E. Congenital coagulation factor X deficiency: genetic analysis of five patients and functional characterization of mutant factor X proteins. Haemophilia. 2018;24(5):774–85.
42. Epstein DJ, Bergum PW, Bajaj SP, Rapaport SIJAjocp. Radioimmunoassays for protein C and factor X: Plasma antigen levels in abnormal hemostatic states. 1984;82(5):573–81.
43. Fareed J, Hoppensteadt DA, Fareed D, Demir M, Wahi R, Clarke M, et al., editors. Survival of heparins, oral anticoagulants, and aspirin after the year 2010. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2008.
44. Thelwell C, Hogwood J, Daniels S, Rigsby P. A new WHO reference reagent for activated blood coagulation factor X (FXa), human (15/102). J Thromb Haemost. 2020;18(1):255–7.
45. Ferrarese M, Baroni M, Della Valle P, Spiga I, Poloniato A, D'Angelo A, et al. Missense changes in the catalytic domain of coagulation factor X account for minimal function preventing a perinatal lethal condition. Haemophilia. 2019;25(4):685–92.
46. F10[gene]. 2022. https://www.ncbi.nlm.nih.gov/clinvar/?term=F10[gene].
47. Mota L, Shetty S, Idicula-Thomas S, Ghosh K. Molecular basis of factor X deficiency cases from India. Haemophilia. 2010;16(4):693–7.
48. Herrmann FH, Navarette M, Salazar-Sanchez L, Carillo JM, Auerswald G, Wulff K. Homozygous Factor X gene mutations Gly380Arg and Tyr163delAT are associated with perinatal intracranial hemorrhage. J Pediatr. 2005;146(1):128–30.
49. Saunders RE, O'Connell NM, Lee CA, Perry DJ, Perkins SJ. Factor XI deficiency database: an interactive web database of mutations, phenotypes, and structural analysis tools. Hum Mutat. 2005;26(3):192–8.
50. Factor X gene (F10) variant database. 2021. http://www.factorx-db.org/index.php.

51. Girolami A, Cosi E, Santarossa C, Ferrari S, Girolami B, Lombardi AM. Factor X friuli coagulation disorder: almost 50 years later. Clin Appl Thromb Hemost. 2018;24(1):33–40.
52. F10 gene. https://ghr.nlm.nih.gov/gene/F10#location.
53. Zhang X, Chen K, Wang G, Zhang C, Zhao B, Liu X, et al. Molecular mechanism of a novel Ser362Asn mutation causing inherited FX deficiency in a Chinese family. Int J Hematol. 2020;112(1):8–16.
54. Fallah A, Shams M, Agi E, Jazebi M, Baghaipoor MR, Naderi T, et al. Genotypes and phenotypes characterization of 17 Iranian patients with inherited factor X deficiency: identification of a novel mutation: Leu487Phe. Blood Coagul Fibrinolysis. 2022;33(2):75–82.
55. Borhany M, Buthiau D, Rousseau F, Guillot O, Naveena F, Abid M, et al. Genotyping of five Pakistani patients with severe inherited factor X deficiency: identification of two novel mutations. Blood Coagul Fibrinolysis. 2018;29(7):622–5.
56. Lu S, Lin W, Ji H, Su M, Zhao X, Wang C. A compound heterozygosis of two novel mutations causes Factor X deficiency in a Chinese pedigree. Acta Haematol. 2021;144(2):176–81.
57. Reynolds SB, Maghavani DP, Hashmi H. Acquired factor X deficiency in a patient with multiple myeloma: a rare case highlighting the significance of comprehensive evaluation and the need for antimyeloma therapy for bleeding diathesis. BMJ Case Rep CP. 2019;12(9):e230249.
58. Ichinose A, Osaki T, Souri M. Autoimmune coagulation factor X deficiency as a rare acquired hemorrhagic disorder: a literature review. Thromb Haemost. 2022;122(03):320–8.
59. Vaidya K, Arnott C, Biscoe A, Eggleton S, Brighton T. Differential diagnosis and therapeutic limitations in a rare case of acquired factor X deficiency. Case Rep Int Med. 2015;2(4):55.
60. Shapiro A. Plasma-derived human factor X concentrate for on-demand and perioperative treatment in factor X-deficient patients: pharmacology, pharmacokinetics, efficacy, and safety. Expert Opin Drug Metab Toxicol. 2017;13(1):97–104.
61. Batsuli G, Kouides P. Rare coagulation Factor deficiencies (factors VII, X, V, and II). Hematol/Oncol Clin. 2021;35(6):1181–96.
62. Peyvandi F, Auerswald G, Austin SK, Liesner R, Kavakli K, Román MTÁ, et al. Diagnosis, therapeutic advances, and key recommendations for the management of factor X deficiency. Blood Rev. 2021;50:100833.
63. Bolton-Maggs P, Perry D, Chalmers E, Parapia L, Wilde J, Williams M, et al. The rare coagulation disorders–review with guidelines for management from the United Kingdom Haemophilia Centre doctors' organisation. Haemophilia. 2004;10(5):593–628.
64. Payne J, Batsuli G, Leavitt AD, Mathias M, McGuinn CE. A review of the pharmacokinetics, efficacy and safety of high-purity factor X for the prophylactic treatment of hereditary factor X deficiency. Haemophilia. 2022;28:523.
65. io Products Laboratory Receives FDA approval for Coagadex® (Coagulation Factor X, Human). 2015. http://www.businesswire.com/news/home/20151021005989/en/Bio-Products-Laboratory-Receives-FDA-approval-Coagadex%C2%AE.
66. FDA Approves Coagadex®, First Factor X Concentrate. 2015. https://www.hemophilia.org/Newsroom/Medical-News/FDA-Approves-Coagadex-First-Factor-X-Concentrate.
67. Nance D, Josephson N, Paulyson-Nunez K, James A. Factor X deficiency and pregnancy: preconception counselling and therapeutic options. Haemophilia. 2012;18(3):e277.
68. Greipp PR, Kyle RA, Bowie E. Factor-X deficiency in amyloidosis: a critical review. Am J Hematol. 1981;11(4):443–50.
69. Mumford AD, Ackroyd S, Alikhan R, Bowles L, Chowdary P, Grainger J, et al. Guideline for the diagnosis and management of the rare coagulation disorders. Br J Haematol. 2014;167(3):304–26.
70. Thompson CA, Kyle R, Gertz M, Heit J, Pruthi R, Pardanani A. Systemic AL amyloidosis with acquired factor X deficiency: a study of perioperative bleeding risk and treatment outcomes in 60 patients. Am J Hematol. 2010;85(3):171–3.
71. Di Paola J, Nugent D, Young G. Current therapy for rare factor deficiencies. Haemophilia. 2001;7(s1):16–22.

Congenital Factor XI Deficiency, Diagnosis and Management

13

Simon Davidson

13.1 Introduction

Factor XI (FXI) deficiency, or hemophilia C, is a rare autosomal recessive injury-related hemorrhagic disorder, but some dominant cases have also been reported. Worldwide prevalence of the disorder is 1:1,000,000, but this rate is higher in some ethnicities such as Ashkenazi and Iraqi Jewish populations with a prevalence of 1:11 for heterozygotes and 1:450 for homozygotes or compound heterozygotes, affecting both males and females similarly [1–4]. The clinical symptoms are highly heterogeneous, and there is no direct relationship between bleeding tendency and residual plasma FXI level. Bleeding is usually observed in homozygotes and combined heterozygotes, while heterozygotes are generally asymptomatic. Sometimes, patients never experience any bleeding diathesis in their life. Bleeding is usually post-traumatic, postsurgical, postpartum, and in areas with high fibrinolytic activity such as the mucosal surface of the oral cavity [3–6]. FXI deficiency is classified into two phenotypes: in type I or CRM−, both FXI coagulant activity (FXI:C) and FXI antigen (FXI:Ag) is low; in type II or CRM+, FXI:Ag is normal, while the FXI:C is lower than the normal range. A wide spectrum of mutations has been identified within the *F11* gene. FXI deficiency in Ashkenazi Jews is mostly due to four common causative mutations that were categorized as types I to IV. The disorder based on FXI:C level is classified into three types, including mild, moderate, and severe [7–9]. FXI level is less than 20 U/dL in severe deficiency, while in mild and moderate deficiency, FXI level is usually between 20 and 70 U/dL. Due to the mild phenotype of the disorder, in most cases, the disorder is diagnosed based on family history or randomly in routine workup [3, 10, 11]. The main therapeutic strategy in

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_13

these patients is on-demand therapy, and the main therapeutic products that can be used to raise FXI levels in these patients are fresh frozen plasma (FFP) preferably solvent detergent treated (FFP-SD), FXI concentrate, and desmopressin [1, 2, 4, 12].

13.2 Factor XI Structure

F11 gene is located at the distal end of the long arm of chromosome 4 (4q35) and consists of 15 exons and 14 introns that span a 23-kb region. FXI protein has an 18 amino acid-leader peptide. Exon 1, exon 2, exons 3–10, and exons 11–15 encode the promoter region, signal peptide, 4 N-terminal apple domains, and C-terminal catalytic domain, respectively [10, 13–16]. FXI has a distinguished structure from other coagulation proteases. It is a 160-kDa homo dimer with a similar 607 amino acid sequence in both subunits. These two subunits are joined together by covalent and noncovalent bonds. Each subunit has four 90–91 amino acid sequences at the N-terminal, named apple domain (Ap1–Ap4) and a catalytic domain with 238 amino acids at the C-terminal with serine protease (SP) role that is homologous to the serine protease domains of other coagulation factors (Fig. 13.1) [15–19].

Apple domains form a disk structure in each subunit that provides a surface for the binding of platelet, high-molecular-weight kininogen (HMWK), and factor IX. Prekallikrein has a homolog structure with FXI in their apple domains. In the monomeric structure of FXI, each apple domain contains seven β-strands that form an antiparallel sheet and an α-helix is attached to the concave side of the sheet by two disulfide bonds. Another bond connects N-terminal to C-terminal. These four apple domains are adhesion sites for other molecules [16, 19, 20]. These include Ap1 and Ap2, which are adhesion sites for thrombin and HMWK, respectively, and Ap3 is a binding site for glycoprotein Ib, factor XI, and heparin. An Ap4 is an adhesion site for activated FXII (FXIIa) [14, 15, 21]. Ap4 is also necessary for

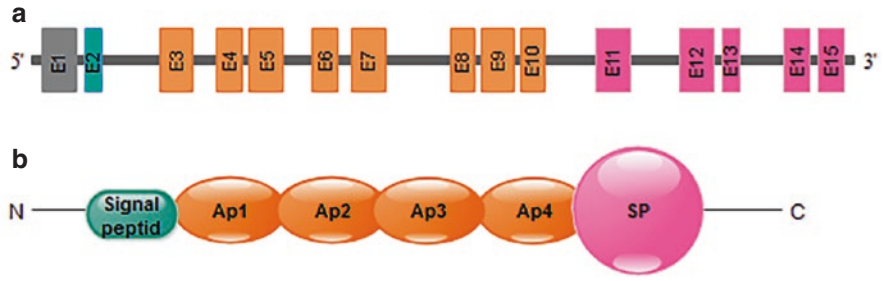

Fig. 13.1 (**a**) Factor XI gene structure: *F11* gene consists of 15 exons and 14 introns. Exons 1 and 2 encode promoter and signal peptide, respectively. Exons 3–10 encode 4 apple domains and exons 11–15 encode the serine protease domain. (**b**) Schematic structure of factor XI protein: Factor XI protein is composed of a signal peptide, 4 apple domains (Ap1–4) at the N-terminal, and a catalytic domain with serine protease activity at C-terminal. *N* N-terminal; *C* C-terminal; *Ap* apple domain; *SP* serine protease domain; *E* exon

dimerization. Catalytic domain that is in C-terminal site on the apple domains in a "cup and saucer" arrangement comprised of His413, Asp462, and Ser557 in a trypsin catalytic domain [20–22].

In circulation, FXI is the only SP in dimeric structure that is active in this form. Ap4 is a major domain for dimerization. Two Ap4 domains develop an interface disulfide bond between two monomer subunits. This bond is formed through Cys321 at a finger-like loop in Ap4 domain. Moreover, hydrophobic interaction between residues including Tyr329, Ile 290, and leu284 of the A4 domain interface and a salt bridge between Lys331 in one subunit and Glu 287 in another subunit is necessary for dimerization [16]. FXI has 5 N-linked glycosylation sites including Asn residues: at positions 72, 108, and 335 in heavy chain and Asn residues at 432 and 473 of light chain. Almost all circulating FXI is in complex with HMWK. HMWK is a multifunctional plasma protein with 6 domains (D1–D6). The D6 domain is the binding site of FXI Ap2 domain that is necessary for optimal binding to the platelet surface and efficient activation of FXI. Optimal binding of FXI to platelet occurs through Ap3 domain, GPIb, and HMWK in the presence of zinc ions [13, 17, 18].

13.3 Factor XI Activation and Function

Plasma activators of FXI are FXIIa, thrombin, meizothrombin, and FXIa (autoactivation). Platelet polyphosphate affects FXI activation via α-thrombin, β-thrombin, and FXIa, and all of them cleave FXI monomers at Arg369-Ile370 site between Ap4 and catalytic domain. The dimeric form of FXI is necessary for the effective activation and function of FXI. FXI activators bind to one monomer and activate another subunit [19]. HMWK is required for optimal FXI binding to GPIb on activated platelet. Indeed, FXI binds to platelet, as a negative charge surface, through one monomer and binds to substrate (FIX, FV, and FVIII) through another subunit, an explanation for its dimeric structure. FXIa is composed of 2 N-terminal heavy chains containing 4 apple domains and 2 C-terminal light chains containing catalytic domains. Dimeric structure of FXIa is maintained by 3 disulfide bonds: heavy chains bind to light chains by 2 bonds in each of the monomers and the two monomers connect together by 1 disulfide bond [20].

Along with the activation of FXI by thrombin and FXII, an intermediate form is generated that has one activation subunit. This intermediate is named 1/2FXIa. Inhibition of FXI by antithrombin in the presence of 1/2 molar heparin shows that each of the FXI subunits has an independent catalytic function, so 1/2FXIa can be the main form of activated FXIa (Fig. 13.2) [21–23].

In the initiation phase of the coagulation cascade, the tissue factor (TF)/FVII complex activates FX and produces a small amount of thrombin. Then a tissue factor pathway inhibitor (TFPI) inhibits TF and blocks the extrinsic coagulation pathway. At this time, the amplification phase begins with the conversion of FXII to FXIIa. Then FXI is converted to FXIa in the intrinsic pathway. Since there is no bleeding tendency in patients with FXII deficiency, this fact demonstrates that an alternative mechanism is present for FXI activation [24]. Thrombin-mediated

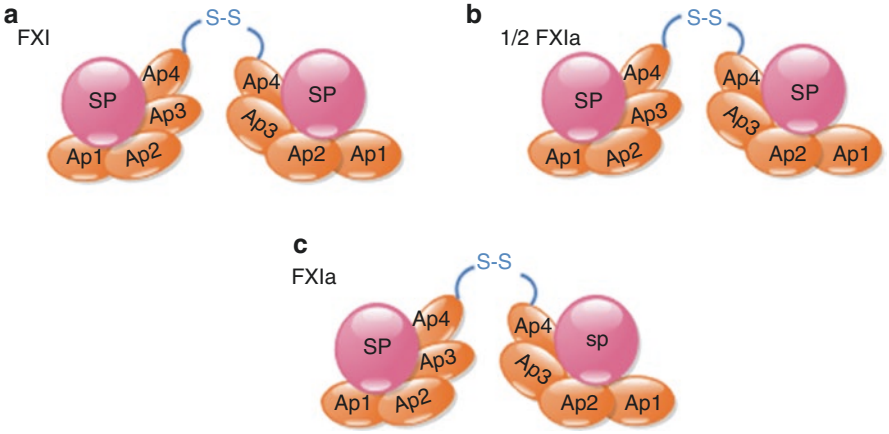

Fig. 13.2 (**a**) Each monomer of factor XI consists of 4 apple domains (Ap1–Ap4) and a serine protease domain (SP). The factor XI dimer is linked by Cys321 interchain disulfide bond between 2 subunits. (**b**) All factor XI activators cleavage factor XI between Arg369-Ile370 site and develop an intermediate form named 1/2FXIa. (**c**) With cleavage in another subunit, 1/2FXIa is changed to activated factor XI. *FXI* factor XI; *FXIa* activated factor XI; *Ap* apple domain; *SP* serine protease domain

activation of FXI reinforces the coagulation common pathway with this thrombin feedback loop [24]. Since in the initiation phase of the coagulation cascade, TF/FVII complex is inhibited by circulating TFPI, only a small amount of thrombin can be produced, and the extrinsic pathway is stopped. At this time, generated thrombin activates platelet, FXI, and FV-FVIII. FXI dimeric structure is necessary because FIX cannot bind to monomeric FXI on the platelet surface; therefore, one of the Apple3 domains is required to bind to the surface of the platelet leaving the AP3 domain on another monomer available for binding of FIX [20]. Therefore, after dimerization and conversion of FXI to FXIa, FXI binding site—amino acids 183–191 at Ap3—is exposed and FXIa can bind to FIX. Then FXIa cleaves FIX at Arg145–Ala146 and Arg180-Val181 sites and releases active peptide (Ala146–Arg180); at this time, FIX converts to its active forms (FIXaα and FIXβ). Thrombin generation will continue even after clot formation. This additional thrombin activates a thrombin-activable fibrinolysis inhibitor that protects the clot from fibrinolysis. Therefore, FXI has both procoagulant and antifibrinolytic activities (refer to Chap. 1) (Fig. 13.3) [9, 19, 20].

Antithrombin, protease nexin-2 (PN-2), C1 inhibitor, aprotinin, leupeptin, P-aminobenzidamin, and protein Z-dependent protease inhibitors are FXI inhibitors. Heparin also binds to serpin and A3 domain of FXI and inhibits FXI. Heparin also inhibits FXI via binding to the catalytic domain through a charge neutralization mechanism. PN-2 that is released from active platelets inhibits FXI through the binding of its Kunitz-type domain to FXI catalytic domain [20, 22].

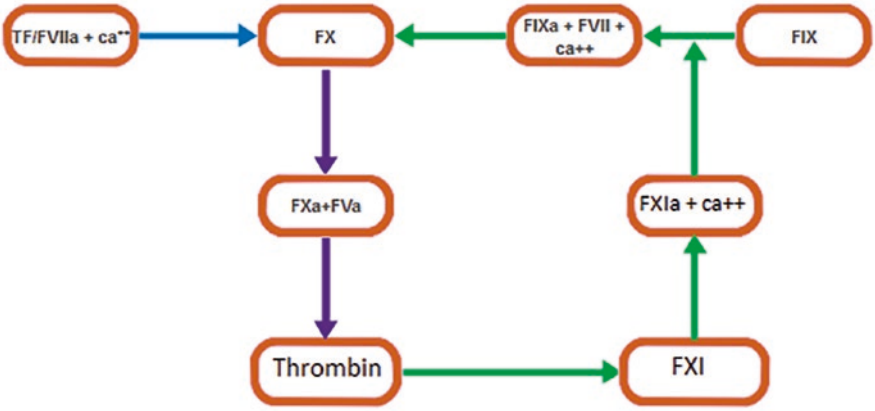

Fig. 13.3 Thrombin feedback loop. In the extrinsic coagulation pathway, a small amount of thrombin is generated by tissue factor (TF)/factor (F) VII + Ca^{++} complex in the initiation phase. Then, tissue factor pathway inhibitor (TFPI) inhibits TF/FVII. In the amplification phase, thrombin activates the intrinsic pathway through the conversion of FXI to FXIa. FXIa cleaves FIX to FIXa, and then FIXa activates FX. At this time, a large amount of thrombin is generated in the common pathway. *TFPI* tissue factor pathway inhibitor; *FXI* factor XI; *FXIa* activated factor XI; *FXa* activated factor X; *FIX* factor IX; *FIXa* activated factor IX; *TF* tissue factor; *FVII* factor VII; *FV* factor V

13.4 Factor XI Deficiency

FXI deficiency, hemophilia C, plasma thromboplastin antecedent deficiency, or Rosenthal syndrome is a rare hemorrhagic disorder with variable clinical symptoms that was first described by Rosenthal in 1953 [2]. The incidence of the disorder is equal in both genders and is ~1 per one million in the general population, but it is more common among Ashkenazi Jews with ~5% carriers and 1 per 450 homozygotes [3–5]. The bleeding tendency is mild in this disorder. Patients with FXI deficiency may not experience abnormal bleeding in their lives. Although severe bleeding is rare, menorrhagia and epistaxis are relatively common. Due to high fibrinolytic activity in the oral and nasal cavities or the urinary tract, bleeding risk is higher in these areas. Some patients may experience bleeding after some surgeries such as tooth extraction and tonsillectomy. Postpartum hemorrhage may occur in this disorder [2, 4–7].

The normal range of FXI coagulant activity (FXI:C) is 70–150 U/dL. Severe FXI-deficient patients have 1–20 U/dL of FXI:C level, while those with partially deficient FXI have 20–60 U/dL FXI:C levels and patients with mild deficiency have 61–70 U/dL FXI plasma levels. Individuals with severe FXI deficiency are homozygous or compound heterozygous for causative mutations, while those with partial

deficiency are heterozygous, with one mutated allele [1, 2, 6]. Activated partial thromboplastin time (APTT) is the screening test for FXII-mediated FXI activation and is prolonged in FXI deficiency. Thrombin-mediated FXI activation is assayed via FXIIa-inhibited diluted thromboplastin time (FXIIai DTT). FXI antigen level (FXI:Ag) is measured via enzyme-linked immunosorbent assay (ELISA). Generally, the mainstay of treatment in these patients is on-demand therapy. Replacement therapy is used in invasive operations, but it is not required for minor surgeries such as tooth extraction. Desmopressin elevates endogenous FXI levels and can be used for severe FXI deficiency. Recombinant FVII can be used in patients with inhibitor (plasma FXI level <1%). Oral antifibrinolytic agents such as tranexamic acid or ε-aminocaproic acid can be used in pregnant women and for minor surgeries such as tooth extraction [2, 6, 7]. However, adverse reactions such as thrombosis can occur, and these antifibrinolytics should be avoided in genitourinary tract bleeding [4].

13.5 Molecular Basis

F11 gene is located at the distal end of the long arm of chromosome 4 (4q35) and consists of 15 exons and 14 introns that span a 23-kb region. This gene is expressed in hepatocytes and regulated via transcription factor hepatocyte nuclear factor-4a (HNF4-α). Blood mononuclear cells, granulocytes, pancreas, and kidney also express a little *F11* gene. FXI deficiency is mostly an autosomal recessive injury-related hemorrhagic disorder, but autosomal dominant forms of the disorder have also been reported [25].

FXI deficiency is due to mutation in *F11* gene, and most mutations are associated with CRM− phenotype and fewer associated with CRM+ phenotype. In type I, CRM−, FXI activity (FXI:C), and antigen (FXI:Ag) levels are decreased [3, 5, 19]. In this type, the mutant protein level is low or absent, which can be due to reduced translation, secretion, or stability of the protein. There are three subgroups in type I (CRM−):

1. Mutations that affect protein synthesis; therefore, the production of polypeptide is decreased or stopped. Glu117stop mutation is common in this group.
2. Mutations that disrupt dimerization within two Ap4 domains; therefore polypeptide remains in monomeric form in the intracellular that results in decreased plasma level of FXI. Phe283Leu mutation is common in this group.
3. Mutations that cause the production of nonsecretable homodimers. Mutations that decrease homodimer secretion of wild-type FXI also result in decreased plasma levels of FXI. Ser225Phe and Trp569Ser mutations are common in this group (Fig. 13.4).

In type II, CRM+, the FXI:C is low, but FXI:Ag is normal. In other words, although the activity is decreased, the plasma protein level is normal or close to normal. A total of 8 CRM+ variants, 5 in catalytic domain and 3 in apple domain, have been identified up to now [6, 15].

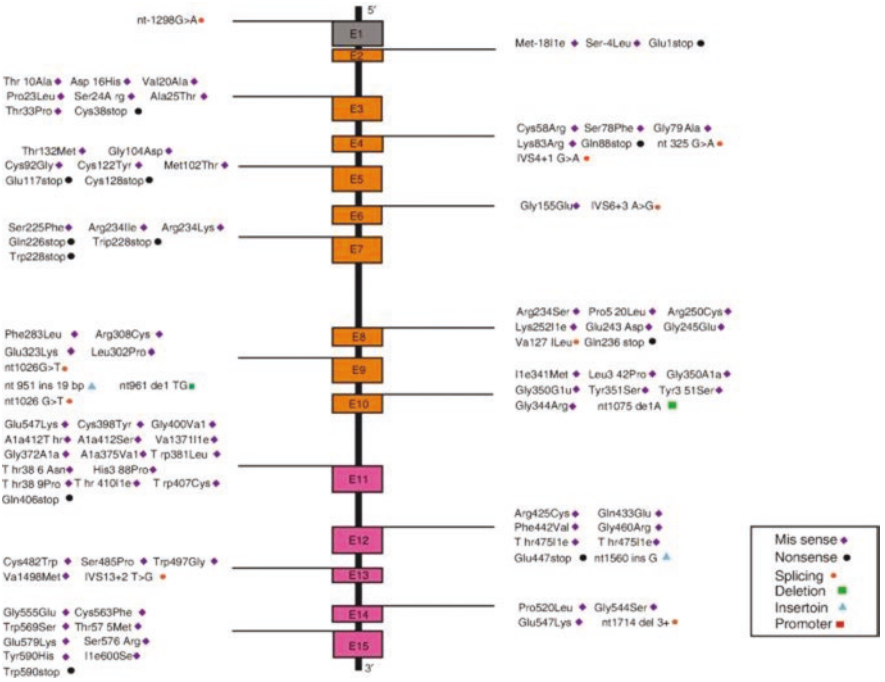

Fig. 13.4 A number of *F11* gene mutations. Missense mutations are the most common mutations within *F11* gene. Most of the mutations occur in the catalytic domain. *E* exon

Inhibitor formation against exogenous FXI is another problem in patients with FXI deficiency, mostly in severely affected patients. Patients with severe deficiency are at higher risk of inhibitor generation. FXI inhibitors are polyclonal IgG alloantibodies against various epitopes of the FXI molecule and inhibit FXI activation. The prevalence of antibodies in patients with FXI deficiency is 3–5%. Most patients with inhibitors are homozygotes, with <1% FXI plasma level and a positive history of replacement therapy or injection of RH immunoglobulin. Glu117Stop is the most common mutation in patients with FXI deficiency and inhibitors [8, 15, 19, 26].

FXI deficiency in the Jewish population is due to four common causative mutations that are categorized as types I to IV. Type I, a point mutation, occurs at the donor splice site of the last intron (-intron N) that is a G → A substitution. Type II, a nonsense mutation, GLU117stop in exon 5 results in early chain termination. Type III, a missense mutation, Phe283Leu substation in exon 9 results in the partial defect in dimerization and intracellular retention of FXI monomers [9]. Type IV is caused by a 14 bp deletion in exon 14/intron N splice site. Types II and III account for >90% of causative mutations in the Jewish population [10]. Other frequent mutations were observed in other populations including type II in Iraqi, Arab, and other Middle Eastern Jews. Type III mutation more frequently is found in recent European origin, Cys88Stopp mutation in French Basques, and the Cys128Stop mutation in the United Kingdom [11, 27]. More than 220 mutations were observed in *F11* gene.

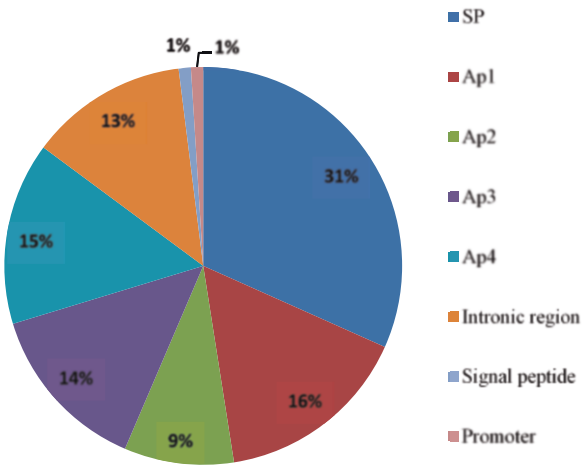

Fig. 13.5 Distribution of different mutations within *F11* gene. *Ap* apple domain, *SP* serin protease domain

FXI mutations include missense (67%), nonsense (11.9%), splice site (10.6%), deletion/insertion (9.4%), and promoter mutations (0.8%) [9, 28].

Most mutations occur at the catalytic domain with 32% frequency and then Ap1, Ap4, Ap3, intronic region, Ap2, signal peptide and linker region, respectively (Fig. 13.5) [19, 21, 22].

13.6 Clinical Manifestations

Patients with FXI deficiency present with variable clinical phenotypes. Patients with homozygote and compound heterozygote mutations usually have less FXI less than 15–20 U/dL, while heterozygotes usually have FXI levels between 20 and 70 U/dL (mild to moderate deficiency). Generally, there is no direct correlation between bleeding tendency and FXI plasma level, bleeding tendency is more profound in homozygotes and compound heterozygotes than heterozygotes, and heterozygotes are usually asymptomatic. Some homozygotes may not experience abnormal bleeding in their lives. FXI deficiency is usually detected in preoperative patient blood workup, in hemostatic challenges, and in patients with a positive family history of FXI deficiency. Although spontaneous bleeding is rare in this disorder, life-threatening bleeds after surgery or post-trauma may occur [28–30]. Excessive bleeding often occurs in tonsillectomy, dental extraction, and sinus surgery. There is a mild to moderate bleeding tendency in hemostatic fluctuations at the sites with high fibrinolytic activity such as oral cavity, nasal cavity, or genitourinary tract. Postpartum hemorrhage only occurs in ~20% of affected women. Abnormal bleeding in women due to obstetric and menstruation can occur [29–33]. Other contributing factors for bleeding tendency are type of mutations, plasmatic factors, platelet

Table 13.1 Clinical manifestations of patients with congenital factor XI deficiency

Bleeding symptom	Santoro et al. (N:95)	Shao et al. [34] (N:57)	Peyvandi et al. [35] (N:18)*
Ecchymosis	28		–
Epistaxis	24	5.3	27
Gastrointestinal bleeding	15	–	–
Hematuria	4	–	0
Hematoma	2	–	22
Menometrorrhagia	7 of women	7.5 of women	0.3 of women
Post-traumatic intracranial hemorrhage	1	–	–
Pulmonary hemorrhage	1	–	–
Gum bleeding	1	–	–
Bleeding from minor wounds	–	1.8	–
Postdental extraction bleeding	–	3.5	–
Postsurgical bleeding	–	2.5 of women	66
Hemarthrosis	–	–	27
Oral cavity bleeding	–	–	78
Easy bruising	–	8.8	–

*Severe/moderate factor XI deficiency

and endothelial disturbance, von Willebrand disease, hemophilia A or B, and other bleeding disorders [33]. These variable clinical symptoms make the diagnosis and management of FXI potentially difficult [36] (Table 13.1).

13.7 Laboratory Diagnosis of Factor XI Deficiency

13.7.1 Overview

Due to the mild phenotype of the disorder, diagnosis of FXI deficiency is difficult, and in most cases, diagnosis is made based on unexplained bleeding episodes, family history, or presurgery laboratory workup. Routine and specific coagulation tests can be used for an appropriate and timely diagnosis of the disorder. These tests include PT, APTT, FXI:C, and FXI:Ag assays. FXIIai DTT can determine thrombin-mediated FXI activation [19, 37, 38]. Other factor deficiencies, specific XI inhibitors, lupus anticoagulants, and other factors in the APTT assay that are commonly found as interferents, e.g., heparin should be excluded.

- Factor XI deficiency should be suspected when PT is normal and APTT is prolonged, and other intrinsic coagulation pathway factor deficiencies have been excluded. Differential diagnosis can be made by FXI:C and FXI:Ag assays.
- Factor XI activity, FXI:C, can be performed using a one-stage factor assay or chromogenic assay.
- FXI plasma level (FXI:Ag) assay can be performed by ELISA [39].
- FXI activity can be measured via a modified APTT and FXIIai DTT [38].

APTT reflects the FXII-mediated FXI activation, and this test is prolonged in FXI deficiency. Patient's plasma is mixed with normal plasma in a 1:1 ratio. The correction percentage shows the amount of FXI in patient's plasma [4].

Factor XIIa-inhibited dilute thromboplastin time (FXIIa iDTT): Rabbit Thromboplastin is diluted with normal saline to make different dilutions. Corn trypsin inhibitor (CTI) is an FXII inhibitor that is added immediately before the assay. Diluted thromboplastin and plasma are mixed in identical proportions and incubated at 37 °C for 2 min. Then, prewarmed $CaCl_2$ is added to the mixture and clotting time is measured. FXIIai DTT is markedly prolonged in severe FXI deficiency at 1:1000 concentration of thromboplastin and has a good correlation with the severity of bleeding [19, 38].

Thrombin generation assay and thromboelastometry can be used to measure the efficacy of treatment with FFP-SD or FXI concentrate in patients with FXI deficiency undergoing surgery and monitor the treatment with recombinant activated FVII (FVIIa) [40–43].

APTT mixing study is used to detect inhibitors in patients with severe FXI deficiency (<1%). The lack of APTT correction after mixing of patient and control plasma suggests the presence of an inhibitor. Incubation of patient and control plasma at varying dilutions from 100% patient plasma to 0% at 37 °C up to 2 h can be required. Repeating the APTT at 30-min intervals.

A specific inhibitor assay should be performed, e.g., modified Nijmegen Bethesda assay [44–46].

13.7.1.1 Functional Assay

The One-Stage APTT-Based FXI Activity Assay

A one-stage APTT-based FXI activity assay is used for the measurement of FXI activity in plasma. In this assay, the FXI activity level is measured based on the ability of test plasma to correct or shorten the APTT of FXI-deficient plasma. One-stage factor assay is normally fully automated even on small coagulation instrumentation.

Box 13.1 The Principle of the One-Stage APTT-Based FXI Activity Assay

Serial dilutions (1/10, 1/20, 1/40, etc.) of the standard plasma (a value of 100% of FXI) are prepared and mixed with an equal volume of substrate plasma (FXI-deficient plasma) and the APTT is measured for each dilution. Then the APTT values are plotted against the dilutions in the semi-log paper either manually or via computer. The test plasma (patient plasma) is also treated in the same way as standard plasma (i.e., preparation of the serial dilutions followed by mixing with substrate plasma). Following the addition of phospholipids and an activating agent, the calcium ions are added in order to start the coagulation reaction and the APTT is measured (Fig. 13.6). The results of APTT are plotted on the graph. Since the substrate plasma lacks the FXI, the difference between the dilutions of standard plasma and the test plasma is determined as the FXI activity level [46, 47].

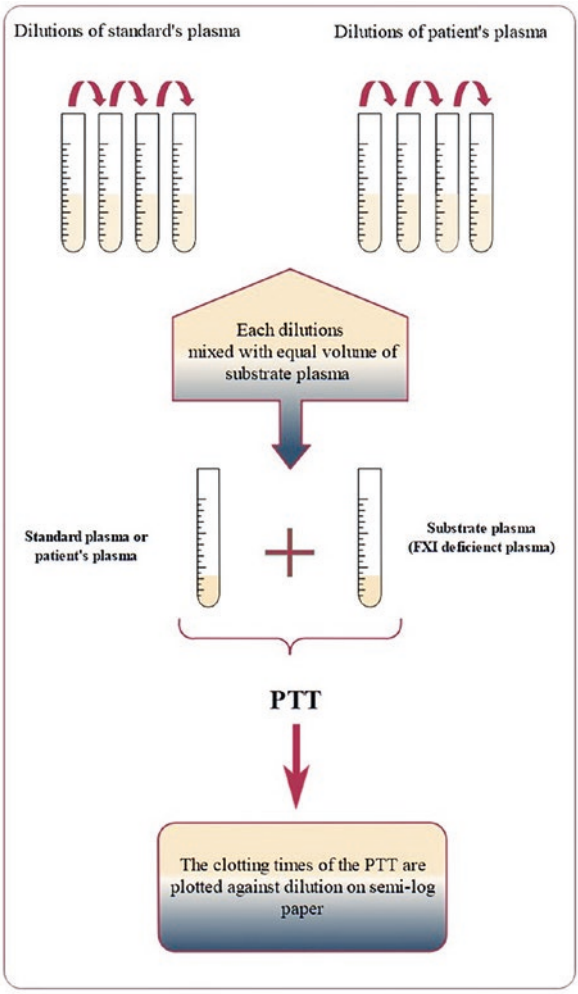

Fig. 13.6 The one-stage APTT-based FXI activity assay. In this assay, serial dilutions of standard and test plasma are prepared and mixed with equal volumes of substrate plasma. Following the mixing, the APTT is measured, and the results are plotted in the semi-log paper

Sample requirements: Blood should be collected into trisodium citrate anticoagulant with a concentration of 105–109 mmol/L (3.1–3.2%) and 1:9 ratio of blood: citrate. The collected blood was transported to the laboratory at a temperature of 18–25 °C in 4 h of collection. The samples can be kept at −20 °C for 2 weeks or at −40 to −70 °C for 3–6 months. Since thawing the frozen samples results in precipitation of the coagulation factors and abnormal results, the frozen sample should be thawed at 37 °C and inverted gently [47].

The interfering variables: This assay can be influenced by all the pre-analytical variables, which affect the APTT. Hemolysis, lipemia, icterus samples, underfilling tube, the presence of clot, and the use of incorrect sample tubes [48].

The Chromogenic Factor XI Assay

The chromogenic assay for measurement of the FXI:C in plasma is based on the two-stage assay and can be fully automated [49].

Box 13.2 The Principle of the Chromogenic Factor XI Assay

In this assay, plasma is treated with acetone to destroy inhibitors against FXIIa and FXIa in the plasma. Then the contact system is activated with an activating agent (kaolin), which results in the activation of FXI by FXIIa. Following the activation step, the FXIIa is inhibited with CTI. Then the FXI level in the plasma is determined by the ability of FXI to cleave the chromogenic substrate (substrate-pNA) and release p-nitroaniline (pNA). This can be measured photometrically; the intensity is proportional to the FXI concentration (Fig. 13.7).

Sample requirement: The plasma is collected in the 105–109 mmol/L (3.1–3.2%) concentration of Trisodium citrate. The plasma samples should be assayed within 2 h of blood collection or stored frozen at −20 °C.

Factor XII-Inhibited Dilute Thromboplastin Time

The FXIIai DTT-based assay reflects the thrombin generation of FXIa and is used for the determination of FXI:C [38].

Box 13.3 The Principle of the Factor XII-Inhibited Dilute Thromboplastin Time

In this assay, different dilutions of thromboplastin in saline are prepared. Before starting the assay, in order to inhibit FXII, CTI is added. The diluted thromboplastin and plasma are mixed and incubated for 2 min at 37 °C. Following the incubation, the prewarmed Cacl2 is added and the clotting time is recorded. In patients with FXI deficiency, the FXII ai DTT is prolonged at a 1:1000 concentration of thromboplastin and has a better correlation between FXI level and the severity of bleeding episodes.

Fig. 13.7 The principle of the chromogenic factor XI assay

13.7.1.2 Immunological Factor XI Assay

Although measurement of FXI antigen level is not a routine assay in most of the laboratories, distinguishing between the quantitative and qualitative defects of FXI deficiency is necessary. The concentration of FXI antigen in plasma, serum, or other biological fluids is estimated with an ELISA assay.

Box 13.4 The Principle of Factor XI Immunological Assay

In this test, a specific monoclonal antibody against FXI is adsorbed onto the wells of the 96-well microtiter plate. Following the preparation of dilution of the plasma sample and addition to the wells, the FXI antigen in the plasma binds to the precoated antibody. After appropriate washing steps for omitting the unbound conjugates, the peroxidase-labeled detecting antibody is added and binds to the captured FXI. Following another washing step, a solution of TMB (the peroxidase substrate, tetramethylbenzidine) is applied. TMB is catalyzed by streptavidin-peroxidase and develops blue-colored products that change to yellow upon quenching the reaction by acid (Fig. 13.8).

The color developed is measured spectrophotometrically at 450 nm. The absorbance at 450 nm is directly proportional to the quantity of FXI antigen captured on the plate (Fig. 13.9) [19, 49].

Sample requirement: The required samples are in a standard 105–109 mmol/L (3.1–3.2%) concentration of citrate anticoagulants. The assay should be done immediately or in 5 days when the samples are stored at 2–8 °C. Otherwise, the samples should be kept at −20 °C (≤1 month) or −80 °C (≤2 months). Before performing the assay, samples should be defrosted at 37 °C for 5 min and gently inverted to mix.

Interfering variables: Hemolysis, Lipemia, and Icterus blood samples can affect the result. However, the conditions of the performing assay, including the operator, pipetting and washing, and temperature of the laboratory, can affect the results of the test.

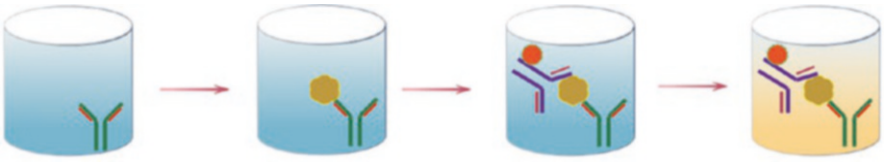

Fig. 13.8 The principle of the FXI antigen assay. The antibodies against FXI is precoated. Following the addition of the test plasma, the FXI binds to precoated antibodies. Then the labeled binds to the captured FXI. Following the addition of TMB solution, the yellow color developed, which directly related to FXI

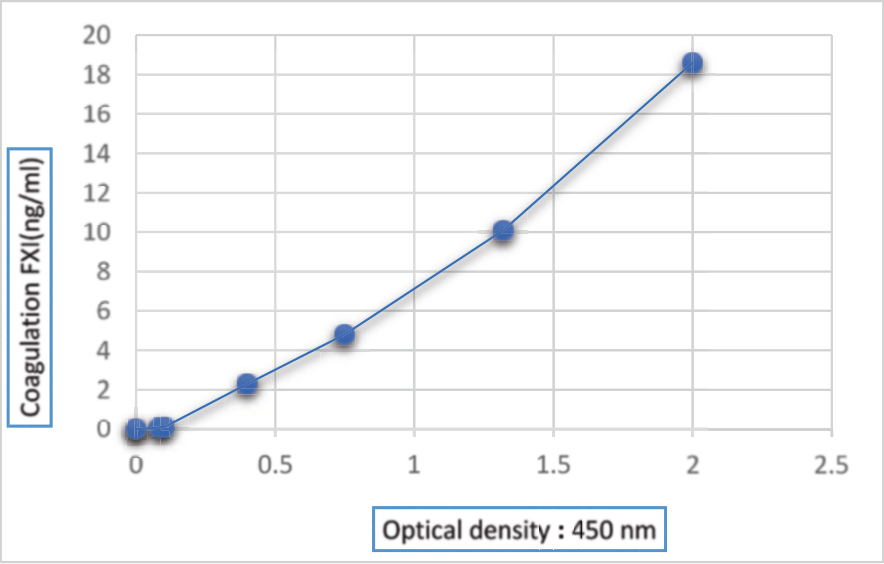

Fig. 13.9 Typical standard curves for measurement of FXI antigen level. In order to determine the FXI antigen level in the plasma sample, the OD values of the standard are plotted against the known concentration of the standard plasma

13.8 Global Hemostasis Assays

Since the bleeding tendency in patients with FXI deficiency is unpredictable and there is a lack of relationship between the FXI activity level and the severity of bleeding episodes, the use of reliable assays is required to correctly identify patients who are prone to bleeding [49]. Thrombin generation assay and thromboelastography/rotational thromboelastometry are widely used for the assessment of bleeding tendency in congenital FXI deficiency. Furthermore, these assays are used for the determination of the treatment's effectiveness, while the coagulation assays are not able to correlate with the severity of bleeding. However, due to the small sample size, the variable FXI activity level, and conflicting results in different studies, the ability of these assays to predict the bleeding tendency is a matter of debate depending on the sample conditions [49].

13.9 Molecular Diagnosis

FXI deficiency can be affected by some specific mutations including heterozygous large deletions mutations which are located in regions that cannot be detected by conventional mutational screenings (promoter region far from the transcription start site or deep intronic mutations). The other mutations in *F11* gene are the nucleotide

variations including those that occur in untranslated regions of the mRNA and alter the regulation of the gene expression; therefore, molecular diagnosis of FXI deficiency is a challenge [49]. However, more than 220 mutations were reported in *F11* gene. These mutations include missense as the most common type of mutation (67%) followed by nonsense (11.9%), splice site (10.6%), deletion/insertion (9.4%), and promoter mutations (0.8%) [49]. There are four common types of causative mutations in the Jewish population that are commonly affected by FXI deficiency. Type I is a point mutation (G → A substitution) that occurs at the donor splice site of the last intron. Type II is a nonsense mutation in exon 5 (Glu117stop) that results in early termination. Type III is another missense mutation that occurs in exon 9 (Phe283Leu) causing a partial defect in dimerization [49]. Type IV which results from a 14-bp deletion in exon 14/intron N splice site. Among all, types II and III accounted for more than 90% of the causative mutations in the Jewish population. Moreover, type II mutation is reported in Iraqi and Arab populations [10, 49]. Type III mutation, Cys88Stopp, and Cys128Stop are more frequently reported in the population of European origin, French Basques, and United Kingdom, respectively [11, 27].

13.10 Recommendations and Precautions in Laboratory Investigation

Hemolytic, Icterus, and lipemic plasma may affect some coagulation tests in a number of automated analyzers. Fasting is not necessary for routine coagulation tests. Patients should avoid stressful conditions, strenuous exercise, and some drugs that can affect the results before blood samples are taken for investigation. Anticoagulant drugs can also affect the results of coagulation tests [50].

Different APTT reagents have been shown to give rise to variations in the APTT sensitivity; therefore, reference ranges should be established locally [51–53].

Some mutations such as large deletions or mutations in those regions that are not covered by conventional mutational screening may not be detected and should be analyzed either by reverse transcription–PCR on suitable patient specimens (when available) or by using in vitro approaches based on the transfection of appropriate minigene construct containing the nucleotide variation under investigation [25, 54].

Differential diagnoses should exclude the presence of lupus anticoagulant, liver dysfunction, heparin contamination, von Willebrand disease, and other coagulation factor deficiencies or coagulation factor inhibitors [19].

13.11 Treatment

Most of the patients with FXI deficiency have few bleeding problems during their life. Spontaneous bleeding except for menorrhagia is rare and usually subsides with treatment. The mainstay of treatment in FXI deficiency, like most other rare bleeding disorders, is on-demand treatment to stop the bleeding as soon as possible. The

presence of FXI inhibitors should be excluded in patients with severe deficiency (<1%), particularly those that have received plasma, FXI concentrates, or immunoglobulin. Assessment of thrombotic risk in specific types of surgeries should carefully be evaluated when selecting specific treatment options. Age and underlying diseases such as heart and renal disorders can also affect the treatment [4, 55, 56].

For patients undergoing major surgery, replacement therapy with FFP-SD or FXI concentrate is used to achieve trough levels of 45 U/dL for 5–7 days prior to surgery. In areas with high fibrinolytic activity such as nose, tonsils, oral cavity, and urinary tract the use of antifibrinolytics should be used. The use of FFP-SD may result in circulatory overload in patients with congestive heart failure and chronic renal failure. **FXI concentrates** have several advantages including shorter infusion times, the level of other coagulation factors is not increased, and a lower rate of transfusion-related reactions. Special precautions should be considered when these products are used in elderly adults and patients with cardiovascular disease because of their potent thrombotic risk. The dose should not exceed 30 U/kg, and peak FXI levels should not exceed 70 U/dL. Tranexamic acid should be avoided in patients receiving FXI concentrates because it may pose a thrombotic risk [55, 56]. **Recombinant FVII (rFVII)** can be used in patients with FXI deficiency and inhibitors (see below). Replacement therapy is not necessary for minor surgeries. For minor surgeries such as tooth extraction and cataract extraction, antifibrinolytic agents such as **tranexamic acid** at a dose of 1 g daily for 12 h before surgery until 7 days after surgery are suitable. Epsilon aminocaproic acid can be used at 5–6 g four times daily for the same period of time [55, 56]. **Fibrin glue** can be used in resection of skin lesions and local hemostasis; however, there is a lack of clinical trials showing efficacy and risk [55, 56]. Prophylactic treatment is not necessary in patients without a bleeding history. **Desmopressin (DDAVP)**, a synthetic analog of the natural antidiuretic hormone vasopressin, as a supplementary agent can elevate endogenous FXI level that can be used in patients with a bleeding history (refer to Chap. 3). Indeed, desmopressin can normalize coagulation parameters in heterozygous patients with FXI deficiency by slightly increasing both FXI activity and antigen levels. Replacement therapy is not necessary in patients undergoing surgery without a history of bleeding tendency, but tranexamic acid and/or fibrin glue is used in high-risk surgeries such as prostatectomy [12, 56].

Menorrhagia: *Oral antifibrinolytic agents* such as **tranexamic acid** are used in women with prolonged menorrhagia [57].

Pregnant women: Women with partial FXI deficiency without previous bleeding history do not mean that they will not bleed during pregnancy. Bleeding assessment tools should be used and can help in targeting women with potential bleeding risk. In women with a significant bleeding history, tranexamic acid is used for 3 days with the first dose being administered during vaginal delivery. FXI concentrate can be prescribed for women with severe FXI deficiency during vaginal delivery or cesarean section. Recombinant VIIa can also be used for severe FXI deficiency. Tranexamic acid can be used in FXI deficiency patients with levels 15–40% either having a vaginal or c-section birth. As with all of the potential treatments, there is a

potential thrombotic risk in this patient group. FFP-SD is advisable only when there is excessive bleeding during cesarean or vaginal delivery [30, 32, 36].

Circumcision: FXI deficiency may be observed first in this procedure. It is important to check newborn males in areas with high rates of FXI deficiency such as Ashkenazi and Iraqi Jewish populations. For diagnosis of FXI deficiency in infants, a factor FXI level of <10 U/dL is indicative of deficiency. FXI level should be rechecked until 6 months. If the FXI level remained low, the procedure should be performed by cover of either FXI concentrate or FFP-SD. The boys with FXI levels >10 U/dL should receive tranexamic acid at a dose of 15 mg/kg every 8 h for 3 days under circumcision procedures [30, 36, 58].

Patients with an inhibitor: Patients with severe FXI deficiency may develop an inhibitor after replacement therapy. **RFVIIa** or **prothrombin complex** is used in these patients RFVIIa is effective at a low dose of 15–30 μg/kg with **oral tranexamic acid** for major surgeries. It is also used in patients with a history of allergic reactions to replacement therapy or those with IgA deficiency. **Epsilon aminocaproic acid** is used at a dose of 5–6 g four times daily [59, 60].

References

1. O'Connell NM. Factor XI deficiency-from molecular genetics to clinical management. Blood Coagul Fibrinolysis. 2003;14:S59–64.
2. Peyvandi F, Lak M, Mannucci PM. Factor XI deficiency in Iranians: its clinical manifestations in comparison with those of classic hemophilia. Haematologica. 2002;87(5):512–4.
3. Asakai R, Chung DW, Davie EW, Seligsohn U. Factor XI deficiency in Ashkenazi jews in Israel. N Engl J Med. 1991;325(3):153–8.
4. Lewandowska MD, Connors JM. Factor XI deficiency. Hematol Oncol Clin. 2021;35(6):1157–69.
5. Lupo H, Lanir N, Brenner B, Shpilberg O, Seligsohn U, Peretz H, et al. The two common mutations causing factor XI deficiency in Jews stem. Blood. 1997;90(7):2654–9.
6. Bolton-Maggs PH. Factor XI deficiency—resolving the enigma? ASH Educ Program Book. 2009;2009(1):97–105.
7. Saunders RE, O'Connell NM, Lee CA, Perry DJ, Perkins SJ. Factor XI deficiency database: an interactive web database of mutations, phenotypes, and structural analysis tools. Hum Mutat. 2005;26(3):192–8.
8. Salomon O, Zivelin A, Livnat T, Dardik R, Loewenthal R, Avishai O, et al. Prevalence, causes, and characterization of factor XI inhibitors in patients with inherited factor XI deficiency. Blood. 2003;101(12):4783–8.
9. Hancock JF, Wieland K, Pugh RE, Martinowitz U, Schulman S, Kakkar VV, et al. A molecular genetic study of factor XI deficiency. Blood. 1991;77(9):1942–8.
10. Shpilberg O, Peretz H, Zivelin A, Yatuv R, Chetrit A, Kulka T, et al. One of the two common mutations causing factor XI deficiency in Ashkenazi Jews (type II) is also prevalent in Iraqi Jews, who represent the ancient gene pool of Jews. Blood. 1995;85:429.
11. Zivelin A, Bauduer F, Ducout L, Peretz H, Rosenberg N, Yatuv R, et al. Factor XI deficiency in French Basques is caused predominantly by an ancestral Cys38Arg mutation in the factor XI gene. Blood. 2002;99(7):2448–54.
12. Franchini M, Manzato F, Salvagno GL, Montagnana M, Lippi G. The use of desmopressin in congenital factor XI deficiency: a systematic review. Ann Hematol. 2009;88(10):931–5.
13. Emsley J, McEwan PA, Gailani D. Structure and function of factor XI. Blood. 2010;115(13):2569–77.

14. Jin L, Pandey P, Babine RE, Gorga JC, Seidl KJ, Gelfand E, et al. Crystal structures of the FXIa catalytic domain in complex with ecotin mutants reveal substrate-like interactions. J Biol Chem. 2005;280(6):4704–12.
15. Berber E. Molecular characterization of FXI deficiency. Clin Appl Thromb Hemost. 2011;17(1):27–32.
16. Cheng Q, Sun MF, Kravtsov D, Aktimur L, A, Gailani D. Factor XI apple domains and protein dimerization. J Thromb Haemost. 2003;1(11):2340–7.
17. Gailani D, Smith S. Structural and functional features of factor XI. J Thromb Haemost. 2009;7:75–8.
18. Bouma BN, Griffin JH. Human blood coagulation factor XI. Purification, properties, and mechanism of activation by activated factor XII. J Biol Chem. 1977;252(18):6432–7.
19. He R, Chen D, He S. Factor XI: hemostasis, thrombosis, and antithrombosis. Thromb Res. 2012;129(5):541–50.
20. Sinha D, Marcinkiewicz M, Lear JD, Walsh PN. Factor XIa dimer in the activation of factor IX. Biochemistry. 2005;44(30):10416–22.
21. McVey JH, Lal K, Imanaka Y, Kemball-Cook G, Bolton-Maggs PH, Tuddenham EG. Characterisation of blood coagulation factor XIT475I. Thromb Haemost. 2005;93(06):1082–8.
22. Zucker M, Zivelin A, Landau M, Rosenberg N, Seligsohn U. Three residues at the interface of factor XI (FXI) monomers augment covalent dimerization of FXI. J Thromb Haemost. 2009;7(6):970–5.
23. Smith SB, Verhamme IM, Sun M-f, Bock PE, Gailani D. Characterization of novel forms of coagulation factor XIa: independence of factor XIa subunits in factor IX activation. J Biol Chem. 2008;283(11):6696–705.
24. Hoffman M. A cell-based model of coagulation and the role of factor VIIa. Blood Rev. 2003;17:S1–5.
25. Dai L, Rangarajan S, Mitchell M. Three dominant-negative mutations in factor XI-deficient patients. Haemophilia. 2011;17(5):e919–e22.
26. Zucker M, Zivelin A, Teitel J, Seligsohn U. Induction of an inhibitor antibody to factor XI in a patient with severe inherited factor XI deficiency by Rh immune globulin. Blood. 2008;111(3):1306–8.
27. Bolton-Maggs P, Peretz H, Butler R, Mountford R, Keeney S, Zacharski L, et al. A common ancestral mutation (C128X) occurring in 11 non-Jewish families from the UK with factor XI deficiency. J Thromb Haemost. 2004;2(6):918–24.
28. Moser SS, Chodick G, Ni YG, Chalothorn D, Wang M-D, Shuldiner AR, et al. The association between factor XI deficiency and the risk of bleeding, cardiovascular, and venous thromboembolic events. Thromb Haemost. 2022;122(05):808–17.
29. Bolton-Maggs P, Patterson D, Wensley R, Tuddenham E. Definition of the bleeding tendency in factor XI-deficient kindreds–a clinical and laboratory study. Thromb Haemost. 1995;73(02):194–202.
30. Santoro C, Di Mauro R, Baldacci E, De Angelis F, Abbruzzese R, Barone F, et al. Bleeding phenotype and correlation with factor XI (FXI) activity in congenital FXI deficiency: results of a retrospective study from a single Centre. Haemophilia. 2015;21(4):496–501.
31. Salomon O, Steinberg DM, Tamarin I, Zivelin A, Seligsohn U. Plasma replacement therapy during labor is not mandatory for women with severe factor XI deficiency. Blood Coagul Fibrinolysis. 2005;16(1):37–41.
32. Wheeler AP, Hemingway C, Gailani D. The clinical management of factor XI deficiency in pregnant women. Expert Rev Hematol. 2020;13(7):719–29.
33. Bolton-Maggs P, Perry D, Chalmers E, Parapia L, Wilde J, Williams M, et al. The rare coagulation disorders–review with guidelines for management from the United Kingdom Haemophilia Centre Doctors' Organisation. Haemophilia. 2004;10(5):593–628.
34. Shao Y, Cao Y, Lu Y, Dai J, Ding Q, Wang X, Xi X, Wang H. Clinical manifestations and mutation spectrum of 57 subjects with congenital factor XI deficiency in China. Blood Cells, Molecules, and Diseases. 2016;1:58:29–34.

35. Peyvandi F, Lak M, Mannucci PM. Factor XI deficiency in Iranians: its clinical manifestations in comparison with those of classic hemophilia. Haematologica. 2002;1;87(5):512–4.
36. Mumford AD, Ackroyd S, Alikhan R, Bowles L, Chowdary P, Grainger J, et al. Guideline for the diagnosis and management of the rare coagulation disorders: a United Kingdom Haemophilia Centre Doctors' Organization guideline on behalf of the British Committee for Standards in Haematology. Br J Haematol. 2014;167(3):304–26.
37. Wheeler AP, Gailani D. Why factor XI deficiency is a clinical concern. Expert Rev Hematol. 2016;9(7):629–37.
38. He R, Xiong S, He X, Liu F, Han J, Li J, et al. The role of factor XI in a dilute thromboplastin assay of extrinsic coagulation pathway. Thromb Haemost. 2001;85(06):1055–9.
39. Wilmot H, Hockley J, Rigsby P, Gray E. Establishment of the World Health Organization 2nd international standard for factor XI, plasma, human. Front Med. 2017;4:28.
40. Pike G, Cumming A, Thachil J, Hay C, Bolton-Maggs P, Burthem J. Evaluation of the use of rotational thromboelastometry in the assessment of FXI deficency. Haemophilia. 2017;23(3):449–57.
41. Pike GN, Cumming A, Thachil J, Hay CR, Burthem J, Bolton-Maggs PH. Evaluation of the use of global haemostasis assays to monitor treatment in factor XI deficiency. Haemophilia. 2017;23(2):273–83.
42. Désage S, Dargaud Y, Meunier S, Le Quellec S, Lienhart A, Negrier C, et al. Report of surgeries, their outcome and the thrombin generation assay in patients with factor XI deficiency: a retrospective single-Centre study. Haemophilia. 2022;28(2):301–7.
43. Riddell A, Abdul-Kadir R, Pollard D, Tuddenham E, Gomez K. Monitoring low dose recombinant factor VIIa therapy in patients with severe factor XI deficiency undergoing surgery. Thromb Haemost. 2011;106(09):521–7.
44. Franchini M, Marano G, Mengoli C, Piccinini V, Pupella S, Vaglio S, et al., editors. Inhibitors in patients with congenital bleeding disorders other than hemophilia. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2018.
45. Franchini M, Lippi G, Favaloro EJ, editors. Acquired inhibitors of coagulation factors: part II. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2012.
46. Bortoli R, Monticielo OA, Chakr RM, Palominos PE, Rohsig LM, Kohem CL, et al., editors. Acquired factor XI inhibitor in systemic lupus erythematosus—case report and literature review. Seminars in arthritis and rheumatism. Elsevier; 2009.
47. Kitchen S, Adcock DM, Dauer R, Kristoffersen AH, Lippi G, Mackie I, et al. International Council for Standardization in Haematology (ICSH) recommendations for processing of blood samples for coagulation testing. Int J Lab Hematol. 2021;43(6):1272–83.
48. Lippi G, Blanckaert N, Bonini P, Green S, Kitchen S, Palicka V, et al. Haemolysis: an overview of the leading cause of unsuitable specimens in clinical laboratories. Clin Chem Lab Med. 2008;46(6):764–72.
49. Rodgers S, Duncan E. Chromogenic factor VIII assays for improved diagnosis of hemophilia A. Hemostasis and thrombosis. Springer; 2017. p. 265–76.
50. Lippi G, Salvagno GL, Montagnana M, Lima-Oliveira G, Guidi GC, Favaloro EJ, editors. Quality standards for sample collection in coagulation testing. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2012.
51. Srivastava A, Santagostino E, Dougall A, Kitchen S, Sutherland M, Pipe SW, et al. WFH guidelines for the management of hemophilia. Haemophilia. 2020;26:1–158.
52. Lawrie A, Kitchen S, Purdy G, Mackie I, Preston F, Machin S. Assessment of actin FS and actin FSL sensitivity to specific clotting factor deficiencies. Clin Lab Haematol. 1998;20(3):179–86.
53. Salloum-Asfar S, María E, Esteban J, Miñano A, Aroca C, Vicente V, et al. Assessment of two contact activation reagents for the diagnosis of congenital factor XI deficiency. Thromb Res. 2018;163:64–70.
54. Podmore A, Smith M, Savidge G, Alhaq A. Real-time quantitative PCR analysis of factor XI mRNA variants in human platelets. J Thromb Haemost. 2004;2(10):1713–9.
55. Tabatabaei T, Dorgalaleh A. Congenital factor XI deficiency. Congenital bleeding disorders. Springer; 2018. p. 291–306.

56. Duga S, Salomon O, editors. Congenital factor XI deficiency: an update. seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2013.
57. Seligsohn U. Factor XI deficiency in humans. J Thromb Haemost. 2009;7:84–7.
58. Davies J, Kadir R, editors. The management of factor XI deficiency in pregnancy. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2016.
59. Kenet G, Lubetsky A, Luboshitz J, Ravid B, Tamarin I, Varon D, et al. Lower doses of rFVIIa therapy are safe and effective for surgical interventions in patients with severe FXI deficiency and inhibitors. Haemophilia. 2009;15(5):1065–73.
60. Livnat T, Tamarin I, Mor Y, Winckler H, Horowitz Z, Korianski Y, et al. Recombinant activated factor VII and tranexamic acid are haemostatically effective during major surgery in factor XI-deficient patients with inhibitor antibodies. Thromb Haemost. 2009;102(09):487–92.

Congenital Factor XIII Deficiency, Diagnosis, and Management

14

Akbar Dorgalaleh

14.1 Introduction

Congenital factor XIII (FXIII) deficiency is an ultra-rare hemorrhagic disorder with an estimated incidence of one per two million in the general population [1]. The disorder is more frequent in areas with high rates of consanguinity, such as Iran, India, and Pakistan. In a recent study in southeast Iran, a prevalence of 0.2% homozygotes and 3% heterozygotes was found, named Khash FXIII [2]. FXIII deficiency, with an overall mortality rate of ~15% due to intracranial hemorrhage (ICH), umbilical cord bleeding (UCB), and miscarriage, is one of the most severe congenital hemorrhagic disorders [3]. Indeed, in the absence of a prophylaxis program, approximately one-third of patients experience fatal ICH prior to the onset of middle age [1, 3]. Therefore, primary prophylaxis is mandatory for all patients with severe FXIII (FXIII activity <5%) upon diagnosis [4]. Early diagnosis, performed by an FXIII functional assay, and timely management of the disorder are crucial [4, 5]. In the absence of this assay in a considerable number of laboratories, a traditional clot solubility test is the only diagnostic test for the detection of FXIII deficiency [6]. Primary prophylaxis can be provided using a wide range of therapeutic agents, including traditional choices, fresh frozen plasma (FFP), cryoprecipitate, or the more advanced options of plasma-derived FXIII and recombinant FXIII concentrates [7, 8]. Although the rate of life-threatening bleeding is high in FXIII deficiency, with timely diagnosis and appropriate therapeutic regimen, the rate of morbidity and mortality can be significantly reduced. Clinical presentations, family history, and laboratory findings should be used for timely diagnosis, and the most suitable therapeutic regimen should be instituted for proper management of the disorder.

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_14

14.2 Classification

Coagulation FXIII is a heterotetramer (FXIII-A_2B_2) composed of two catalytic (FXIII-A_2) and two carrier subunits (FXIII-B_2) that are encoded by two separate genes, *F13A* and *F13B* [9]. Depending on the underlying gene defect, FXIII is classified into two types: FXIII-A and FXIII-B deficiencies [9, 10]. Due to the carrier role of FXIII-B_2, the deficiency of this FXIII subunit is accompanied by mild bleeding episodes. On the other hand, FXIII-A deficiency is one of the most severe congenital bleeding disorders, which is further classified into type I, with a concomitant decrease in FXIII-A antigen and activity levels, and type II FXIII-A deficiency, with normal or near-normal FXIII antigen levels and decreased FXIII activity [5] (Table 14.1).

Table 14.1 Classification of congenital factor XIII deficiency (adapted from [5])

FXIII deficiency		FXIII-A antigen	FXIII-B antigen	FXIII-A_2B_2 antigen	FXIII activity	Platelet FXIII-A antigen	Platelet FXIII activity
FXIII-A deficiency	Type I	↓↓↓	>30%	↓↓↓	↓↓↓	↓↓↓	↓↓↓
	Type II	↓ OR N	>30%	↓ OR N	↓↓↓	↓ OR N	↓↓↓
FXIII-B deficiency		↓↓	↓↓↓	↓↓↓	↓↓	N	N

The classification is based on the recommendations of the Scientific and Standardization Committee (SSC), Factor XIII and Fibrinogen Subcommittee, International Society for Thrombosis and Haemostasis (ISTH). *N* normal

14.3 Clinical Manifestations

Patients with congenital FXIII deficiency present a wide range of clinical manifestations, including UCB, ICH, impaired wound healing, epistaxis, gum bleeding, etc. UCB is a hallmark of FXIII deficiency and afibrinogenemia and is the most common presentation, observed in about 80% of neonates with severe FXIII deficiency [11]. In the absence of timely diagnosis and appropriate management, FXIII deficiency can result in fatal consequences in a considerable number of such neonates [2]. ICH is the most dreaded occurrence, and the main cause of death, in FXIII deficiency, observed in approximately one-third of patients [3]. In fact, ICH is more common in FXIII deficiency than any other congenital bleeding disorder [12, 13]. Generally, ICH has two main consequences: death and neurological complications. Most often (>90%), ICH occurs intraparenchymally, with the rest located in epidural and subdural spaces [3]. In about two-thirds of patients, ICH causes neurological complications, some of which are serious and disruptive to the patient's life [3]. ICH can occur spontaneously or following minor head trauma. Trauma-related ICH is more common in children, while spontaneous ICH is more frequent in adults [3]. Recurrent miscarriage is a common finding in women with severe FXIII deficiency, and generally, these women cannot have a successful delivery in the absence of replacement therapy [3, 14]. In one study, about one-third of patients experienced recurrent miscarriages, with two patients experiencing 13 spontaneous abortions each. Another interesting finding of this study was the high rate of mortality due to UCB: about one-fifth of deaths [3]. Due to the crucial role of coagulation FXIII in the healing process, impaired wound healing is a relatively common finding; about one-third of patients experience this diathesis [15–18] (Table 14.2).

Although heterozygous individuals are generally asymptomatic, post-traumatic bleeding has been reported in these individuals more frequently than in the general population. Indeed, heterozygous FXIII deficiency is mainly a hemorrhagic complication for women experiencing hemostatic challenges. Spontaneous bleeding does not occur in heterozygous FXIII deficiency, and almost all bleeding events occur in hemostatic challenges such as pregnancy, surgery, childbirth, and trauma. Among individuals with heterozygous FXIII deficiency, postoperative bleeding, postpartum hemorrhage, and miscarriage are the most common presentations [19–24].

Table 14.2 Clinical manifestations of patients with congenital factor XIII deficiency [15–18]

	Dorgalaleh et al. (N:218)[a]	Bouttefroy et al. (N:33)	Ivaskevicius V et al. (N:104)	Shetty et al. (N:96)
Umbilical cord bleeding	82.5%	57.6%	56%	73%
Intracranial hemorrhage	17%	27.3%	34%	19%
Miscarriage	~10%	3%[b]	NR	NR
Hematoma	53%	15.2%	49%	30%
Ecchymosis	13%	15.2%	NR[e]	58%
Hemarthrosis	4%	3%	36%	7%
Delayed wound healing	31%	NR	29%[d]	NR
Postsurgical bleeding	3%	3%[c]	40%	19%
Gum bleeding	17%	6%	NR	13%
GI bleeding	NR	NR	6%	4%
Menorrhagia	5%	3%[f]	NR	94%
Epistaxis	14%	NR	NR	25%
Genitourinary tract bleeding	NR	NR	NR	15%
Lacerations	NR	NR	NR	89%

NR Not reported
[a] Combination of several Iranian reports
[b] Spontaneous abortion
[c] Bleeding during surgery
[d] Prolonged wound bleeding
[e] Subcutaneous: 57%
[f] Menometrorrhagia

14.4 Molecular Basis

Severe congenital FXIII-A deficiency is due to homozygote or compound heterozygote *F13A* gene variants, while FXIII-B deficiency is due to *F13B* gene variants [9]. These variants can affect the synthesis of the protein, decrease its stability, and subsequently cause intracellular degradation of the protein [9, 21, 25, 26]. A total of 172 variants have been reported in the *F13A* gene, while 25 variants have been observed within *F13B*. About half of the variants in *F13A* (48.8%) and *F13B* (52%) genes are missense; most *F13A* variants (55.3%) occur within the catalytic core [9, 21, 28–30] (Table 14.3).

While there is no hotspot for *F13A* and *F13B* genes, a few recurrent variants have been reported in different nationalities [9]. Although Sanger sequencing is the most commonly available molecular method, in 5% of cases it cannot detect the underlying variant(s). However, next-generation sequencing or high-throughput sequencing can improve this situation [9]. The spectrum of *F13* gene variants in heterozygous FXIII deficiency is similar to that of homozygote FXIII deficiency. A total of 49 variants have beendetected in heterozygous FXIII deficiency, most of them missense (n:30, 61.2%), nonsense (n:6, 12.2%), small deletions (n:6, 12.2%), splice site (n:4, 8.2%), and large deletions (n:3, 6.2%) [21, 25–36].

Table 14.3 Spectrum of *F13A* and *F13B* gene variants (adapted from [9])

Gene	Number of variants	Type of variants (number)	Exonic site (number)	Protein site (number)	Comment
F13A	172	Missense (84) Insertion/deletion (45) Splice site (25) Nonsense (18)	Intronic (23) Exon 2 (6) Exon 3 (13) Exon 4 (14) Exon 5 (11) Exon 6 (13) Exon 7 (15) Exon 8 (8) Exon 9 (14) Exon 10 (7) Exon 11 (6) Exon 12 (11) Exon 13 (5) Exon 14 (13) Exon 15 (10)	Catalytic core (95) Beta sandwich (27) Barrel-1 (12) Barrel-2 (26) Undetermined (12)	
F13B	25	Missense (13) Splice site (4) Frameshift (8)	Intronic (4) Exon 2 (1) Exon 3 (3) Exon 4 (1) Exon 5 (1) Exon 7 (2) Exon 8 (3) Exon 9 (1) Exon 12 (1) Undetermined (8)	Sushi 1 (2) Sushi 2 (3) Sushi 3 (2) Sushi 4 (3) Sushi 6 (2) Sushi 7 (4) Sushi 8 (1) Sushi 12 (1) Undetermined (7)	

14.5 Diagnosis

Routine coagulation laboratory tests, including bleeding time, prothrombin time, activated partial thromboplastin time, thrombin time, and platelet count, are normal in FXIII deficiency [37–41]. Therefore, a more specific test should be used for the detection and confirmation of FXIII deficiency [42–47]. Although the clot solubility test is not a standard procedure, and is not further recommended for the detection of FXIII deficiency, it is the most commonly used diagnostic test worldwide and is used as a primary screening test in most developing, and 20% of developed, countries [46, 48]. In this assay, fibrin clot solubility is assessed in 5 M urea, 2% acetic acid, or 1% monochloroacetic acid solutions. In normal samples, the clot is stable for 1 day or more, whereas in FXIII-deficient samples, the clot is dissolved within a few minutes to a maximum of 1 h [37, 46]. The clot solubility test is not standardized, and various parameters influence the assay, including the type and concentration of clotting agent, the time of clotting, the type and concentration of solubilizing agents, and the time of detection of solubility [37, 46].

Precise diagnosis and classification of FXIII deficiency are achieved through quantitative assays. These are based on two principles: (1) measurement of FXIII activity level (functional activity assays) and (2) measurement of FXIII antigen level (immunological assays). FXIII activity assay is able to detect acquired and inherited forms of the disease and can quantify the FXIII level [4, 37]. Functional FXIII assay, which is used as a first-line assay for diagnosis, measures the residual FXIII activity. The measurement of FXIII activity is conducted through methods such as ammonia-release assay, or amine incorporation assay [37], which the former is the most commonly used assay. In this test, activated FXIII (FXIIIa) crosslinks a substrate to an oligonucleotide, which contains glutamine. Then, via glutamate dehydrogenase-mediated indicator reaction, NADH or NADPH in combination with one molecule of ammonia is released. FXIII activity is determined by the photometric absorbance at 340-nm wavelength. At a low FXIII activity level, photometric absorbance at 340 nm is decreased [37] (Fig. 14.1).

Due to the presence of other ammonia-producing and NADH-consuming reactions independent of FXIIIa, the use of a plasma blank is necessary to avoid overestimation of FXIII activity level [37, 45]. Since patients with a lower level experience severe clinical manifestations, the application of a plasma blank, for more reliable measurement of low FXIII activity, is inevitable [37]. FXIII antigen assay can be used for the classification of FXIII deficiency. There are several methods of measuring antigen levels, among which enzyme-linked immunosorbent assay is the most sensitive and reliable [37, 47]. One of the most important limitations of the assay is that it cannot identify type II FXIII deficiency [37].

Based on Clinical and Laboratory Standards Institute (CLSI) guidelines, three points should be considered for FXIII antigen assay: (1) interference of non-complex FXIII-B in the FXIII-A_2B_2 antigen assay should be prevented; (2) in subunit

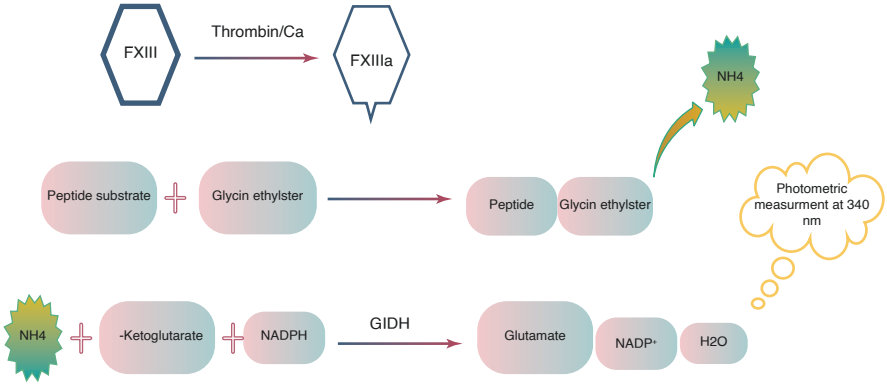

Fig. 14.1 The principle of the ammonia-release FXIII activity assay. Following activation, FXIIIa crosslinks a substrate to a glutamine-containing oligonucleotide, which releases NADH or NADPH with one molecule of ammonia via GIDH reaction. Finally, photometric absorbance at 3400 nm demonstrates FXIII activity. *FXIII* factor XIII; *FXIIIa* activated factor XIII; *GIDH* glutamate dehydrogenase-mediated indicator; *NADPH* nicotinamide adenine dinucleotide phosphate; *nm* nanometer

assays, both free and complex antigenic forms should react with antibodies to the same extent; and (3) interference of fibrinogen concentration in the antigen assay should be prevented [1].

A low level of FXIII in plasma does not necessarily result from congenital FXIII deficiency, as it can be related to an antibody against FXIII or other acquired conditions [49–53]. There are two types of autoantibodies, including neutralizing, which inhibits the activation of FXIIIa, and non-neutralizing, which accelerates the elimination of FXIIIa from the circulation. The former can be detected by a mixing study, while the latter can be diagnosed by a binding assay [54–57]. Neutralizing anti-FXIII-A antibodies cause a significant decrease in FXIII activity, whereas FXIII-A_2 and FXIII-A_2B_2 antigen levels are normal or slightly decreased. In contrast to neutralizing, non-neutralizing antibodies result in a significant reduction in all these parameters. In the presence of neutralizing and non-neutralizing anti-FXIII-A antibodies, the plasma FXIII-B_2 antigen level is more than 30%. In the presence of an anti-FXIII-B antibody, there is a reduction of FXIII-A and FXIII-A_2B_2 levels with a considerable reduction in FXIII-B_2 antigen [37] (Fig. 14.2).

A Bethesda assay is performed in order to achieve the approximate quantification of the inhibitors against FXIII. The Nijmegen modification of the Bethesda assay has more sensitivity and specificity compared with the Bethesda assay alone. If the residual FXIII activity is <25%, further dilution is recommended. Moreover, FXIII activity >75% excludes the presence of an inhibitor [26, 27]. An inhibitor level ≥0.6 BU/mL is considered to be of clinical significance. In addition, inhibitor levels are defined as low responding (‘5 BU/mL) and high responding (≥5 BU/mL) [37, 58].

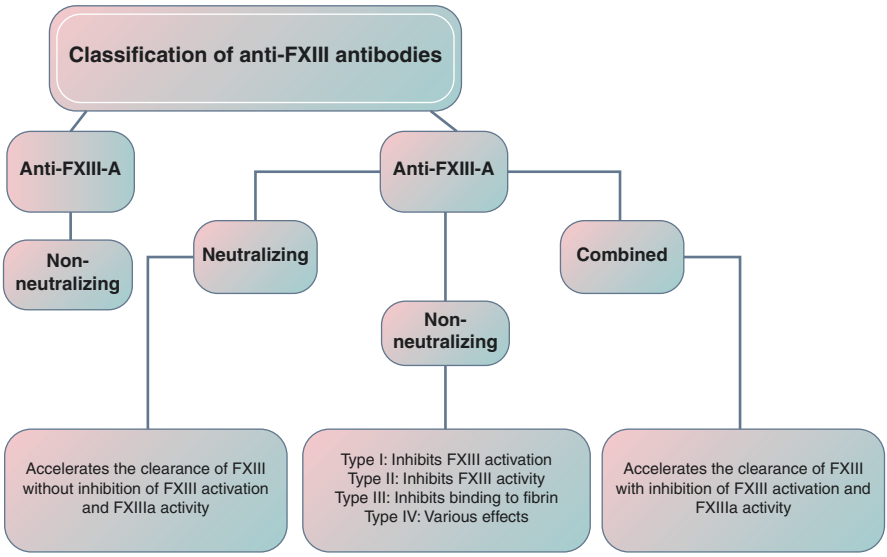

Fig. 14.2 The classification of different types of anti-factor XIII antibodies. *FXIII* factor XIII; *FXIIIa* activated factor XIII

14.6 Management

Due to the high rate of life-endangering hemorrhages, particularly ICH, primary life-long prophylaxis from the moment of diagnosis is mandatory for all patients with severe congenital FXIII deficiency [1, 4, 59]. Several therapeutic options are available, including FFP, cryoprecipitate, plasma-derived FXIII concentrate, and recombinant FXIII-A_2 (rFXIII-A_2) concentrate [59–65]. In spite of the severity of FXIII deficiency and its high rate of life-threatening bleeding, it can be managed easily due to the long half-life of FXIII (11–14 days) and its low hemostatic level. Although the treatment is relatively easy, however, treatment abandonment may result in fatal ICH in a considerable number of patients, who should be warned about this possibility [3]. Precise hemostatic levels of FXIII that prevent spontaneous major and minor bleeding, and manage major and minor surgeries, are not clear. Although plasma-derived FXIII and rFXIII are the preferred options, FFP and cryoprecipitate are the only therapeutic choices in a considerable number of countries. All available options are discussed below [60, 61] (Table 14.4).

For prophylaxis in FXIII deficiency, both 10–26 IU/kg every 4–6 weeks and 40 IU/kg every 4 weeks have been successfully used, but the rate of bleeding episodes was lower in the latter regimen. For surgical management of patients, a dose range from 25 to 40 IU/kg was used [64]. For the management of FXIII deficiency, rFXIII-A_2, in a dose of 35 IU/kg every 4 weeks, significantly reduced the rate of

Table 14.4 Therapeutic regimens for management of patients with congenital factor XIII deficiency (adapted from [15])

Therapeutic option	Type of treatment	Dose	Intervals
FFP	Prophylaxis	10 mL/kg	Every 4–6 weeks
Cryoprecipitate		1 bag/10 kg	
Pd-FXIII concentrate		10–26 IU/kg	
		40 IU/kg	Every 4 weeks
rFXIII-A_2 concentrate		35 IU/kg	Every 4 weeks
Pd-FXIII concentrate	ICH	1. Initially 30 IU/kg 2. 10–26 IU/kg for 10 days	
Pd-FXIII concentrate	Successfully delivery	1. 12 IU/dL (range 3–70 IU/dL) during pregnancy 2. 35 IU/dL (range 19–62 IU/dL) during labor 3. 10 IU/kg for 2 weeks	
Tranexamic acid	Minor surgery	15–20 mg/kg or 1 g	Four times daily alone
Pd-FXIII concentrate	Major surgery	10–40 IU/kg	Depending on the interval since the last prophylaxis and severity of bleeding
Pd-FXIII concentrate	PPH	1. 250 IU weekly early in pregnancy until 23rd week 2. 500 IU per week 3. For labor and delivery, a booster dose of 1000	

FFP fresh frozen plasma; *Pd-FXIII concentrate* plasma-derived factor XIII concentrate; *ICH* intracranial hemorrhage; *rFXIII* recombinant factor XIII; *PPH* postpartum hemorrhage; *IU* international units; *kg* kilogram

bleeding [65]. With the administration of this dose, the trough level of FXIII activity remained above 10% in all patients with congenital FXIII-A deficiency [65, 66]. Interestingly, the assay was performed in these patients in the absence of a plasma blank, meaning that the through level of FXIII activity was overestimated by about 5–8% of real FXIII activity levels [4]. It means that even a very low plasma level of FXIII (probably 5%) is sufficient to prevent bleeding [11].

Management of surgery is a challenge in FXIII deficiency, and separate therapeutic doses have been proposed for major and minor surgeries. It should be kept in mind that the management of surgical interventions can be affected by a number of elements and the same hemostatic level target cannot be used for all types of minor, and especially major, surgeries [15]. These include type, duration, and complexity of surgery [64]. A wide range of FXIII-level targets, ranging from 5% to >100%, has been proposed [15, 64, 67]. However, during prolonged and complicated surgeries, an FXIII level even as high as >100% cannot guarantee hemorrhagic prevention. Therefore, close monitoring of patients, especially by determination of FXIII activity during surgery, should be a matter of course for the prevention of intraoperative hemorrhage and related consequences. Close collaboration between hematologist, anesthesiologist, and surgeon is thus necessary. Although, as expected, the management of minor surgeries is less complex, as simple a developmental process as teething may result in life-threatening bleeding that requires medical intervention and factor replacement therapy [68]. Fadoo et al. recommended a dose of 10–20 U/kg FXIII concentrate for 2–3 days in the case of minor surgeries [69]. Although Curnow et al. recommended only tranexamic acid, with a dose of 15–20 mg/kg for the management of minor surgeries in rare bleeding disorders (RBDs) including FXIII deficiency, it seems that tranexamic acid cannot prevent bleeding in all types of minor surgeries [70]. For dental extraction, as an example, a minimum of 5% of FXIII level in an adult has been shown to be sufficient to prevent bleeding; other studies confirm this [1, 71]. In a large Iranian case series, single doses of 10 IU/kg, 30 IU/kg, and 50 IU/kg FXIII concentrate, respectively, were administered for minor surgery, major surgery or circumcision, and neurosurgery. Although preoperative FXIII activity was not ascertained, this study's authors expected that these doses would increase FXIII levels to 25%, 75%, and >100%, respectively [13]. In the absence of definitive guidelines, management of surgical intervention, especially major, is challenging in patients with congenital FXIII deficiency. With consideration of all risk factors, and close collaboration between medical teams, and close monitoring of the patient during surgery, the risk of intraoperative hemorrhage can be decreased significantly and patients' quality of life can be improved. A suitable therapeutic regimen should prevent intraoperative hemorrhage and promote successful wound healing.

Acknowledgment I highly appreciate the valuable work of Daisy Morant (ORCID #0000-0002-4055-0715) in improving the English language of the manuscript. I also appreciate the assistance of Seyed Mehrab Safdari in double-checking the presented data.

References

1. Dorgalaleh A, Naderi M, Safa M. Congenital factor XIII deficiency. In: Congenital bleeding disorders. Springer; 2018. p. 307–24.
2. Dorgalaleh A, Tabibian S, Shams M, Majid G, Naderi M, Casini A, et al. Editors. A unique factor XIII mutation in southeastern Iran with an unexpectedly high prevalence: Khash factor XIII. Semin Thromb Hemost. 2019;45(01):043–9.
3. Dorgalaleh A, Naderi M, Shamsizadeh M. Morbidity and mortality in a large number of Iranian patients with severe congenital factor XIII deficiency. Ann Hematol. 2016;95(3):451–5.
4. Muszbek L, Katona E. Diagnosis and management of congenital and acquired FXIII deficiencies. Semin Thromb Hemost. 2016;42:429–39.
5. Kohler H, Ichinose A, Seitz R, Ariens R, Muszbek L, et al. Diagnosis and classification of factor XIII deficiencies. J Thromb Haemost. 2011;9(7):1404–6.
6. Dorgalaleh A. The history of factor XIII deficiency. Semin Thromb Hemost. 2013;3(4):164.
7. Fisher S, Rikover M, Naor S. Factor 13 deficiency with severe hemorrhagic diathesis. Blood. 1966;28:34–9.
8. Nugent D. Corifact™/Fibrogammin® P in the prophylactic treatment of hereditary factor XIII deficiency: results of a prospective, multicenter, open-label study. Thromb Res. 2012;130(Suppl 2):S12–4.
9. Dorgalaleh A, Bahraini M, Shams M, Parhizkari F, Dabbagh A, Naderi T, Fallah A, Fazeli A, Ahmadi SE, Samii A, Daneshi M. Molecular basis of rare congenital bleeding disorders. Blood Rev. 2022;9:101029.
10. Biswas A, Ivaskevicius V, Thomas A, et al. Eight novel F13A1 gene missense mutations in patientswithmild FXIII deficiency: in silico analysis suggests changes in FXIII-a subunit structure/function. Ann Hematol. 2014;93(10):1665–76.
11. Naderi M, Dorgalaleh A, Alizadeh S, Tabibian S, Hosseini S, Shamsizadeh M, et al. Clinical manifestations and management of life-threatening bleeding in the largest group of patients with severe factor XIII deficiency. Int J Hematol. 2014;100(5):443–9.
12. Dorgalaleh A, Yadolah F, Haeri K, Baradarian Ghanbari O. Risk of intracerebral hemorrhge in patients with bleeding disorders. Semin Thromb Hemost. 2019;45(01):043–9.
13. Tabibian S, Motlagh H, Naderi M, Dorgalaleh A. Intracranial hemorrhage in congenital bleeding disorders. Blood Coagul Fibrinolysis. 2018;29(1):1–11.
14. Sharief LA, Kadir RA. Congenital factor XIII deficiency in women: a systematic review of literature. Haemophilia. 2013;19(6):e349–57.
15. Dorgalaleh A, Rashidpanah J. Blood coagulation factor XIII and factor XIII deficiency. Blood Rev. 2016;30:461–75.
16. Ivaskevicius V, Seitz R, Kohler HP, Schroeder V, Muszbek L, Ariens RA, et al. International registry on factor XIII deficiency: a basis formed mostly on European data. Thromb Haemost. 2007;98(06):914–21.
17. Shetty S, Shelar T, Mirgal D, Nawadkar V, Pinto P, Shabhag S, et al. Rare coagulation factor deficiencies: a countrywide screening data from India. Haemophilia. 2014;20(4):575–81.
18. Bouttefroy S, Meunier S, Milien V, Boucekine M, Chamouni P, Desprez D, Harroche A, Hochart A, Thiercelin-Legrand MF, Wibaut B, Chambost H. Congenital factor XIII deficiency: comprehensive overview of the FranceCoag cohort. Br J Haematol. 2020;188(2):317–20.
19. Duckert F, Jung E, Shmerling DH. A hitherto undescribed congenital haemorrhagic diathesis probably due to fibrin stabilising factor deficiency. Thromb Diath Haemorr. 1960;5(02):179–86.
20. Egbring R, Seitz R, Gürten GV, et al. Bleeding complications in heterozygotes with congenital Factor XIII deficiency. In: Mosesson MW, et al., editors. Fibrinogen 3. Biochemistry, biological functions, gene regulation and expression. Amsterdam: Elsevier; 1988. p. 341–6.
21. Dorgalaleh A. Novel insights into heterozygoud factor XIII deficiency. Semin Thromb Hemost. 2013;3(4):164.

22. Egbring R, Rohner I, Lerch L, Fuchs G, Kröniger A, Seitz R. Bleeding complications in patients with heterozygous FXIII subunit a deficiency? Blood Coagul Fibrinol. 1995;6:340.
23. Egbring R, Kröniger A, Seitz R. Factor XIII deficiency: pathogenic mechanisms and clinical significance. Semin Thromb Hemost. 1996;22(5):419–25.
24. Fisher S, Rikover M, Naor S. Factor 13 deficiency with severe hemorrhagic diathesis. Blood. 1966;28(1):34–9.
25. Ivaskevicius V, Windyga J, Baran B, Schröder V, Junen J, Bykowska K, Seifried E, Kohler HP, Oldenburg J. Phenotype–genotype correlation in eight polish patients with inherited factor XIII deficiency: identification of three novel mutations. Haemophilia. 2007;13(5):649–57.
26. Deng J, Li D, Mei H, Tang L, Wang HF, Hu Y. Novel deep intronic mutation in the coagulation factor XIII a chain gene leading to unexpected RNA splicing in a patient with factor XIII deficiency. BMC Med Gen 2020;21(1):9.
27. Shen MC, Chen M, Chang SP, Lin PT, Hsieh HN, Lin KH. Segmental uniparental disomy as a rare cause of congenital severe factor XIII deficiency in a girl with only one heterozygous carrier parent. Ped Hemato Onco. 2018;35(7–8):442–6.
28. Jia S, He Y, Lu M, Liao N, Lei Y, Lauriane N, Liang K, Wei H. Identification of novel pathogenic F13A1 mutation and novel NBEAL2 gene missense mutation in a pedigree with hereditary congenital factor XIII deficiency. Gene. 2019;70(2):143–7.
29. Moret A, Zúñiga Á, Ayala JM, Liquori A, Cid AR, Haya S, Ferrando F, Blanquer A, Cervera J, Bonanad S. Factor XIII deficiency in two Spanish families with a novel variant in gene F13A1 detected by next-generation sequencing; symptoms and clinical management. J Thromb Thromboly. 2020;50(3):686–8.
30. Borhany M, Handrková H, Cairo A, Schröder V, Fatima N, Naz A, Amanat S, Shamsi T, Peyvandi F, Kohler HP. Congenital factor XIII deficiency in Pakistan: characterization of seven families and identification of four novel mutations. Haemophilia. 2014;20(4):568–74.
31. Farah RA, Al Danaf JZ, Chahinian RA, Braiteh NT, Al Ojaimi NF, Cairo A, Farhat H, Mantoura JR. Spontaneous epidural hematoma in a child with inherited factor XIII deficiency. J Ped Hemat Onco. 2014;36(1):62–5.
32. Sun L, Yan Q, Wang Y, Luo H, Du P, Hassan R, Liu L, Jiang W. Pathogenicity analysis of variations and prenatal diagnosis in a hereditary coagulation factor XIII deficiency family. Hematology. 2018;23(8):501–9.
33. Handrkova H, Borhany M, Schroeder V, Fatima N, Hussain A, Shamsi T, Kohler HP. Identification of two novel missense mutations causing severe factor XIII deficiency. Haemophilia. 2015;21(3):e253–6.
34. Souri M, Biswas A, Misawa M, Omura H, Ichinose A. Severe congenital factor XIII deficiency caused by novel W187X and G273V mutations in the F13A gene; diagnosis and classification according to the ISTH/SSC guidelines. Haemophilia. 2014;20(2):255–62.
35. Halverstadt A, Walsh S, Roth SM, Ferrell RE, Hagberg JM. Identification of a novel mutation combination in factor XIII deficiency: genetic update to the first reported case in the United States. Int J hemato. 2006;83(2):144–6.
36. Castaman G, Giacomelli SH, Ivaskevicius V, Schröder V, Kohler HP, Dragani A, Biasioli C, Oldenburg J, Madeo D, Rodeghiero F. Molecular characterization of five Italian families with inherited severe factor XIII deficiency. Haemophilia. 2008;14(1):96–102.
37. Dorgalaleh A, Tabibian S, Hosseini MS, Farshi Y, Roshanzamir F, Naderi M, et al. Diagnosis of factor XIII deficiency. Hematology. 2016;21(7):430–9.
38. Shanbhag S, Shetty S, Kulkarni B, Ghosh K. An improved, semi quantitative clot based assay for factor XIII. Haemophilia. 2011;17(4):718–20.
39. Hsu P, Zantek ND, Meijer P, Hayward CP, Brody J, Zhang X, et al., editors. Factor XIII assays and associated problems for laboratory diagnosis of factor XIII deficiency: an analysis of international proficiency testing results. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2014.
40. Dorgalaleh A, Tabibian S, Shams M, Tavasoli B, Gheidishahran M, Shamsizadeh M. Laboratory diagnosis of factor XIII deficiency in developing countries: an Iranian experience. Lab Med. 2016;47(3):220–6.

41. Loewy AG, Dunathan K, Kriel R, Wolfinger HL, Fibrinase I. Purification of substrate and enzyme. J Biol Chem. 1961;236(10):2625–33.
42. Bohn H. Isolation and characterization of the fibrin stabilizing factor from human thrombocytes. Thromb Diath Haemorrh. 1970;23(3):455.
43. Sigg P. The monoiodoacetate (MIA) tolerance test, a new quantitative method for the fibrin stabilizing factor (factor XIII) assay. Thromb Haemost. 1966;15(01):238–51.
44. Bohn H, Haupt H. A quantitative determination of factor 13 with anti-factor 13 serum. Thromb Diath Haemorrh. 1968;19(3):309.
45. Katona É, Pénzes K, Molnár É, Muszbek L. Measurement of factor XIII activity in plasma. Clin Chem Lab Med. 2012;50(7):1191–202.
46. Jennings I, Kitchen S, Woods T, Preston F. Problems relating to the laboratory diagnosis of factor XIII deficiency: a UK NEQAS study. J Thromb Haemost. 2003;1(12):2603–8.
47. Katona É, Ajzner É, Tóth K, Kárpáti L, Muszbek L. Enzyme-linked immunosorbent assay for the determination of blood coagulation factor XIII A-subunit in plasma and in cell lysates. J Immunol Methods. 2001;258(1–2):127–35.
48. Dorgalaleh A, Tabibian S, Hosseini S, Shamsizadeh M. Guidelines for laboratory diagnosis of factor XIII deficiency. Blood Coagul Fibrinolysis. 2016;27(4):361–4.
49. Ichinose A, Group TJCR. Inhibitors of factor XIII/13 in older patients. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2014.
50. Muszbek L, Pénzes K, Katona É. Auto-and alloantibodies against factor XIII: laboratory diagnosis and clinical consequences. J Thromb Haemost. 2018;16(5):822–32.
51. Penzes K, Vezina C, Bereczky Z, Katona E, Kun M, Muszbek L, et al. Alloantibody developed in a factor XIII a subunit deficient patient during substitution therapy; characterization of the antibody. Haemophilia. 2016;22(2):268–75.
52. Kun M, Szuber N, Katona É, Pénzes K, Bonnefoy A, Bécsi B, et al. Severe bleeding diatheses in an elderly patient with combined type autoantibody against factor XIII a subunit; novel approach to the diagnosis and classification of anti-factor XIII antibodies. Haemophilia. 2017;23(4):590–7.
53. Pénzes K, Rázsó K, Katona E, Kerenyi A, Kun M, Muszbek L. Neutralizing autoantibody against factor XIII a subunit resulted in severe bleeding diathesis with a fatal outcome–characterization of the antibody. J Thromb Haemost. 2016;14(8):1517–20.
54. Lorand L, Velasco P, Rinne J, Amare M, Miller L, Zucker M. Autoimmune antibody (IgG Kansas) against the fibrin stabilizing factor (factor XIII) system. Proc Natl Acad Sci. 1988;85(1):232–6.
55. Ajzner É, Schlammadinger Á, Kerényi A, Bereczky Z, Katona É, Haramura G, et al. Severe bleeding complications caused by an autoantibody against the B subunit of plasma factor XIII: a novel form of acquired factor XIII deficiency. Blood. 2009;113(3):723–5.
56. Ichinose A. Autoimmune acquired factor XIII deficiency due to anti-factor XIII/13 antibodies: a summary of 93 patients. Blood Rev. 2017;31(1):37–45.
57. Osaki T, Sugiyama D, Magari Y, Souri M, Ichinose A. Rapid immunochromatographic test for detection of anti-factor XIII a subunit antibodies can diagnose 90% of cases with autoimmune haem (orrhaphilia XIII/13). Thromb Haemost. 2015;113(06):1347–56.
58. Miller C, Platt S, Rice A, Kelly F, Soucie J, Investigators* HIRS. Validation of Nijmegen–Bethesda assay modifications to allow inhibitor measurement during replacement therapy and facilitate inhibitor surveillance. J Thromb Haemost. 2012;10(6):1055–61.
59. Ashley C, Chang E, Davis J, Mangione A, Frame V, Nugent DJ. Efficacy and safety of prophylactic treatmentwith plasma-derived factor XIII concentrate (human) in patients with congenital factor XIII deficiency. Haemophilia. 2015;21(1):102–8.
60. Nugent DJ, Ashley C, García-Talavera J, Lo LC, Mehdi AS, Mangione A. Pharmacokinetics and safety of plasma-derived factor XIII concentrate (human) in patients with congenital factor XIII deficiency. Haemophilia. 2015;21(1):95–101.
61. Lusher J, Pipe SW, Alexander S, Nugent D. Prophylactic therapy with fibrogammin P is associated with a decreased incidence of bleeding episodes: a retrospective study. Haemophilia. 2010;16(2):316–21.

62. Lovejoy AE, Reynolds TC, Visich JE, et al. Safety and pharmacokinetics of recombinant factor XIII-A2 administration in patients with congenital factor XIII deficiency. Blood. 2006;108(1):57–62.
63. Fujii N, Souri M, Ichinose A. A short half-life of the administered factor XIII (FXIII) concentrates after the first replacement therapy in a newborn with severe congenital FXIII deficiency. Thromb Haemost. 2012;107(3):592–4.
64. Janbain M, Nugent DJ, Powell JS, St-Louis J, Frame VB, Leissinger CA. Use of factor XIII (FXIII) concentrate in patients with congenital FXIII deficiency undergoing surgical procedures. Transfusion. 2015;55(1):45–50.
65. Inbal A, Oldenburg J, Carcao M, Rosholm A, Tehranchi R, Nugent D. Recombinant factor XIII: a safe and novel treatment for congenital factor XIII deficiency. Blood. 2012;119(22):5111–7.
66. Brand-Staufer B, Carcao M, Kerlin BA, et al. Pharmacokinetic characterization of recombinant factor XIII (FXIII)-A2 across age groups in patients with FXIII a-subunit congenital deficiency. Haemophilia. 2015;21(3):380–5.
67. Colin W, Needleman HL. Medical/dental management of a patient with congenital factor XIII. Pediatr Dent. 1985;7(3):227–30.
68. Golpayegani MV, Behnia H, Araghi MA, Ansari G. Factor XIII Deficiency, Review of the literature and report of a case. Journal of comprehensive Pediatrics 2016;7(4).
69. Fadoo Z, Merchant Q, Rehman KA. New developments in the management of congenital factor XIII deficiency. J Blood Med. 2013;4:65.
70. Curnow, et al. Managing and supporting surgery in patients with bleeding disorders. Semin Thromb Hemost. 2017;43:653–71.
71. Salcioglu Z, Tugcu D, Akcay A, Sen HS, Aydogan G, Akici F, et al. Surgical interventions in childhood rare factor deficiencies: a single-center experience from Turkey. Blood Coagul Fibrinolysis. 2013;24(8):854–61.

Part IV

Inherited Platelet Function Disorders

Glanzmann Thrombasthenia: Diagnosis and Management

15

Man-Chiu Poon and Seyed Mehrab Safdari

15.1 Introduction

Glanzmann thrombasthenia (GT) is a rare congenital bleeding disorder of platelet function with an estimated incidence of one per one million in the general population. In regions where consanguineous marriage is common, the incidence may be as high as 1:40,000–100,000 [1]. Although GT is a rare disorder, it is the most prevalent inherited platelet function disorder (IPFD) with a severe bleeding phenotype. GT is due to mutations in the *ITGA2B* and/or *ITGB3* genes that encode αIIbβ3 (glycoprotein (GP) IIb/IIIa) resulting in qualitative or quantitative integrin defects. αIIbβ3 is a fibrinogen receptor and plays a crucial role in platelet aggregation; therefore, platelet aggregation is impaired in GT. Bleeding tendency is highly variable among the affected patients. Homozygous and double heterozygous patients may be prone to life-threatening bleeding events, while heterozygous patients are mainly asymptomatic. Patients with GT usually present with mucocutaneous bleeding early in life. Purpura, epistaxis, and gum bleeding are the most common clinical presentations. Menorrhagia is one of the most common presentations among women with GT [2–4]. Diagnosis of this disorder is made based on clinical manifestations, family history, and appropriate laboratory assessment. In laboratory diagnosis, patients with GT have an impaired response to all physiological agonists but with a normal response to ristocetin. In flow cytometric analysis, αIIbβ3 (CD41/CD61) is

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_15

decreased, but in the variant form of GT, nonfunctional αIIbβ3 is expressed in a nearly normal amount [5]. Patients with GT are inherited autosomal recessively and generally have a normal platelet count and platelet morphology. Rare gain-of-function variants may be inherited autosomal dominantly and may have macrothrombocytopenia because of αIIbβ3 mutations affecting megakaryopoiesis and platelet production [5]. A wide spectrum of mutations is identified in both *ITGA2B* and *ITGB3* genes, although there are more mutations affecting *ITGA2B* [6]. Different therapeutic options are available for the management of patients with GT including local and systemic measures, and the use of antifibrinolytics, platelet concentrates, and recombinant activated factor VII (rFVIIa). More advanced curative options are stem cell transplantation and gene therapy. Currently, platelet transfusion is the standard treatment for patients with GT, but for mild bleeds, local and antifibrinolytic agents can be used. Repeated platelet transfusions can result in alloimmunization against platelet human leukocyte antigen [HLA] and/or αIIbβ3 antigens resulting in platelet refractoriness. αIIbβ3 antibodies can also cross the placenta during pregnancy resulting in harm (bleeding, thrombocytopenia) to the fetus/neonate. HLA-matched leukocyte-reduced platelet concentrates, particularly from single donors, can minimize the risk of HLA alloimmunization. rFVIIa can be used for the management of patients with platelet antibodies and platelet refractoriness [7–10]. rFVIIa is also efficacious and safe in GT patients without platelet antibodies or platelet refractoriness [11]. In some countries (e.g., the European Union and Canada), rFVIIa is approved for any GT patients when platelet concentrates are not readily available.

15.2 Integrin αIIbβ3 (GPIIb/IIIa) Structure and Function

15.2.1 Biosynthesis Pathway

αIIbβ3 and αvβ3 are two members of the β3 subfamily with a common beta subunit with 36% amino acid sequence identity of the alpha subunits and having similar biosynthetic pathways. αIIbβ3 consists of α and β subunits that are noncovalently bonded to each other. Initially, αIIb (GPIIb) is synthesized as a pro-αIIb moiety, which consists of light (L) and heavy (H) chains that are connected to each other. β3 (GPIIIa) is produced as a single chain. β3 undergoes N-linked glycosylation and complexes with pro-αIIb in the endoplasmic reticulum (ER). The heterodimers are then transported into the Golgi system. The intracellular transport, stability, and processing of the αIIb subunit are dependent on the complex formation with β3. Almost all translated αIIb make a complex with β3 and appear on the cell surface, only 40% of the translated β3 binds to αIIb to form the αIIbβ3 complex; the other 60% remains inside the megakaryocytes (MK). It is suggested that αIIb and the alpha subunit (αv) of vitronectin receptors (αvβ3) compete for complex formation with the β3 subunit within ER (Fig. 15.1) [10, 12].

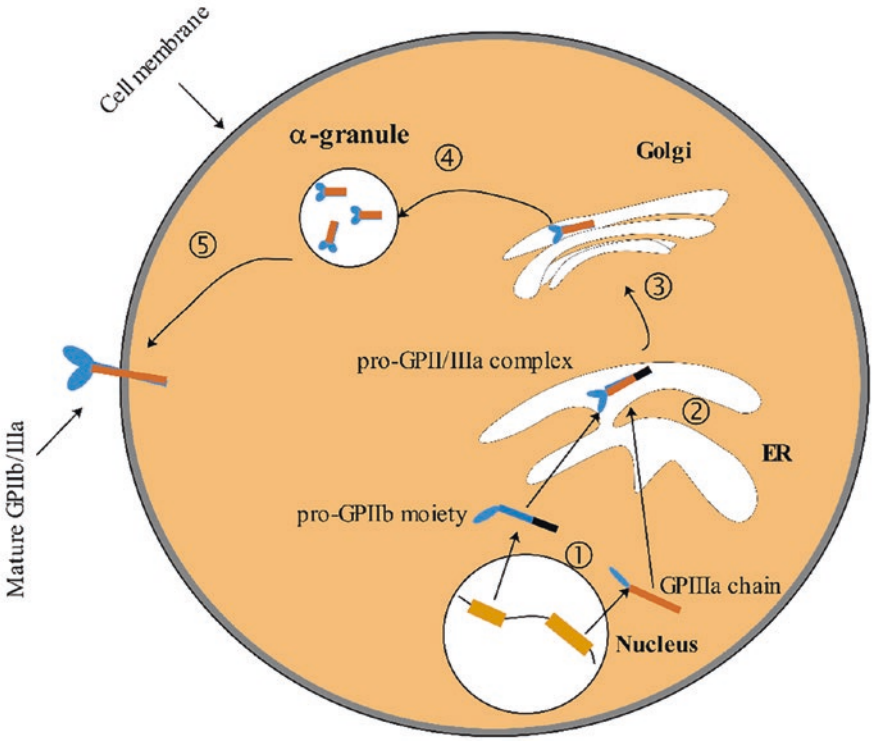

Fig. 15.1 Biosynthesis pathway of integrin αIIbβ3 (GPIIb/IIIa). (1) Two subunits of integrin αIIb (GPIIb) and β3 (GPIIIa) are encoded by *ITGA2B* and *ITGB3* genes located on the long arm of chromosome 17. The synthesis of these subunits differs; GPIIb is synthesized as a pro-GPIIb moiety, which consists of a heavy (H) and light (L) chain, while GPIIIa is synthesized as a single chain. (2) Within the ER, the GPIIIa undergoes post-translational glycosylation and makes a complex with pro-GPIIb moiety. In addition, high mannose N-linked oligosaccharide processing and degradation of uncompleted subunits occur in the ER. (3) The formed pro-GPIIb/IIIa (αIIbβ3) complex is transported into the Golgi apparatus. Further N-linked oligosaccharide processing and proteolytic cleavage of pro-αIIb occur in the Golgi complex. (4) After final processing, mature αIIbβ3 (GPIIb/IIIa) complexes are stored within alpha granules. (5) Mature αIIbβ3 (GPIIb/IIIa) complexes appear on the cell surface. *ER* endoplasmic reticulum, *Golgi* complex

15.2.1.1 Integrin αIIbβ3 (GPIIb/IIIa) Structure

αIIbβ3 complex is the most abundant glycoprotein (GP) on the platelet surface forming ~1% of the platelet's weight. Nearly 70% of αIIbβ3 are distributed randomly on the platelet surface, while the remaining 30% are located between the closed canalicular membrane and alpha granules' membrane. When platelets are suspended in a buffer containing calcium chelators, the αIIbβ3 structure is changed leading to the separation of the monomeric subunits, which can no longer support the platelet aggregation response. Activation of platelets in the presence of strong agonists (thrombin and collagen) or weak agonists (adenosine diphosphate (ADP)

Table 15.1 Characteristics of αIIbβ3 (GPIIb/IIIa) subunits

Characterization	Subunit	
	GPIIb	GPIIIa
Number of amino acids	1146	747
Glycosylation sites	5	6
Number of cysteine residues	17	56
Number of calcium-binding domains	4	1
Cytoplasmic domain length	37	45
Transmembrane domain length	23	22

and epinephrine (EP)) results in a conformation change of αIIbβ3 on the platelet surface to function as active fibrinogen receptors [12, 13] (Table 15.1).

15.2.1.2 αIIb (GPIIb) Subunit

ITGA2B, the gene encoding αIIb spans 17.2 kb and consists of 30 exons. It is located on the long arm of chromosome 17. αIIb with a molecular weight of 139 kDa consists of two polypeptide chains, L and H, bound through disulfide bonds. The H chain consists of 871 amino acids with a molecular weight of 125 kD, while the L chain consists of 37 amino acids and has a molecular weight of 23 kDa. The L chain consists of one transmembrane domain with 25 amino acids, while the H chain is entirely extracellular. About 15% of αIIb includes mannose-rich complex carbohydrates. The H chain of αIIb has a 12 amino acid sequence containing the calcium/calmodulin-binding domain, which can bind γ-chain dodecapeptide sequences of fibrinogen. In addition, there are four calcium-binding sites on it [12–14].

15.2.1.3 β3 (GPIIIa) Subunit

ITGB3, the gene encoding β3 spans 65 kb and consists of 15 exons, is also located on the long arm of chromosome 17. The single chain β3 with a molecular weight of 92 kDa has 747 amino acids and contains a 26-amino acid polypeptide in the N-terminal and a large external domain with 56 cysteine residues. The transmembrane domain and cytoplasmic tail of β3 consist of 29 and 41 amino acids, respectively. About 15% of β3 molecular weight is carbohydrates.

15.2.1.4 Ligand Binding to αIIbβ3

After platelet activation, the αIIbβ3 complex undergoes conformational changes and binds to fibrinogen and other physiological agonists such as ADP, adrenalin, thrombin, and platelet-activating factor (PAF). Each αIIbβ3 complex binds to one fibrinogen molecule. Upon ADP stimulation, approximately 15,400–82,500 (mean: 38,000) fibrinogen molecules can bind to αIIbβ3 (GPIIb/IIIa) complexes on the platelet surface. In addition to fibrinogen, other ligands such as von Willebrand factor (VWF), fibronectin, and vitronectin can bind the platelet surface and activate the αIIbβ3 complex with a similar mechanism. Amino acid sequences that mediate these interactions with αIIbβ3 include a sequence of three amino acids: arginine, glycine, and asparagine (RGD). RGD is the adhesive sequence originally identified

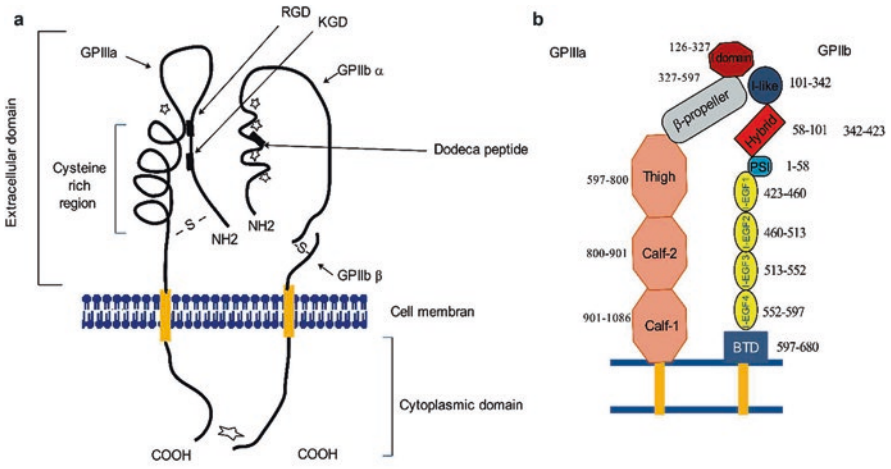

Fig. 15.2 (**a**) Structure of integrin αIIbβ3 (GPIIb/IIIa). (**b**) Domain structure of integrin αIIbβ3. GP: Glycoprotein; Dodecapeptide (DXDXD/NGXX/DXXD) is a fibrinogen-binding site on αIIb (GPIIb) subunit; GPIIbα: the heavy chain of GPIIb; GPIIb β: the light chain of GPIIb; RGD: Arg-Gly-Asp sequence is a fibrinogen-binding site on GPIIIa subunit; KGD: Lys-Gly-Asp sequence is a fibrinogen-binding site on GPIIIa subunit; BTD: β-tail domain; IEGF1–4: integrin epidermal growth factor domain; PSI: plexin-semaphorin-integrin domain; Hybrid: β-sandwich hybrid domain; I-like: β I-like domain; I domain: A-type domain; β-propeller: N-terminal domain of GPIIIa; Thigh and calf-1-2: cysteine-rich region and protease-resistant domains; The star shape indicates cation-binding sites

in fibronectin molecules and is also identified in fibrinogen, VWF, and vitronectin. Fibrinogen has two RDG sequences (at amino acid sequences 95–97 and 572–574), on its alpha chain, while other ligands only have one RDG sequence. The other important area in the fibrinogen molecule participating in αIIbβ3 interaction is the 12-peptide sequence (HHLGGAKQAGDV) located at the carboxylic tail of its gamma chain (Fig. 15.2). This dodecapeptide sequence is not found in other αIIbβ3-interacting adhesive molecules [12–16].

15.2.1.5 Signaling Pathway for αIIbβ3 (GPIIb/IIIa) Activation

αIIbβ3 plays a pivotal role in platelet aggregation and platelet activation, and the acquisition of a capacity to bind soluble ligands is necessary for this function. Two main mechanisms, outside-in and inside-out signaling, are involved in αIIbβ3 affinity to ligands. The former is triggered by the ligands binding to the extracellular domains of αIIbβ3 and the latter is triggered by the factors binding to the cytoplasmic domains of αIIbβ3 following platelet activation.

The inside-out signaling generated by the activation of platelets leads to conformational changes in αIIbβ3 and alters the relative orientation of αIIb and β3 subunits to each other. The biochemical events involved in the signaling pathway of αIIbβ3 (GPIIb/IIIa) are classified into the following processes: (1) binding of platelet agonists ADP, collagen, serotonin, thrombin, EP, thromboxane A2 (TxA2), or

PAF to their receptors on the platelet surface leads to platelet activation and signal transduction via the agonist receptor's cytoplasmic domains. (2) Signal transduction from the agonist receptor cytoplasmic domain to the cytoplasmic domains of αIIbβ3. (3) Signal transduction from αIIbβ3 cytoplasmic domains to its extracellular domains leads to integrin conformational changes and increased integrin affinity. (4) Platelet-activating secondary signal leads to cytoplasmic granular release.

The various platelet agonists (ADP, PAF, lysophosphatidic acid (LPA), TxA2, serotonin, and thrombin) transmit signaling through the G-proteins. G-proteins comprise three subunits, α, β, and γ, in a heterotrimeric complex that binds the G-protein-coupled receptors (GPCRs). GPCRs are a large family of seven-transmembrane domain receptors on the cell surface. Based on α-subunits similarity, G-proteins have been divided into four subfamilies: Gq, G12, Gi, and Gs. Binding of ligands to the G-protein results in the conversion of the inactive GDP-bound form to the active GTP-bound form of the α-subunit, and the separation of the β/γ complex from the activated α-subunit. The activated α-subunit and β/γ complex can interact with downstream targets in the G-protein signaling pathway. Several changes induced by the platelet agonists include platelet deformation, granule contents release, clot contraction, and platelet adherence to the vascular endothelial cells. All these events are considered as a result of the following biological processes (Fig. 15.3):

1. G-proteins activate phospholipase C (PLC) and A2 (PLA2) that lead to the production of secondary messengers including diacylglycerol (DAG), inositol triphosphate (IP3), and arachidonic acid (AA).
2. The assembled integrin activates serine/threonine kinases and phosphatases, which lead to phosphorylation/dephosphorylation of cytoplasmic proteins such as lipid kinase (phosphatidyl inositol 3 and 4 kinases (PI3 and PI4k)).
3. PLC converts phosphatidylinositol 4, 5-bisphosphate (PIP2) to DAG, and IP3 leading to the entrance of calcium from outside cells and the ER into the cytoplasm.
4. Phosphokinase C (PKC) phosphorylates Ras-proximate-1/guanosine diphosphate (Rap1/GDP) which has been bound to the cytoplasmic tail of αIIb. Calcium and DAG-regulated guanine nucleotide exchange factor 1 (CalDAG/GEF1) serve as cofactors for this function.
5. Activation of antiporter of Na^+/K^+ leads to elevation of cytoplasm pH as well as phosphorylation of pleckstrin through PKC.
6. Rap1/GDP interacts with the Rap1-GTP-interacting adaptor molecule (RIAM).
7. RIAM recruits talin1 and kindlin-3, which interact with the cytoplasmic tail of β3.
8. The rearrangement of cytoskeletal proteins leads to the conformational change in extracellular domains of both αIIb and β3, which enables αIIbβ3 to bind to other ligands.

Talin1 with a molecular weight of 270 kDa is one of the cytoskeletal proteins that connect the cytoplasmic domains of the integrin β subunit to actin filaments. Talin1 is composed of talin-H (a globular N-terminal head domain) and talin-R (a flexible

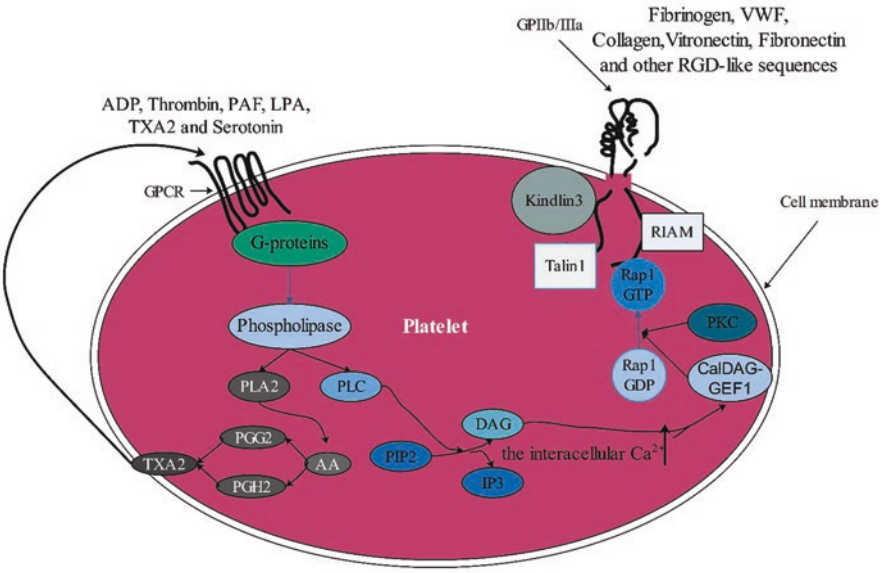

Fig. 15.3 The molecular mechanisms involved in the signaling pathway of integrin αIIbβ3 (GPIIb/IIIa). G-protein-mediated signaling pathway plays a critical role in the activation of αIIbβ3, which is initiated by the binding of ADP and some other agonists such as thrombin, PAF, TXA2, and serotonin to their receptors. The downstream molecules of G-protein-mediated signaling such as phospholipase A2 (PLA2), phospholipase C (PLC), etc. have cooperated in this activation. The downstream molecules including calcium and DAG-regulated guanine nucleotide exchange factor 1 (CalDAG/GEF1), Ras-proximate-1/guanosine diphosphate, Rap1/GTP (Rap1/GDP), Rap1-GTP-interacting adaptor molecule (RIAM) and protein kinase C (PKC) are considered as important molecules in activation of αIIbβ3. *ADP* adenosine diphosphate; *PAF* platelet-activating factor; *LPA* lysophosphatidic acid, *TxA2* thromboxane A2; *GPCR* G-protein-coupled receptors; *AA* arachidonic acid; *PGG2* prostaglandin G2; *PGH2* prostaglandin H2; *PIP2* phosphatidylinositol biphosphate; *DAG* diacylglycerol; *IP3* inositol trisphosphate; *VWF* von Willebrand Factor; *RGD* arginine, glycine, and asparagine

C-terminal domain). Talin-H binds the NPLY motif of the integrin β subunit, which initiates a conformational change in the extracellular domains of integrin αIIbβ3. The interaction between talin1 and cytoplasmic domains of the integrin β subunit is a key element in αIIbβ3 activation [12, 17–20].

Kindlin-3 is an integrin co-activator that binds the cytoplasmic domains of the integrin β subunit and contributes to αIIbβ3 activation. Kindlin-3 binds the NITY motif of the integrin β subunit. The integrin-binding protein and calcium-binding protein then interact with the cytoplasmic domains of the integrin β subunit and cooperate with kindlin-3 and talin 1 in the activation of αIIbβ3. When αIIbβ3 is activated, it can bind with high affinity to fibrinogen and VWF. These ligands serve as bridges between adjacent platelets to form platelet aggregates [19, 21].

The importance of the αIIbβ3 signaling pathways for primary hemostasis is reflected by the description that patients with mutations in CalDAG/GEF1 and Kindlin-3 have thrombasthenia-like bleeding symptoms [3].

15.2.2 Integrin αIIbβ3 Antagonists

The integrin αIIbβ3 binding to ligands results in cross-linking between adjacent platelets, resulting in aggregation and subsequent clot formation. Although platelet aggregation and clot formation are considered as natural mechanisms to prevent bleeding following tissue damage, uncontrolled platelet aggregation can result in thrombus formation giving rise to various morbidities. Such overactivation and aggregation can be prevented by antagonists targeting the αIIbβ3 receptor. There are three groups of such inhibitors: abciximab, eptifibatide, and tirofiban. Abciximab, an irreversible noncompetitive inhibitor is a Fab fragment of the chimeric monoclonal antibody 7E3, that binds to the αIIbβ3 and inhibits cell–ligand interactions. Eptifibatide and tirofiban are antagonists that bind to the alpha subunit of αIIbβ3 in a competitive reversible manner. Tirofiban contains the RGD sequence, while in eptifibatide, the arginine residue of the RGD motif is replaced by a lysine amino acid [22, 23].

15.3 Glanzmann Thrombasthenia

15.3.1 General Overview

GT is a rare autosomal recessive bleeding disorder that most often presents with spontaneous mucocutaneous bleeding early in life. Bleeding tendency is highly variable among the affected patients, ranging from asymptomatic conditions to potentially life-threatening bleeds including intracranial hemorrhage. Purpura, epistaxis, gum hemorrhage, and menorrhagia are the most common clinical presentations. Generally, the severity of bleeding is decreased with age. This finding is contrary to Bernard–Soulier syndrome in which the bleeding tendency worsens in adult age. Most of the patients are diagnosed early in life with mucocutaneous bleeding. Timely diagnosis and appropriate management of the disorder are crucial. Diagnosis of GT can be done based on clinical presentations, physical examinations, family history, and appropriate laboratory assessments. For patients without a family history of GT, a differential diagnosis that should be considered includes malignancies and autoantibody-induced acquired GT or the use of αIIbβ3 antagonist medications. All routine coagulation tests are generally normal in GT except for a prolonged bleeding time (BT)/closure time (Platelet Function Analyzer [PFA]-100/200®). Platelet count and platelet morphology are generally normal, although rare gain-of-function variants may have macrothrombocytopenia. Confirmatory investigations will show the distinct feature of impaired aggregation response to all physiological agonists while ristocetin-induced platelet agglutination is normal. Flow cytometry analysis reveals a decrease of αIIbβ3 (CD41/CD61) in GT but may be quantitatively normal (but dysfunctional) in variant GT forms (Table 15.2) [2, 3, 8].

GT is due to mutations in αIIbβ3 (GPIIb/IIIa) encoded by two separated genes, ITGA2B (for αIIb) and *ITGB3* (for β3). A wide spectrum of mutations has been

Table 15.2 Classification and characteristics of Glanzmann thrombasthenia

Type	Percent	Platelet integrin αIIbβ3 expression	Platelet integrin αIIbβ3 (%) (Flow cytometry)	Platelet aggregation[a]	Platelet agglutination[b]	BT/CT (PFA-100/200)	Clot retraction
Type I	~75%	Undetectable or trace	5	Absent	Normal	Prolonged	Absent
Type II	~15%	Significantly reduced	5–20	Absent	Normal	Prolonged	Subnormal
Variant	~10%	Nearly normal	>20	Absent/ abnormal	Normal	Prolonged	Variable

BT bleeding time; *CT* closure time; *PFA-100/200* platelet function analyzer-100/200. (Adapted from Poon et al. [8] with permission from the publisher)

[a] Platelet aggregation to thrombin, collagen, or adenosine diphosphate and epinephrine

[b] Platelet agglutination to ristocetin

identified, more so in *ITGA2B* than in *ITGB3*. Family-specific mutations can be used for carrier detection and prenatal diagnosis (PND) in the affected family. Mutation analysis is also useful in identifying αIIbβ3 mutations that may be at risk for the development of αIIbβ3 alloimmunization following platelet transfusion [24].

15.3.2 Clinical Manifestations

Patients with GT have variable bleeding tendencies, some patients have quite mild bleeding symptoms, while others experience life-threatening hemorrhages. Bleeding symptoms usually occur in homozygotes and double heterozygotes, while heterozygotes with an about half-normal concentration of αIIbβ3 usually are asymptomatic. Rare exceptions are GT with gain-of-function αIIbβ3 mutations inherited autosomal dominantly [3, 25]. These patients usually have macrothrombocytopenia.

Although the disorder usually manifests early in life, some may not be diagnosed until much later. The majority of GT patients are diagnosed before the age of 5 years (with first bleed symptoms occurring at a median age of 1 year, mean 5.6 years [8] with recurrent epistaxis and gingival bleeds. In the majority of patients with bleeding, symptoms may be sufficiently severe to require a blood transfusion. With increasing age in GT, the bleeding tendency is generally decreased, but the incidence of severe bleeds is not. The bleeding symptoms in GT are mostly mucocutaneous, with purpura, epistaxis, gum bleeding, and menorrhagia nearly constant features of this disorder, while gastrointestinal bleeding and hematuria are less frequent bleeding symptoms. There is no correlation or little relationship between platelet αIIbβ3 concentration (hence GT types) and severity of hemorrhagic tendency. Some patients with undetectable levels of αIIbβ3 may have a mild bleeding phenotype, while others with 10–15% αIIbβ3 levels may have severe life-threatening hemorrhagic manifestations. It seems that an absence of αvβ3 in vascular cells can contribute to the severity of bleeding symptoms (Table 15.3) [2, 12, 26].

Table 15.3 Clinical manifestations of patients with Glanzmann thrombasthenia

Bleeding symptoms	Iranian (n:382) [26]	France (n:177)[a] [27]	Pakistan (n:43) [28]	GTR (n:216) [1]
Epistaxis	50%	73%	62.5%	79%
Gingival bleeding	23%	55%	56.4%	62%
Ecchymosis, easy bruising, purpura, petechiae	15%	86%	76.6%	43%
Hematuria	–	6%	8%	7%
Gastrointestinal bleeding	4.7%	12%	7.4%	23%[b]
Hematoma	4.7%	1%	–	13%[c]
Menorrhagia	12.9%	98%	70%	74%
Post-circumcision bleeding	3.6%	–	43.4%	5%
Post-surgical bleeding	2.8%	–	–	–
Post-trauma bleeding	–	–	47.2%	–
Bleeding at injection sites	2%	–	–	1.4%[d]
Umbilical cord bleeding	0.3%	–	5.5%	–
Excessive bleeding at delivery	0.5%	–	–	74%
Central nervous system bleeding	0.3%	2%	–	1.8%
Hemarthrosis	0.3%	3%	–	6%

GTR Glanzmann's Thrombasthenia Registry; *n* number

[a] Data from 113 patients from literature and 64 patients from France

[b] Twenty-three percent for general gastrointestinal bleeding (additional 6% for hemorrhoidal bleeding)

[c] 13% for muscle hematoma (additional 38% for subcutaneous hematoma)

[d] Bleeding from the vaccination injection site

There is also a poor relationship between underlying αIIbβ3 gene defect and severity of bleeding tendency, and bleeding tendency can be variable even among family members. In males, diagnosis of the disorder may occur after excessive post-circumcision bleeding, while in women diagnosis may be delayed until menarche when severe menorrhagia requires management that may include blood transfusion. Delivery is a special concern in women with GT, as these women are at risk of severe life-threatening hemorrhage at this time.

Single-nucleotide polymorphisms (SNPs) may be associated with a decrease or increase in the bleeding tendency. *ITGA2* C807T SNP is associated with a higher density of α2β1 and has been shown to confer a milder phenotype of GT. Although GT is a severe bleeding disorder, the prognosis is generally good if careful supportive care is provided [29].

15.3.3 Molecular Basis

GT is an extremely rare bleeding disorder that is inherited in an autosomal recessive manner due to mutations in the *ITGA2B* and/or *ITGB3*. Mutations in both genes have been identified in different populations including the French Gypsy, Japanese, African, American, Iranian, Chinese, and mixed Caucasian [2, 12, 26].

ITGA2B gene encoding the alpha chain of αIIbβ3 complex consists of heavy (H) and light (L) chains connected by disulfide bonds. *ITGB3* encodes the beta chain of the αIIbβ3 complex. The *ITGA2B* gene comprises 30 exons that is transcribed to a 3.1 kb mRNA and is then translated to a precursor protein with 1146 amino acids. The *ITGB3* gene consists of 15 exons is transcribed to a 2.3 kb mRNA and is translated to a 747 amino acid precursor protein. Unlike the alpha chain (αIIb) that is restricted to the megakaryocyte lineage, the beta chain (β3) is distributed in a wide range of tissues (Figs. 15.4 and 15.5) [12].

15.3.3.1 The *ITGA2B* and *ITGB3* Genes Mutations

Missense, nonsense, and frameshift mutations are common causes of GT. Based on mutation locations, they have different effects on αIIbβ3, for example, c.433G>T (D145Y) and c.2332T>C (S778P) mutations in the ITGB3 gene involve the fibrinogen-binding site and signaling pathway of β3 subunit of αIIbβ3. Over 500 mutations have now been reported in GT patients [3, 30, 31]. A continually updated list of the *ITGA2B* and *ITGB3* mutations can be found at https://glanzmann.mcw.edu/. Depending on the nature of the mutations, patients may have a total absent expression of αIIbβ3 (type I GT), decreased αIIbβ3 (type II GT), or qualitatively defective αIIbβ3 (variant GT). Causative mutations of some variant GT are listed in Table 15.4.

Mutations causing GT occur worldwide, but since the rate of consanguineous marriages is significantly higher in certain ethnic groups such as Iranian, Jewish, Jordanian, Arabs, Iraqi, and Palestinian, the disease is expected to be more prevalent in these ethnicities [2, 26]. The prevalence in Madinah, Saudi Arabia, has been reported as high as 1:40,000–100,000 [1].

Mutations Affecting the αIIb Subunit

There are more GT-causing mutations affecting the *ITGA2B* gene than the *ITGB3 gene*. A prevalent GT-causing mutation in the Israeli-Arab population is the 13 bp deletion of the *ITGA2B* gene, which leads to six amino acid deletions of the αIIb subunit of the αIIbβ3 complex. This founder mutation involves the acceptor splice site of exon 4 resulting in a lack of expression of the αIIbβ3 complex [32]. Certain important causative mutations occurring in the *ITGA2B* gene include:

KW-Milwaukee Patient

A large homozygote deletion (IVS1 → 9del4.5 kb) in exon 1 of the *ITGA2B* gene leads to premature termination of mRNA that translates into a shortened αIIb subunit consisting of 88 amino acids. This aberrant αIIb subunit is not able to complex with the β3 subunit for expression on the platelet surface [12].

SK I and SK II Patient

Two nonsense mutations in the *ITGA2B* gene, more common in the Japanese population, include c.1750C>T mutation in exon 17 resulting in p.Arg584stop amino acid substitution (SK I patient) and IVS25(-3) C>G mutation in exon 26 (SK II

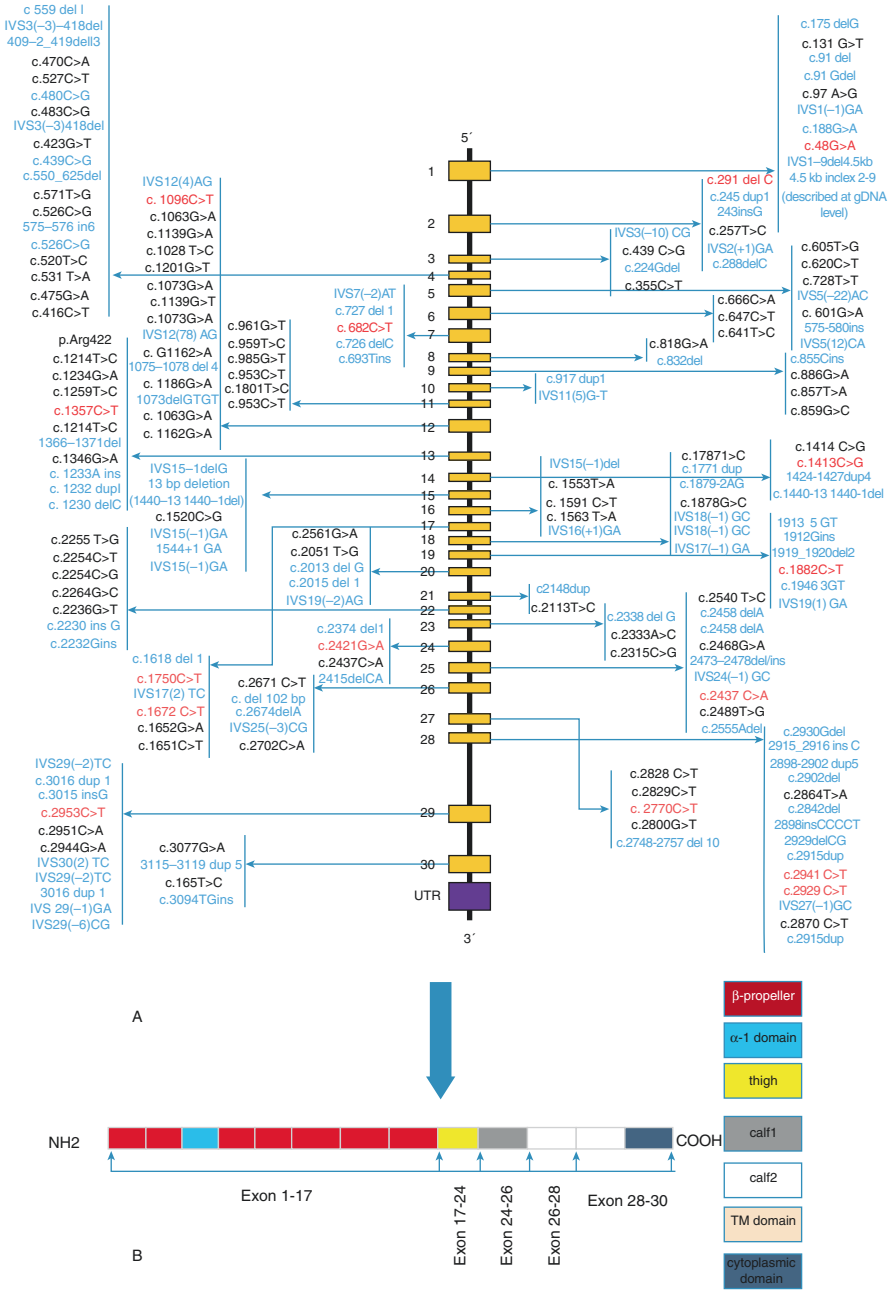

Fig. 15.4 (**a**) Schematic representation of *ITGA2B* gene structure comprising 30 exons with genetic abnormalities that lead to Glanzmann thrombasthenia. Approximately 350 mutations, including 163 missense (in black letters), 130 frameshift (in blue letters), and 41 nonsense (in red letters) mutations, have now been reported to the Glanzmann thrombasthenia database (https://glanzmann.mcw.edu/). (**b**) The domain structure of GPIIb. *UTR* untranslated region; *inv* inversion; *del* deletion; *ins* insertion; *dup* duplication

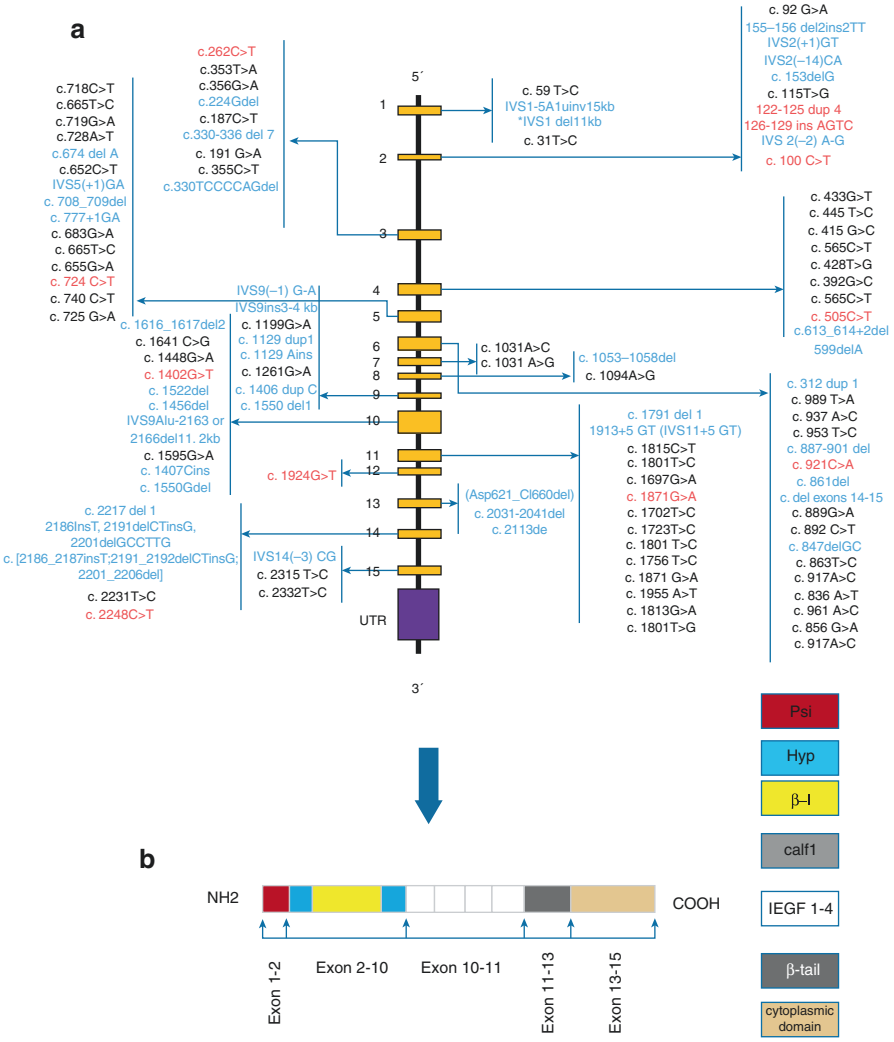

Fig. 15.5 (**a**) Schematic representation of *ITGB3* gene structure comprising 15 exons with genetic abnormalities that lead to Glanzmann thrombasthenia. Approximately 134 mutations, including 62 missense (in black letters), 47 frameshift (in blue letters), and 11 nonsense (in red letters) mutations have now been reported to the Glanzmann thrombasthenia database (https://glanzmann.mcw.edu/). (**b**) The domain structure of GPIIIa. *UTR* untranslated region; *inv* inversion; *del* deletion; *ins* insertion; *dup* duplication

Table 15.4 Glanzmann thrombasthenia (GT) variant causative mutations and their effect on phenotypic characteristics of the disease

Exon	AAS	Mutation	subunit	Genotype	Phenotype	Bleeding	Aggregation	Leads to
4	Y174H	c.520T>C	αIIb	Hetero	Missense	Mild	Absent	Residual alteration of the β-propeller domain
5	T207I	c.620C>T	αIIb	Homo	Missense	Severe	Absent	Residual alteration of the β-propeller domain
30	R1026Q	c.3077G>A	αIIb	Hetero	Missense	Mild	Reduced	Thrombocytopenia
4	D145Y	c.433G>T	β3	Homo	Missense	Severe	Absent	Alteration of Fg binding site
5	R240Q	c.719G>A	β3	Homo	Missense	Severe	Absent	Unstable integrin/alteration of Ca2+ binding site
4	S188L	c.563C>T	β3	Homo	Missense	Severe	Absent	Refractory integrin poorly expressed
6	L288P	c.863T>C	β3	Hetero	Missense	Moderate	Absent	Production of an unstable integrin
15	S778P	c.2332T>C	β3	Hetero	Missense	Mild	Absent	Signaling alteration of β3 CT
14	R750X	c.2248C>T	β3	Homo	Nonsense	Severe	Absent	Signaling alteration of β3 CT
11	C575R	c.1723T>C	β3	Homo	Missense	Severe	Absent	Production of a constitutively active receptor
11	C586R	c.1756 T>C	β3	Homo	Missense	Moderate	Much reduced	Production of a constitutively active receptor
14	L718P	c.2231T>C	β3	Hetero	Frame shift	Severe	Much reduced	Aggregation and secretory defects

AAS amino acid substitution; *Hetero* heterozygous; *Homo* homozygous; *Del* deletion; *Fg* fibrinogen; *CT* cytoplasmic tail

patient). These mutations lead to the absence of αIIb proteolytic cleavage site and decrease the expression of αIIbβ3 on the platelet surface [12, 33].

LM Patient

A missense mutation in exon 13 (c.1346G>A) leads to p.Gly418Asp and involves the fourth calcium-binding site in the αIIb subunit. The integrin αIIbβ3 is formed but not expressed on the platelet surface [12].

UP Patient

A missense mutation, c.818G>A, in exon 8 of the *ITGA2B* gene leads to p.Gly273Asp, which involves the first calcium-binding site in the αIIb subunit. Abnormal αIIb subunit complexes with β3 subunit, but the formed complex is not transported from the ER to the Golgi apparatus and αIIbβ3 is hence not expressed on the platelet surface [12].

KJ Patient

A homozygote missense mutation, c.1037G>A, in exon 12 which leads to p.Arg327His occurs in the ligand binding site in the αIIb subunit. This represents one of the mutations affecting the β-propeller domain that are found in German patients. Platelets of these patients have shown a decreased level of integrin αIIbβ3 [3, 12].

Iranian Patient

A homozygous single-base exchange mutation, c. 1392A>G, in exon 13 with no amino acid change, occurs in the splice region of the *ITGA2B* gene. This mutation affects the mRNA splicing pattern even though it does not change the amino acid sequence. This mutation in the splice region of the *ITGA2B* gene was shown to break the splicing site and is considered pathogenic for the GT bleeding phenotype [34].

A premature termination that resulted from a homozygous deletion of one nucleotide in exon 1 of *ITGA2B* (113: Del A) has been described. This mutation results in a shortened and malformed protein due to the creation of an ectopic premature stop codon (thymine–guanine–adenine; TGA) at the start of exon 1 of *ITGA2B* [35].

Tunisian Patients

Homozygous nonsense mutation in the *ITGA2B* gene including c.2702C>A mutation in exon 26 resulting in p.S901 stop codon. Furthermore, a new intronic homozygous mutation at position g.13944G>A in intron 14 presents the c.2702C>A mutation. Heterozygous single-nucleotide intronic variations, rs116003115 (intron 12), rs148067822 (intron 18), and rs79203039 (intron 26) have also been seen in this population [36].

Turkish Patients
Four missense mutations in the *ITGA2B* gene, including c. 507 T>G (p.G159V, exon 4), c. 1277 T>A (p.S416R, exon 13), c. 1291 T>G, (p.V420L, exon 13), and c. 1921 A>G (p.T646A, exon 19), are seen in the Turkish patients [37].

Swiss Patient
A missense heterozygous mutation in exon 4 (c.555T>G, p.I154M) of the *ITGA2B* gene affecting the extracellular β-propeller region of αIIb was found in a Swiss patient [38].

Kosovo Patient
A missense heterozygous mutation in exon 8 (c.842C>T, p.T250I) affecting the β-propeller region of αIIb was found in Kosovo patients [38].

Caucasian Patient
A nonsense heterozygote mutation in exon11(c.957T>A, p.Y288X) of the *ITGA2B* gene affecting the β-propeller region of αIIb was described. The affected αIIb protein is significantly shortened and is incapable of forming a functional complex with β3 [38].

Caucasian Patient
A heterozygous mutation from an amino acid duplication without frameshift was found in exon 23 (c.2326_2331dup GAGGCC) leading to p.E745_A746dup. In another patient, a two-amino-acid duplication (p.E745 A746dup) without frameshift was found in the Calf2 domain of *ITGA2B*. This duplication did not cause a reading frameshift but critically affects the mutant αIIb protein by creating the complex with αIIbβ3 [38].

Arabs and Caucasian Patients
Two heterozygous frameshift *ITGA2B* exon 30 mutations, c.3091delC and c.3092delT, leading to p.L1000delC and p.L1000delT, respectively, were described. Each of these mutations results in the deletion of residue leucine 1000 in the αIIb cytoplasmic tail. In each case, the affected αIIb protein is longer than the wild-type αIIb protein. The frameshift results in no more stop codons in the remaining coding area [38].

The β-propeller domain is considered a mutation-rich domain of the integrin αIIb subunit which plays a critical role in the biogenesis of αIIbβ3 complex. The GT database (https://glanzmann.mcw.edu/) showed that in this domain, there are about 150 different mutations including 83 missense, 60 frameshift, 13 nonsense, and 13 others.

Mutations Affecting the β3 Subunit
The mutations such as inversion, deletion, frameshift, and splice site can change the ability and stability of β3 to make a complex with αIIb. Examples of causative mutations in *ITGB3* genes include:

Strasbourg I Variant
A homozygous mutation, c.718C>T, in exon 5 leads to p.Arg214Trp in the β3 subunit. This mutation occurring in the ligand binding site of the β3 subunit was reported in the French population. Unlike the other GT patients, the affected patients showed the absence of platelet aggregation in response to ristocetin [12].

ET Variant
A missense mutation, c.719G>A, in exon 5 of the ITGB3 gene leads to p.Arg214Gln in the β3 subunit, which was reported in the Australian population [3, 12].

Cam Variant
A homozygous missense mutation in the β3 subunit, c.433G>T, in exon 4 of *ITGB3* leads to p.Asp119Tyr. This mutation involving the ligand binding site and calcium-binding site of the β3 subunit was reported in the Guam population. The mutant αIIbβ3 is not able to bind RGD-containing ligands [3, 12].

German Patients
Three heterozygous *ITGB3* missense mutations, c.31T>C (p.W-15R) in exon 1 and c.1458T>C (p.C460W) and c.1458T>C (p.C460) in exon 10 of ITGB3, were found in the German patients. These two exon 10 mutations affect the EGF domains 1–2 [38].

Two other heterozygous mutations in exon 10 (c.1594T>C, p.C506R) and intron 12 (c.2014+1G>A, which is likely an alternative splicing or splice site mutation) were found. The exon 10 mutation affects the EGF domains 1–2, while the intron 12 mutation is found within the donor splice site causing a splicing abnormality [38].

German (Mother) Jugoslav (Father) Patient
Two heterozygous *ITGB3* frameshift mutations in exon 7 (c.1030dupT) and in intron 8 (c.1126–72C>T, likely alternative splicing or splice site mutation) were present. The exon 7 frameshift mutation results in premature termination at *ITGB3* nucleotide position 322 as a result of the single-nucleotide duplication (c.1030dupT), which leads to a reading frameshift beginning at amino acid 318 (p.Y318L) [38].

Caucasian Patient
A heterozygous missense *ITGB3* mutation c.118C>T in exon 2 leads to p.Q14X. This mutation affects the β3 PSI (plexins, semaphorins, and integrins) domain [38].

15.4 Diagnosis of GT

15.4.1 General Overview

Since a considerable number of patients with GT may present with severe life-threatening bleeds, early and timely diagnosis of the disorder is crucial for their appropriate management. Diagnosis of GT can be made based on family history,

clinical presentations, and appropriate physical examination as well as suitable laboratory assessments. In such autosomal recessive bleeding disorders, a positive family history is a very important diagnostic clue that can help in the rapid diagnosis of the patient. In a patient with a history of excessive bleeding and a positive family history of GT, more direct and specific tests can be used. On the other hand, in those patients without a family history of GT but with signs of primary hemostasis disorders, more general tests could be used. Clinical presentations are other important clues for the diagnosis of GT.

15.4.2 Laboratory Diagnosis

According to the Platelet Physiology Subcommittee of the International Society on Thrombosis and Haemostasis (ISTH) guidelines; the first step for diagnosis of inherited platelet function disorders (IPFDs) should include blood smear examination and light transmission aggregometry (LTA) using a limited number of agonists as well as assessment of platelet granule release and flow cytometric analysis of major platelet glycoproteins (Fig. 15.6) [39–41].

15.4.2.1 Primary Tests

Platelet Count and Peripheral Blood Smear

In classic GT, complete blood count usually shows a normal platelet count with normal platelet size and morphology [42].

Rare GT variants, however, may be accompanied by thrombocytopenia or macrothrombocytopenia [3]. Thrombocytopenia with anisocytosis was reported to be associated with β3 Leu718Pro while macrothrombocytopenia was reported to be associated with αIIb Arg995Gln, αIIb Gly991Cys, αIIb Phe 993delαIIb Arg995Trp, β3 Asp723His, β3 Leu718Pro, β3 Cys560Arg, β3Asp331Ser, and β3 c.2134 + 1G > C + 40aa deletion (β3 Arg621_Glu660del) mutations [3, 43–47]. These thrombocytopenic and macrothrombocytopeniic variants have gain-of-function αIIbβ3 mutations—their αIIbβ3 expression is deficient (accounting for their bleeding phenotype), but it is constitutively activated on the platelet membrane surface [3, 46]. This leads to the permanent triggering of αIIbβ3-mediated outside-in signaling that arrests actin turnover at the stage of polymerization. The resulting impaired cytoskeletal reorganization was thought to cause platelet enlargement [46] and apparently also leads to impaired proplatelet formation and hence thrombocytopenia [3, 45, 48]. These variants have autosomal dominant inheritance (instead of autosomal recessive inheritance for classical GT).

Routine Coagulation Tests (Activated Partial Thromboplastin Time, Prothrombin Time, Thrombin Time, Bleeding Time)

Routine tests that assess secondary hemostasis, including activated partial thromboplastin time, prothrombin time, and thrombin time, are all within the reference intervals in GT.

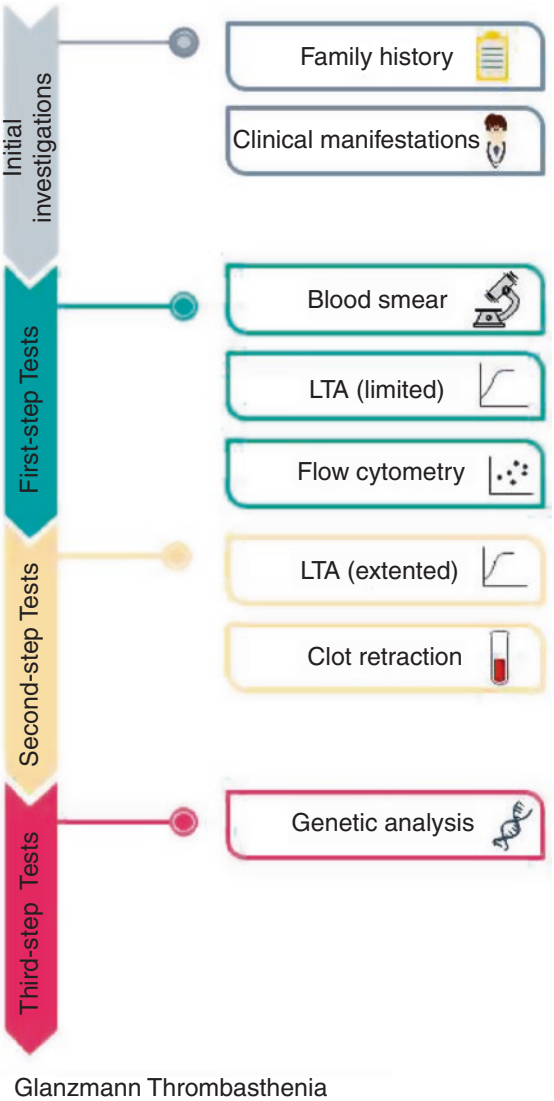

Fig. 15.6 Schematic diagram for the diagnosis of Glanzmann thrombasthenia. First-step tests are screening tests and mainly include assessment of the blood film, aggregation study by LTA* with limited agonists, and flow cytometry analysis of αIIbβ3 glycoprotein. Second-step tests are recommended when first-line screening tests fail to diagnose the disorder. These tests include LTA* with further specific agonists and clot retraction assay. If the disease is still undiagnosed, genetic studies as third-step tests must be conducted. **LTA* light transmission aggregometry

In contrast, BT, a screening test of primary hemostasis, is prolonged in GT patients. BT has been traditionally used for the assessment of platelet function, but with the development of more reliable and less invasive assays, it is no longer recommended. Closure time/PFA-100®/200® is the preferred replacement.

Closure Time/PFA-100®/200®

Closure time is defined as the time taken for the formation of the primary platelet plug and cessation of the blood flow in an in vitro condition, using PFA-100®/200® (Box 15.1). Closure time is an in vitro analysis of platelet function that can be considered a substitute for Bleeding Time. A prolonged closure time suggests a defect in primary platelet plug formation but is not specific to a particular platelet or VWF-related disorder.

Box 15.1 The Principle of Closure Time

Platelet function analyzers take advantage of two cartridges, each having a membrane coated by a combination of two platelet agonists: collagen/epinephrine (Col/Epi) or collagen/ADP (Col/ADP). A third cartridge containing prostaglandin E1 and ADP (Innovance PFA P2Y) may also be used but is not available in all countries. PFA-200® is the upgraded model of PFA-100®. PFA-200® is more user-friendly with more advanced software and touchscreen capability, but the model is not available worldwide. The principle of both PFA-100/200 is the same allowing in vitro assessment of platelet function in a flow system with simulated vascular injury. Each cartridge includes a capillary tube connected to a membrane coated with platelet agonists. There is a small aperture in the center of the membrane to imitate the vessel injury. The patient's citrated whole blood is aspirated through the capillary tube with a high shear rate. When platelets contact the membrane, they adhere to the coated agonist and collagen mediated by VWF. The coated agonists result in platelet activation, adhesion, and aggregation, leading to the formation of a platelet plug obstructing the aperture to stop the blood flow. The time from aspirating the patient sample to obstruction of blood flow is reported as closure time. The test is first performed on the Col/Epi cartridge and Col/ADP cartridge will be applied only if the closure time with the Col/Epi cartridge is prolonged [49, 50] (Fig. 15.7).

Reference Intervals: Normal range is <180 s for Col/Epi and <120 s for Col/ADP. In GT, the closure times are prolonged for both cartridges.

Sensitivity and specificity: As a screening test, closure time has a good sensitivity of about 97% for GT diagnosis. Prolonged closure time, however, is not specific to the diagnosis of GT [31].

Sample requirements: The required sample is whole blood, collected in a standard concentration of citrate anticoagulant. The sample should be fresh, and the delay between sampling and performing the test should not be longer than 4 h.

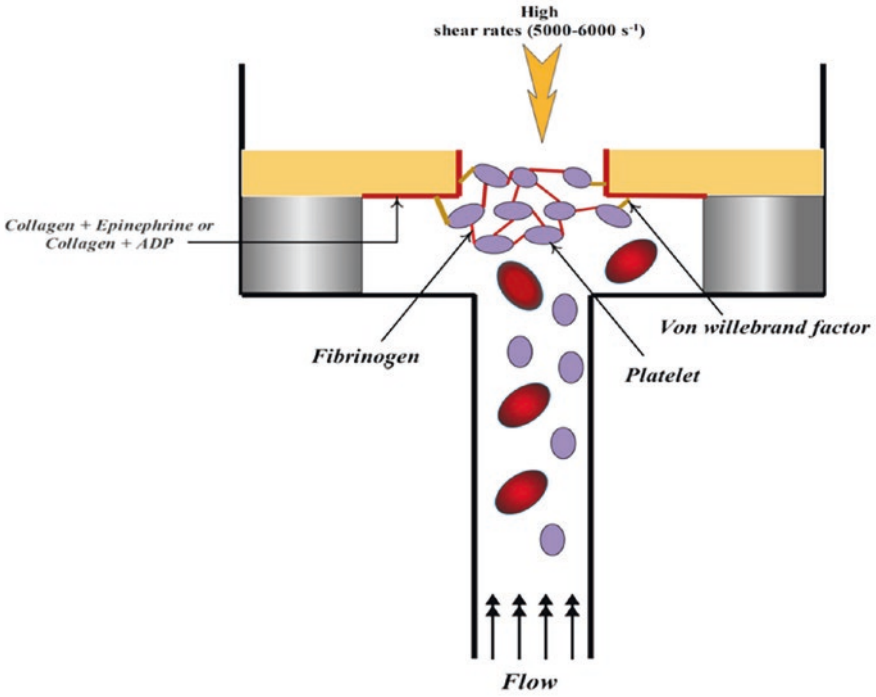

Fig. 15.7 Platelet function analysis (PFA). In this assay, the citrated blood sample is aspirated at a high shear rate and primary hemostasis is triggered by a membrane coated with collagen/epinephrine (C/EPI) or collagen/adenosine diphosphate (C/ADP). Activation of primary hemostasis leads to the occlusion of an aperture in the test cartridge

Interfering variables: Platelet count, hematocrit, citrate concentration, ABO blood group, plasma level of VWF, and time of blood collection are considered as variables that may affect the closure time. It has been reported that platelet counts of $<150 \times 10^9$/L and hematocrit of <35% may increase the closure time [51].

15.4.2.2 Platelet Aggregation Studies

General Overview

Platelet aggregometry is one of the most widely used test procedures for assessing platelet function and was suggested by the Platelet Physiology Subcommittee of the ISTH as a first-line screening test for platelet function disorders. Two types of instruments are available, including (1) light transmission aggregometry (LTA) and (2) whole blood aggregometry (WBA). GT platelets typically have an absent to markedly diminished aggregation response to physiological agonists, but platelet agglutination response to ristocetin in the presence of VWF is present as GPIb receptors on GT platelets are normal.

Light Transmission Aggregometry (LTA)

LTA was developed in the early 1960s and is considered the gold standard method for the evaluation of platelet function in inherited and acquired bleeding disorders. All aggregation studies are generally based on the ability of platelets to aggregate in response to external agonists. LTA is based on the determination of the extent of light transmission change following the addition of an agonist (aggregating agent) to the platelet-rich plasma (PRP) sample (Box 15.2) [52, 53]. The suggested baseline panel of agonists for LTA includes collagen, adenosine diphosphate (ADP), epinephrine (EP), Arachidonic acid (AA), ristocetin, calcium ionophore A23187, and thrombin receptor-activating peptide (TRAP), although other agonists may also be used in specific conditions [54].

Box 15.2 The Principle of LTA

The LTA aggregometry is implemented by platelet aggregometer analyzers. In this method, the PRP sample in a disposable cuvette (incubated at 37 °C while constantly stirring) is situated between a light source and a photocell in an aggregometer analyzer (Fig. 15.8).

In response to the addition of an agonist, the shape of platelets changes from discoid to spherical form initially, leading to temporary slight reduction in the extent of light transmission. Subsequently, as the platelets form aggregates, the PRP becomes less opaque and allows higher light transmission. The light transmission is measured by a photometer placed in front of the cuvette through which light is transmitted. The light transmission will then be converted to a graphic curve and reported as the percentage of aggregation (Fig. 15.9) [49, 53–56].

Changes in the curve tracing during normal platelet aggregation can be observed as shape change, first aggregation wave (primary wave), inflection point, and a secondary wave of aggregation. When an agonist is added, the chain of events is initial aggregation with activation of the platelet which is followed by the release of the

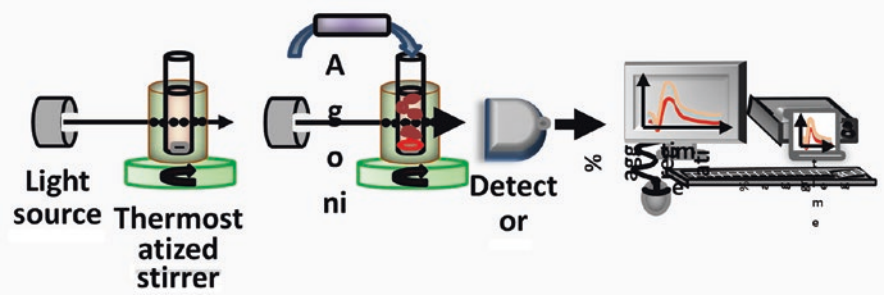

Fig. 15.8 Schematic view of an aggregometer. A cuvette containing PRP that is constantly stirring at 37 °C is placed between a light source and a photocell. After the addition of an agonist, alterations in transmitted light are detected and reported as a curve

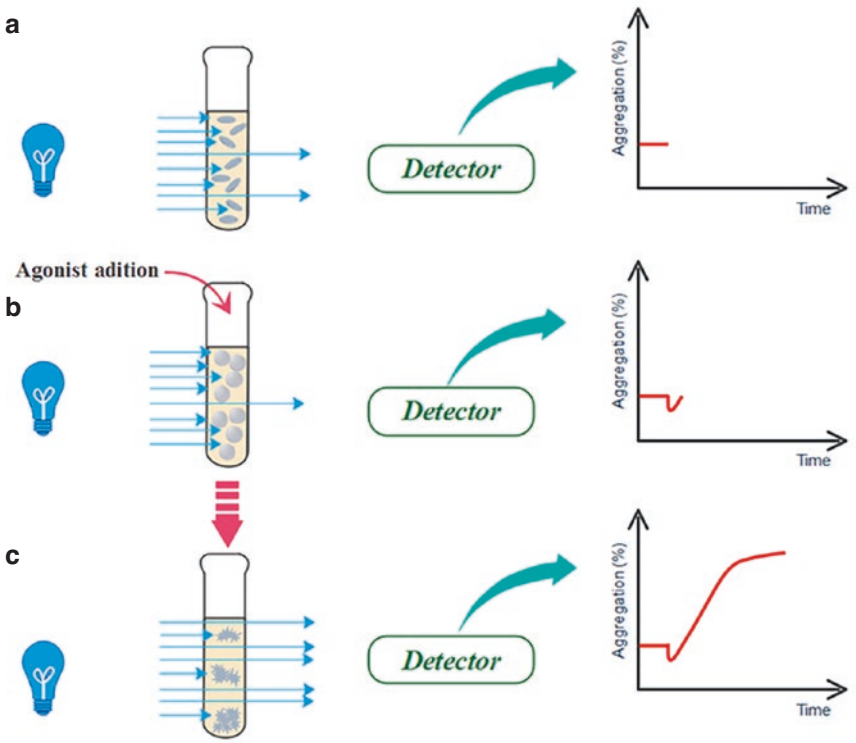

Fig. 15.9 The principle of light transmission aggregometry. (**a**) Before the addition of an agonist, PRP is opaque allowing little light to pass through (baseline transmission). (**b**) Addition of an agonist results in an immediate change in the shape of platelets from discoid to spherical that decreases further the transmitted light and can be observed as a minimal reduction in the aggregation cure. (**c**) Finally, platelet aggregation occurs resulting in a progressive increase in the amount of light transmitted, expressed in the form of an aggregation curve

platelet granular content for further platelet activation and aggregation. If an inadequate dose of the agonist is added, platelets will be activated initially to result in a primary wave formation. Because the stimulation is not strong enough to sufficiently activate the platelet to release its granular content, the secondary wave representing further activation will not be observed. When the concentration of the agonist is sufficiently high to activate the platelets for granular release, the primary wave will be immediately followed by the secondary wave attributed to additional activation by the released granular contents. When the agonist added is strong in higher concentration, only a curve merging both primary and secondary waves will be observed as the strong aggregation due to the agonist stimulation will mask the effect of additional stimulation by granular release (Fig. 15.10) [54, 56]. The slope of the curve and the pattern of the waves are important for interpretation, and their changes will guide the operator to further analysis [54].

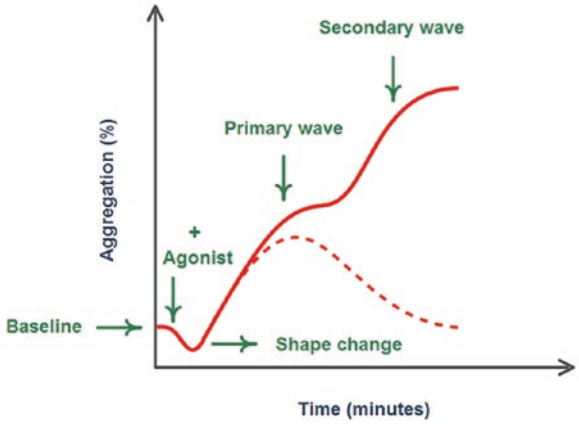

Fig. 15.10 Aggregation curve in response to strong agonists which consists of primary and secondary waves. The primary wave is indicative of initial platelet activation to result in the primary (initial) platelet aggregation. If the agonist is strong enough, it leads to the secretion of platelet granule contents which further activate the platelets, enhancing the platelet aggregation to result in the illustrated secondary wave. A Weak concentration of agonist does not lead to the granule secretion and the primary wave backs down to the baseline (dashes)

Light aggregometry testing is performed by employing platelet-poor plasma (PPP) as a reference for 100% light transmission and platelet rich plasma (PRP) for 0% light transmission. The separated PRP and PPP are placed into a capped plastic tube and stored at room temperature until analyzed.

Platelet Concentration and Adjustment

According to the recommendations for LTA standardization provided by the Platelet Physiology Subcommittee of SSC/ISTH, evaluating the platelet count of PRP samples is essential. The results of LTA for PRP samples with platelet counts below 150×10^9/L are reported as not accurate, and the abnormal results of such samples must be interpreted with caution. Based on the SSC/ISTH recommendations, the PRP samples of patients who have a normal platelet count in whole blood (WB) should not be adjusted with autologous PPP, as it may affect the platelet response to agonists. However, there are controversies about PRP samples with platelet counts above 600×10^9/L [52].

Concentration of Agonists

This is not well standardized and there is no consensus agreement on the concentration of applied agonists. This problem may lead to variable results that cannot be compared in different laboratories. The recommended concentrations of agonists for LTA as provided by the Platelet Physiology Subcommittee of SSC/ISTH are indicated in Table 15.5 [52].

Table 15.5 The recommended concentrations of agonists for LTA

Platelet agonist	Recommended concentrations
Adenosine diphosphate (ADP)	2 μM
Epinephrine	5 μM
Collagen	a low concentration which is sufficient to induce the aggregation of normal platelets
Thrombin receptor-activating peptide (PAR1-AP)	10 μM
Thromboxane A2 mimetic U46619	1 μM
Arachidonic acid	1 mM
Ristocetin	1.2 mg mL^{-1}

Note: In cases of abnormal (low) results with the above concentrations, higher concentrations should be performed

Interfering Variables

LTA may be affected by many variables including pre-analytical conditions (i.e., lipemic plasma, hemolysis, low platelet number, or type of anticoagulant), analytical factors (i.e., type and concentration of working agonists, PRP preparation), and skills and experience of the operator in performing and interpreting the result. Primary lipemia and hemolysis may also affect the opacity of PRP and result in abnormal results [52, 53, 57]. In addition, contamination of the PRP samples with red cells and leukocytes may lead to platelet activation and result in uninterpretable aggregation patterns.

Drugs and supplementation: Patients should avoid consuming foods, supplements, and drugs that may reversibly or irreversibly interfere with platelet function for at least 10 days before testing. Nonsteroidal anti-inflammatory drugs (NSAIDs), aspirin, and specific antiplatelet agents are examples of such drugs. Preferably, the patient should fast overnight and avoid smoking for at least 30 min prior to sampling.

Temperature: Low temperature (<20 °C) or high temperature (>37 °C) may activate platelets and affect the quality of results.

Anticoagulant: According to the current guidelines, the appropriate anticoagulant for LTA is sodium citrate 3.2% with a ratio of 1 part anticoagulant to 9 parts blood. The use of an improper anticoagulant (including EDTA) instead of sodium citrate may change the aggregation patterns. EDTA may dissociate the αIIbβ3 complexes on the platelet surface resulting in an aggregation pattern similar to that of GT platelets. An incorrect anticoagulant ratio may also affect the aggregation patterns.

LTA is a high-cost, laborious, and time-consuming method and is potentially vulnerable to technical errors. The test also requires a large volume of blood samples, which is a challenge to obtain in children and neonates. It also makes limitations in the analysis of cases with thrombocytopenia. Lack of internal and external quality control materials and different interpretations of results among different laboratories due to poor test standardization are other limitations of LTA assay [52, 56, 57]. Thus, much effort must be devoted to standardizing this method. As a

first-line assay, and given that a variety of factors can lead to the development of platelet dysfunction and that there are deleterious effects of a multitude of variables, LTA results should be confirmed by more specialized techniques.

Box 15.3 Light Transmission Aggregometry Diagnostic Criteria for GT
In GT patients, platelet aggregation is absent in response to all agonists, but normal to ristocetin and botrocetin (with occasional ones showing a cyclical aggregation pattern to ristocetin). This pattern of platelet response in aggregometry is typical for GT for diagnosis (Fig. 15.11). Platelet aggregation is the final step of platelet activation in response to the agonists with the aggregation dependent on the function of GP IIb/IIIa. In contrast, ristocetin and botrocetin do not result in platelet activation and granular secretion—the result is platelet agglutination independent of GPIIb/IIIa participation. Thus, in GT, platelet agglutination in response to ristocetin and botrocetin is normal.

15.4.2.3 Luminaggregometry and Whole Blood Aggregometry

General Overview
Lumiaggregometer is a modification of traditional LTA that analyses platelet secretion in parallel with platelet aggregation. The method is based on bioluminescent determination of adenosine triphosphate (ATP). ATP released from platelets reacts with firefly luciferin and luciferase, which leads to light emission. In lumiaggregometry, strong agonists such as thrombin, collagen, and TRAP can induce platelet secretion. A key feature can be used for differential diagnosis between platelet secretion and storage pool disorders. Platelet aggregation, as well as lumiaggregometry, can now be performed with whole blood. For whole blood aggregometry (WBA), Whole blood is diluted 1:1 with saline. Platelet response to an agonist is then measured using an electrode immersed into the blood sample. Several agonists including collagen, ADP, thrombin, ristocetin, and AA are used to get information on the platelet activation pathway. Again, GT platelets show no aggregation to agonists such as collagen, ADP, thrombin, ristocetin, and AA, but ristocetin aggregation is observed. The main advantage of WBA by multiple electrode aggregometry (MEA) over LTA is the elimination of the time-consuming step of blood centrifugation to prepare PRP and PPP. In addition, platelet aggregation is assessed in a normal environment in the presence of other blood cells minimizing the artificial change in platelets, thus improving their functional analysis [39, 58, 59].

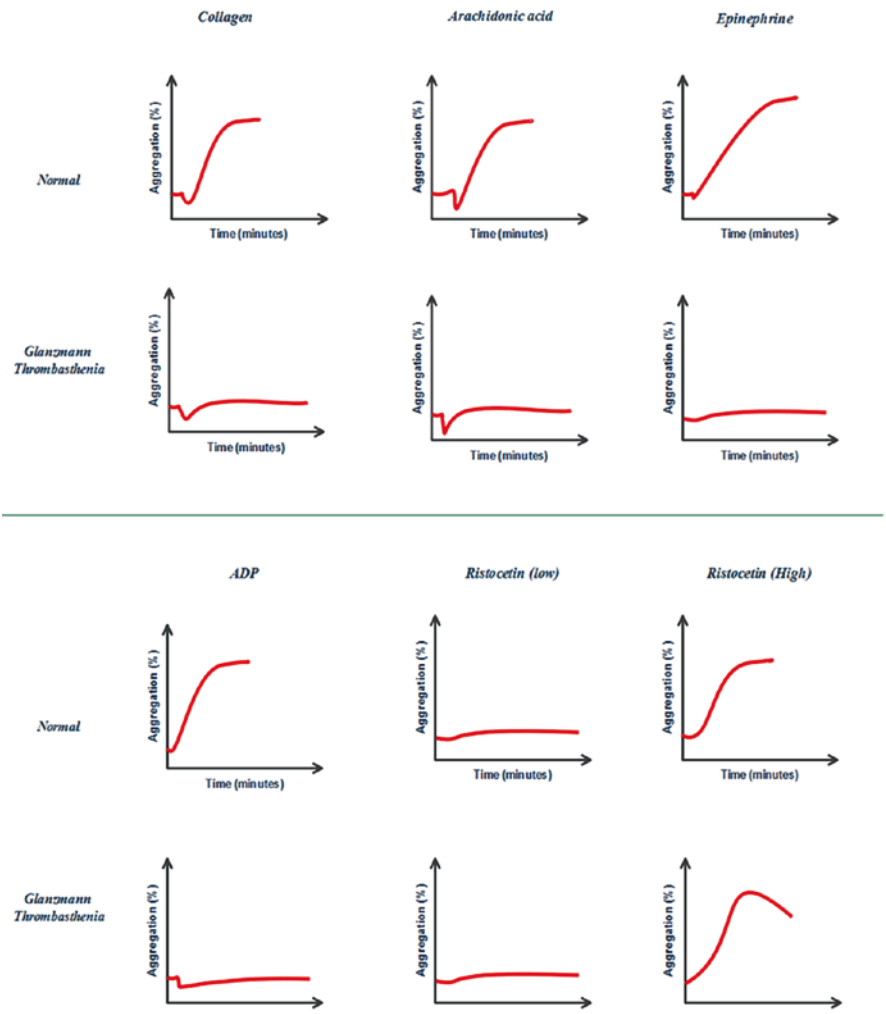

Fig. 15.11 Platelet aggregation panel in GT patients compared with normal platelets. The response to all agonists, except high-dose ristocetin, is absent in GT platelets

Multiple Electrode Aggregometry/Impedance Whole Blood Aggregometry

MEA is a method for evaluating platelet function based on impedance (Box 15.3).

Box 15.4 The Principle of MEA

MEA evaluates platelet function by measuring the changes in electrical impedance following platelet aggregation on the surface of two electrodes that are placed within the sample. In impedance aggregometry, after adding the agonist to a citrated WB sample, the platelets became activated and stuck to the surface of the electrodes (Fig. 15.12a). Platelet aggregation, therefore, leads to an increased electrical impedance recorded in Ohms. Finally, the results are reported as the area under the curve (Fig. 15.12b). Additionally, parameters including lag time, slope, and maximal aggregation can also be obtained from the given curve.

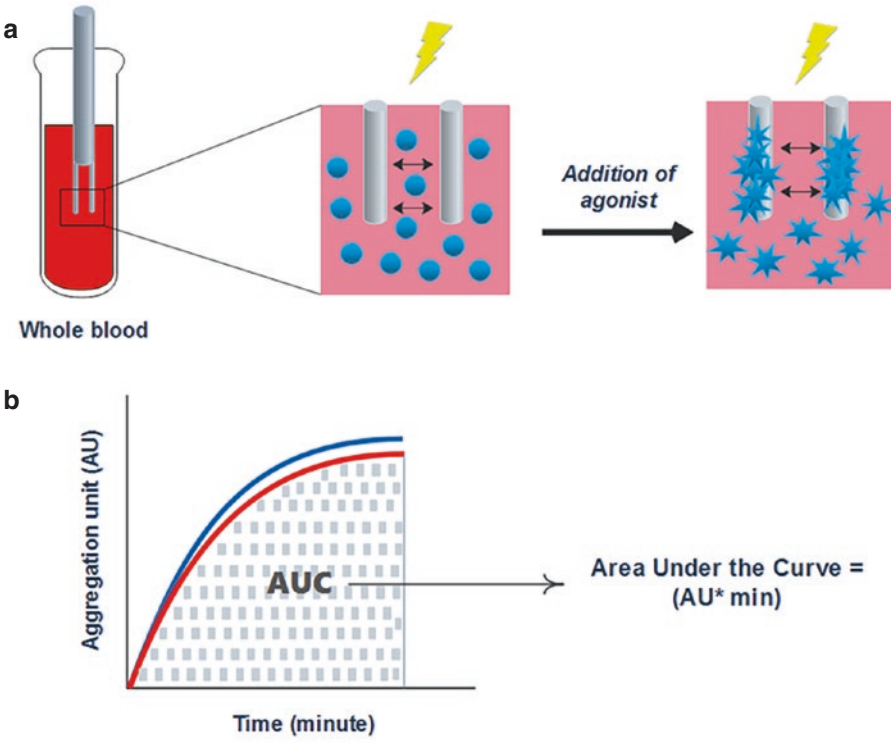

Fig. 15.12 Principle of multiple electrode aggregometry. (**a**) A twin electrode is placed within the whole blood sample. The addition of agonists leads to platelet activation and aggregation on the surface of electrodes resulting in an enhancement of electrical impedance, decreasing the electrical current. (**b**) The results are reported as AUC which is calculated as AU*min. *AUC* area under the curve; *AU* aggregation unit; *min* minute

An advantage of MEA is the measurement of platelet function under a more physiological condition for two reasons: (1) the use of WB allows the presence of other blood elements important for platelet function and (2) platelet adhesion and aggregation to the surface of electrodes is similar to the process of platelet adhesion and aggregation to injured vessels.

Another advantage of MEA is the better standardization of the method. Automated pipetting, standardized and ready-to-use reagents and diluents, and no need for sample processing all make MAE a rapid and user-friendly method. The ability to simultaneous measurement of platelet function in different samples using different agonists is another advantage of MEA. Multiple platelet function analyzers equipped with five channels, each with two pairs of independent electrodes, allow simultaneous determination of platelet aggregation in duplicate. Measuring each sample in duplicate is internal quality control for more reliable results.

MEA technique requires lower amounts of sample and therefore is appropriate for workup in children as well as in cases of thrombocytopenia. MEA is suitable for monitoring cardiovascular patients undergoing antiplatelet therapy for on-treatment platelet reactivity. Despite all the advantages of MEA, it has some restrictions compared with LTA. For example, MEA does not allow the evaluation of alteration in the shape of platelets and of primary and secondary waves of aggregation. Furthermore, the combination of aggregation study with ATP release measurement is not possible in the MEA method.

Optimal 96-Well Plate-Based Aggregometry

Optimal 96-well plate-based aggregometry was introduced in the 1990s. It is based on the principle of LTA but with some modifications. In this method, PRP is added to the wells of a 96-well plate containing various concentrations of agonist and agitated using a shaker. Light absorbance is then simultaneously evaluated by a standard microplate reader and the percentage of aggregation is reported based on the relative absorbance of PPP and PRP controls [57, 60, 61]. The 96-well half-area microplates can save rare and valuable reagents by diminishing the volume of reagents required per well, while still being readable by standard plate readers. In addition, the assay is only performed by adding the PRP or PPP samples to the wells and takes about 10 min. However, similar to MEA, this method is also unable to indicate platelet shape change and secondary aggregation response. Altogether, Optimum 96-well plate-based aggregometry is a cost-effective alternative for high-efficiency platelet function testing [62].

15.4.2.4 Flow Cytometry

Flow cytometry is an effective and reliable technique for identifying platelet impairment providing a rapid and simple means for the diagnosis of homozygous and heterozygous states of platelet membrane glycoprotein (GP) deficiencies, such as GT [63, 64]. Platelet receptor changes or conversion into other surface properties due to response to stimuli can be measured by flow cytometry using specific monoclonal antibodies. Platelet receptor deficiencies are also identifiable using this method [65].

Box 15.5 The Principle of Flow Cytometry

Flow cytometry measures the special characteristics of a large number of individual cells. The cells are labeled fluorescently by binding to a monoclonal fluorescent conjugated antibody (Fig. 15.13a). Suspended single cells pass through a flow cell in the device at a rate of 1000–10,000 events per minute. The laser beam meets the fluorescent conjugated cells and excites the fluorochrome (Fig. 15.13b). The light scattering properties and emission wavelength of each cell are measured. Forward scatter (FSC) and side scatter (SSC) are registered for each cell. FSC evaluates cell size directly and cell transparency indirectly. SSC on the other hand evaluates cell granularity. Each cell is shown as a dot in a dot plot. Two parameters are needed to form a dot plot. For example, we can choose FSC on the X-axis and SSC on the Y-axis, so that the location of the dots is based on the cell size and granularity in the dot plot.

Platelets are commonly conjugated by a monoclonal antibody bound to fluorescein isothiocyanate (FITC) and phycoerythrin (PE). About 5000–10,000 platelets will be checked for positivity of both fluorochromes and light scatter properties. Platelets can be distinguished by their light scatter pattern in WB. However, under special examination conditions, the platelet light scatter gate might include some particles unbound with platelet-specific monoclonal antibodies. Therefore, we recommend using a two-color/two-antibody technique if WB is studied: one monoclonal antibody for platelet discrimination (typically FITC conjugated) and one for measuring the GP of interest (typically PE-conjugated) [66, 67].

WB, PRP, and washed platelet samples could be studied using this method. The sample type should be determined based on the antibody and agonist used in this assay. Generally, WB is preferred because platelets are investigated in a more physiological environment with minimal sample manipulation, which can lead to in vitro platelet activation and platelet subpopulation loss. A very small blood sample, only about 5 μL, is needed making this method appropriate for studying neonates. A subpopulation of as few as 1% of activated platelets in WB can be detected by flow cytometry [68, 69]. Samples for GT studies in flow cytometry should be anticoagulated in sodium citrate. Ethylenediaminetetraacetic acid (EDTA) should be avoided since it can cause dissociation of integrin $\alpha IIb\beta_3$ (GPIIb/IIIa) on the platelet surface [39, 59].

For diagnosis of GT by flow cytometry, monoclonal antibodies specific for αIIb (CD41) and β3 (CD61) GPs are used (Fig. 15.14). This method is reliable not only for the confirmation of αIIbβ3 deficiency but also for zygosity determination based on the level of αIIbβ3 expression [29]. Flow cytometry analysis also allows the characterization of the pattern of reduced αIIbβ3 GP expression on the surface of platelet subpopulations. In heterozygous patients, reduced αIIbβ3 may be observed in the whole platelet population or only in a subpopulation.

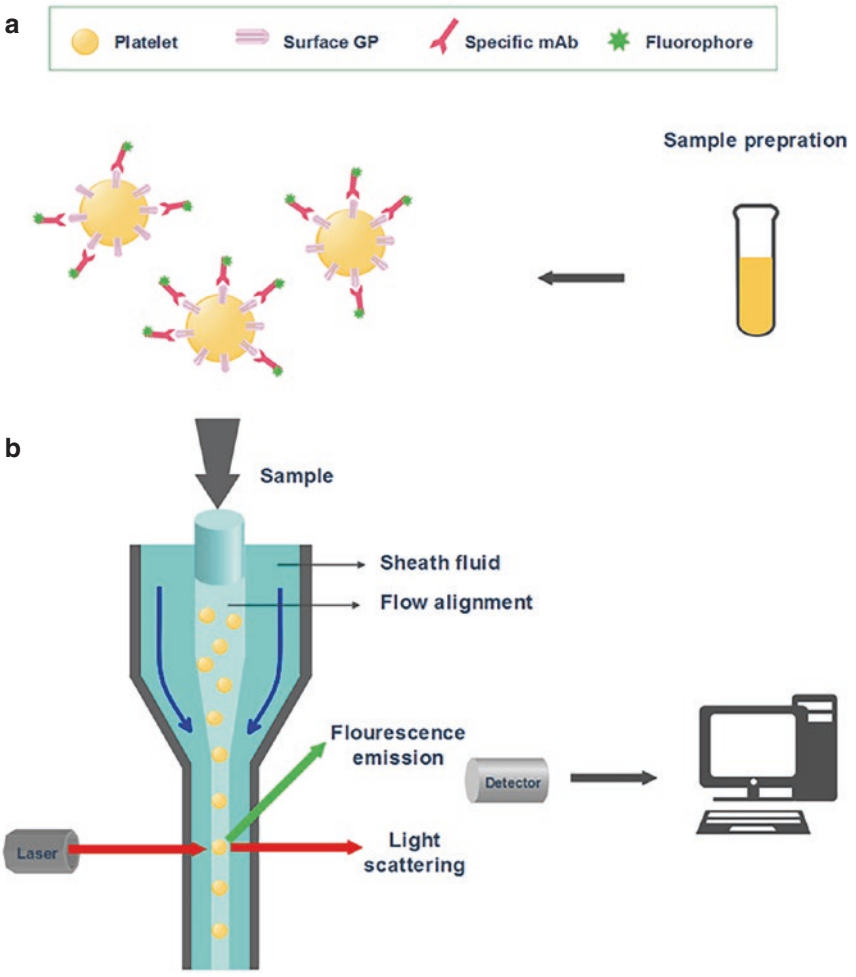

Fig. 15.13 (**a**) Fluorescent conjugated mAbs which are specific for the intended platelet surface GP are added to the sample. (**b**) A large number of cells pass through a flow cell individually. The laser beam meets the conjugated cells and excites the fluorochrome and measurements made of the light scattering (forward and side scatter) and fluorescence emission properties

Variant Glanzmann's Thrombasthenia Detection by Flow Cytometry

The expression level of αIIbβ3 integrins in GT variants is more than 20% and usually more than 50% of normal. Therefore, normal or near-normal results of αIIbβ3 level obtained by flow cytometry in patients suspected of GT should be interpreted with special caution. Other GPIIb/IIIa functional investigations such as LTA must be performed.

To make a diagnosis of variant GT, activation-dependent monoclonal antibodies are required for further investigations. Procaspase-activating compound (PAC-1) developed by Shattil et al. [70] is a specific monoclonal antibody against activated

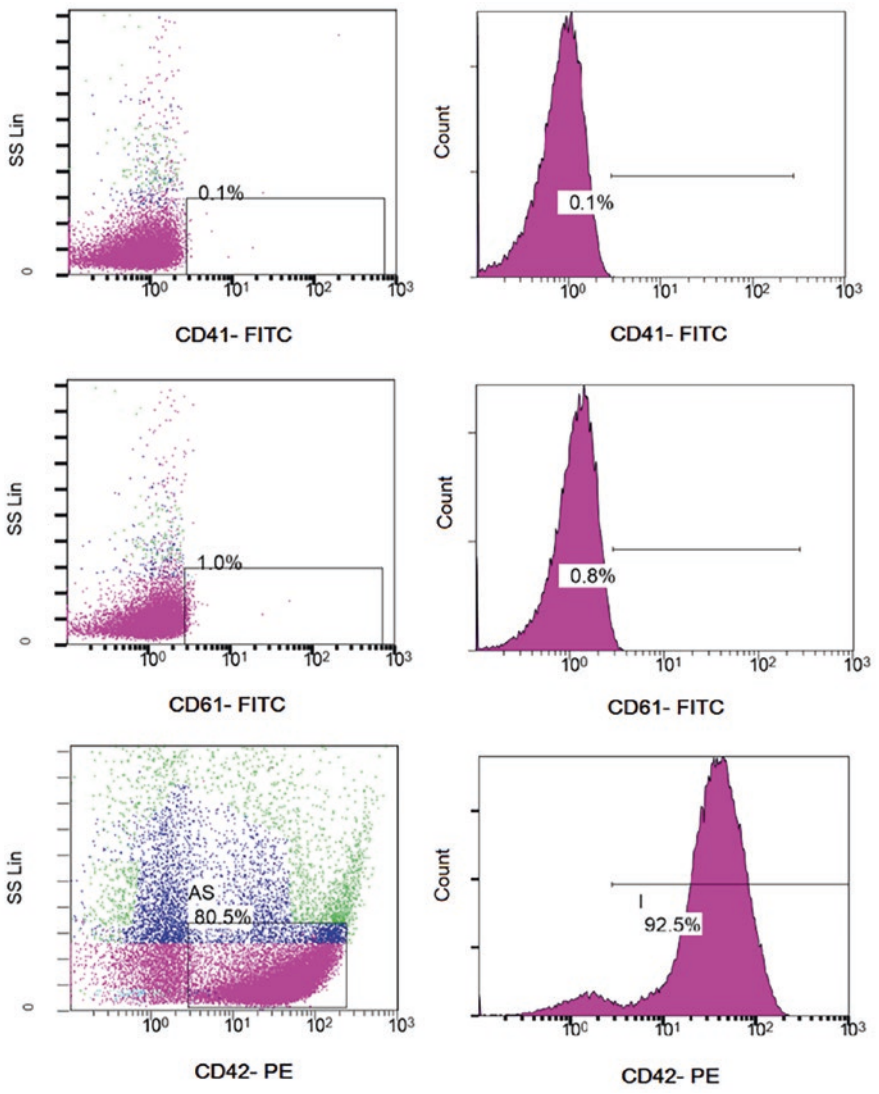

Fig. 15.14 Flow cytometry results of a patient with Glanzmann thrombasthenia. The results indicate that the platelets of patients are negative for both CD41(αIIb) and CD61 (β3) markers, while positive for the platelet marker CD42

αIIbβ3 available commercially [65]. This antibody detects fibrinogen-binding sites exposed by conformational changes in αIIbβ3 complex on the activated platelet surface. PAC-1, therefore, binds to activated platelets only. Platelets of GT variant patients, upon stimulation by agonists, are unable to bind PAC-1 as well as FITC-labeled fibrinogen.

Other activation-dependent monoclonal antibodies specific for αIIbβ3 are (1) ligand-induced binding site antibodies specific for a conformational change induced by the ligand for αIIbβ3 complex and (2) fibrinogen receptor-induced binding site antibodies specific for a conformational change induced by the receptor for binding ligand [66].

Activation of platelets in WB for monoclonal antibody binding without fibrin clot formation can be accomplished with TRAP [66].

Advantages and Disadvantages of Flow Cytometry

Flow cytometry has some advantages in comparison with many methods used for platelet assay. This method allows measurement of the extent of individual platelet activation and differentiates different distinct platelet subpopulations. Recently, commercial platelet flow cytometry kits are available. These kits that provide the required reagents such as antibodies and agonists facilitate the application of flow cytometry in routine coagulation laboratories.

There are disadvantages to this technique. These include the need for expensive instruments and reagents and sample preparation. There is a need for a professional operator, and the samples must be labeled and fixed for analysis within 45 min to avoid in vitro platelet activation [66].

15.4.2.5 Clot Retraction Assay

After clot formation, activated platelets associate with fibrin fibers through their surface receptors and apply a contraction force via their contractile system to result in a reduction in the volume of the established clot. This phenomenon is referred to as clot retraction. Clot retraction results in a more stable clot. β3 integrins and non-muscle myosin IIA are critical components for clot retraction [71]. Following clot retraction, some serum will be expressed. The measurement of the extent of serum expressed forms the basis of the clot retraction assay (Box 15.6).

Box 15.6 The Principle of Clot Retraction Assay

Clot retraction assay is based on the measurement of expressed serum after an indicated time following clot formation [72]. The test is performed by using whole blood with no anticoagulant collected in a graded glass tube. The tube is incubated at 37 °C until clot formation and then for an additional 1 h at 37 °C. The clot is then removed using a wooden stick, and the expressed serum is measured [73]. The result is reported as a percentage using the following formula:

$$\text{Percentage of clot retraction} = \text{Expressed serum volume} / \text{Total initial blood volume} \times 100.$$

Clot retraction is usually absent in GT. In normal conditions, more than 40% serum is expressed. Clot retraction is absent in type I GT, with <5% αIIbβ3 and decreased in type II, with 5–20% αIIbβ3, and in variant (or type III) GT with normal or near-normal (60–100%) expression of dysfunctional receptors (Table 15.5).

This test is not widely available and is no longer required for the diagnosis of GT, as it is not specific and is also impaired in Stormorken syndrome and Wiskott–Aldrich syndrome.

Other Platelet Studies

αIIbβ3 is involved in the uptake of fibrinogen from the peripheral bloodstream to platelet storage granules, so that fibrinogen in the α-granules is absent in type I GT. Expression of functional integrin αIIbβ3 is also necessary for the proper release of the platelet δ-granule contents and abnormality in the release of these granular contents may be observed in GT.

15.4.2.6 Genetic Analysis

Genetic analysis of *ITGA2B* and *ITGB3* mutations will help to confirm GT diagnosis, identify heterozygous carriers for family and prenatal counseling, and, where appropriate, prenatal diagnosis. Identification of genetic variants will also allow genotype–phenotype correlation to help advance understanding of GT and αIIbβ3 integrin biology. Whether mutation analysis may help predict the risk of antiplatelet antibody development (following transfusion and during pregnancy) remains to be studied.

15.4.3 Differential Diagnosis

In a considerable number of patients, the diagnosis of GT is overlooked, being overshadowed by the more common acquired platelet function disorders with their common clinical presentations and laboratory findings. For this reason, it is essential to perform a proper physical examination looking for such common findings as purpura and ecchymoses. We should obtain an appropriate medical history of unprovoked bruising or bleeding episodes or severe bleeds after minor trauma. We recommend the use of the ISTH Bleeding Assessment Tool to systematically assess the patient's bleeding history and bleeding phenotype that may allow prediction of future bleeding risk [74–76] (https://bleedingscore.certe.nl). Family history of GT among other family members should be sought. In isolated cases without a family history, leukemia-associated chromosomal rearrangements and the presence of autoantibodies against integrin αIIbβ3 should be considered as part of the differential diagnosis. Acquired autoantibodies against integrin αIIbβ3 can sufficiently block the platelet function to induce a thrombasthenia-like state [77, 78].

15.4.4 Treatment

Although GT is one of the most severe IPFDs, the prognosis of this disorder is good if appropriate management is provided. The goal of treatment in GT is not only to treat bleeds but also to prevent the occurrence of bleeds and their bleed-related complications to improve patients' quality of life. Management in a comprehensive care center with multidisciplinary expertise on bleeding disorders similar to the management of hemophilia is preferred [79].

On-demand therapy is normally provided to patients with GT and most of the patients experience blood transfusion in their lives. The management approach includes local and adjunctive measures as well as systemic hemostatic agents. Mild bleeds can be managed by local measures and/or antifibrinolytic drugs. But when the use of these agents is not successful or when more severe bleeding occurs or when surgical intervention is required, systemic hemostatic agents such as platelet transfusion or rFVIIa can be used. Platelet transfusion is currently the standard treatment in GT. However, repeated platelet transfusion may result in alloimmunizations against HLA and/or integrin αIIbβ3 antigens and cause refractoriness to future platelet transfusion. To lessen HLA alloimmunization, HLA-compatible single donor apheresis leukocyte-reduced platelet concentrates are preferred for patients with platelet disorder, but these may not always be readily available. In addition, the risk of blood-borne infectious agent transmission is a major concern with the use of human blood products.

rFVIIa is increasingly used for the management of patients with GT and is efficacious and safe in GT with or without antiplatelet antibodies [11, 80]. rFVIIa is approved in the European Union for the management of patients with GT and antibodies against platelets HLA and/or integrin αIIbβ3 with refractoriness to platelets or when platelet is not readily available, while in the United States, this therapeutic agent is approved for the management of patients with refractory to platelet transfusion. In Canada, it is approved for clinical refractoriness and/or antibody against platelet or when platelet is not immediately available [8, 78, 79].

Thus, the following therapeutic options are available for patients with GT:

1. Local measures and/or antifibrinolytic drugs
2. Systemic hemostatic management
3. Curative treatment options

15.4.4.1 Local Measures and/or Antifibrinolytic Drugs

Mild to moderate bleeding episodes can be managed by conservative measures. These include the application of local pressure or local use of topical thrombin or fibrin sealants. Antifibrinolytic agents such as tranexamic acid or epsilon-aminocaproic acid are often effective in the management of bleeding.

Epistaxis is one of the most common bleeding symptoms in GT, especially in children. The most important measures to control epistaxis include compression, topical thrombin, antifibrinolytics, and nasal packing, preferably absorbable gelfoam packing. In severe epistaxis that is not controlled with these conservative

measures, platelet transfusion and/or rFVIIa can be used. In severe cases, surgical procedures such as electrocautery, laser coagulation, septoplasty, and arterial ligation can be used to control bleeding. It is also important to counsel GT patients to refrain from conditions that may predispose them to trauma and bleeding. Antiplatelet agents (e.g., aspirin and NSAIDs) should be avoided [8, 12, 78]. A dry environment may predispose patients with GT (or other bleeding disorders) to nose bleeds. Preventative measures may then include the use of a humidifier or frequent nasal saline spray to supply moisture to the nasal muscle, and/or coating the nasal mucosa with a thin film of inert gel to retain moisture.

15.4.4.2 Systemic Hemostatic Management

When local measures are not successful in stopping hemorrhagic episodes, systemic hemostatic management can be used. These include transfusion of platelet concentrate and administration of rFVII [8, 80].

Platelets Concentrate

Currently, platelet transfusion is the standard treatment for the control of severe hemorrhagic episodes and the management of surgical procedures. Platelet transfusion has two main concerns including the risk of blood-borne infectious disease transmission and alloimmunization against HLA and/or αIIbβ3 antigens. In addition, allergic reaction and transfusion-related acute lung injury are other risks of platelet transfusion. In order to minimize the risk of HLA alloimmunization against platelet, patients should preferably receive HLA-matched leukocyte-reduced platelet concentrates, particularly from a single donor. In GT patients with bleeding, the effectiveness of platelet transfusion is monitored *clinically* for bleeding cessation. In these patients with a baseline normal platelet count, monitoring of platelet count for platelet increment is not perfect, although it may give an estimation of platelet recovery. In GT patients with a deficiency of integrin αIIbβ3, monitoring the increase in platelets bearing αIIbβ3 by flow cytometry (if available) can be used. It should be noted that this method cannot predict the treatment efficacy of the increased platelet, especially in those patients with alloimmunization against platelet. Furthermore, GT platelets have been shown to interfere with the function of transfused normal donor platelets, so sufficient platelets should be given to overcome the interference for proper hemostasis [80–82].

Platelet Refractory

About 50% of patients with nonimmune thrombocytopenia develop anti-HLA following repeated transfusions of platelet concentrate. The risk is 10–15% when leukocyte-reduced platelet concentrate is used.

Due to repeated platelet transfusions in patients with GT, alloantibodies developing against the platelet glycoproteins αIIbβ3 and/or the HLA antigens are common. Thus, platelet refractoriness is a major problem in GT patients. Platelet refractoriness is defined as repeated failure to achieve suitable responses to platelet transfusions. In patients with HLA antibodies and platelet refractoriness, HLA-matched platelets should be used. In nonimmune patients, the risk of HLA alloimmunization

can also be lessened by transfusion of HLA-matched platelets and the use of leukocyte-depleted blood components. It is important that the hemostatic response to platelet transfusions is monitored in GT patients. Clinical improvements in bleeding and laboratory evaluation of platelet function including the use of flow cytometry are monitoring options in GT. Another alternative treatment option for platelet-refractory patients with or without platelet antibodies (to HLA and/or αIIbβ3) is rFVIIa.

Because of the risk of alloimmunization due to repeated platelet transfusions, it has been suggested that rFVIIa be considered as a first-line treatment or prevention of bleeding in GT patients at risk for the development of anti-αIIbβ3. In this context, the genetic information of patients can be very helpful. Patients with GT and disease-causing mutations that result in an absent expression of αIIbβ3 expression on platelets (e.g., mutations leading to premature termination of αIIb or β3) have been shown to be more susceptible to anti-αIIbβ3 development and therefore platelet refractoriness. Thus, genetic findings can be used to help decide whether platelet transfusion or rFVIIa should be used in nonimmune GT patients [8, 12, 78, 83].

Activated Recombinant Factor VII

rFVIIa (NovoSeven/Niastase, Novo Nordisk A/S, Bagsvaerd, Denmark) is produced via recombinant DNA technology in baby hamster kidney cells. In the production process, no human proteins or derivatives are present, so human pathogen transmission is not a risk. rFVIIa was used successfully in platelet function disorders for the first time in 1996 in a 2-year-old boy with GT with epistaxis not responsive to conservative management. Since then, there have been many reports of successful use of rFVIIa in GT and other platelet function disorders. In 2014, the US Food and Drug Administration (FDA) approved rFVIIa for the treatment of bleeding episodes and perioperative management in adults and children with GT who are refractory to platelet transfusions, with or without platelet alloantibodies. As indicated earlier, for patients with type I GT, especially those with severe mutations resulting in the total absence of αIIbβ3 expression, the suggestion is that rFVIIa be preferred to platelet transfusion to prevent αIIbβ3 alloimmunization, reserving platelets to be used effectively for emergency life/limb threatening situations and major surgical procedures. A generic version of rFVIIa was developed by AryoGen (Tehran, Iran) and was first licensed by the Ministry of Health and Medical Education of Iran. AryoSeven is similar to NovoSeven in post-injection rFVIIa activity and clinical efficacy. This Iranian bio generic rFVIIa is currently used by several thousands of patients with hemophilia with inhibitors, acquired hemophilia, congenital FVII deficiency, and Glanzmann's thrombasthenia in different countries. In pregnant women, anti-αIIbβ3 may be transferred across the placenta to result in thrombocytopenia and bleeding in the fetus or the neonates. Thus, platelet transfusion should be avoided during pregnancy, and in women of reproductive age as well as in prepubertal girls. rFVIIa can be used instead for the management of bleeding and surgical procedures [84–86]. Although originally investigated for GT patients with platelet antibodies and/or platelet refractoriness, rFVIIa is equally effective and safe in GT patients without platelet antibodies and/or platelet refractoriness

[11]. Both the EU and Canada approved the use of rFVIIa for any GT patients irrespective of platelet antibody/refractoriness status when platelets are not readily available.

The recommended dose of rFVII is as follows:

Bleeding episodes: 90 mcg/kg q2–6 h until hemostasis is achieved.

Perioperative management:

1. 90 mcg/kg immediately before surgery and repeat q2hr for the duration of the procedure
2. 90 mcg/kg q2–6hr to prevent postoperative bleeding.

Mechanism of Action of rFVIIa

Physiologically, FVIIa exerts its hemostatic action after forming a complex with tissue factor (TF). In normal people, at the site of vascular injury, the FVII-TF complex induces activation of FX and FIX that result in initial thrombin production and subsequent activation of several clotting factors (e.g., FV, FVIII, and FXI) as well as platelets. Activated platelets at the injury site support further coagulation activation that leads to sufficient thrombin generation (thrombin burst). This proper amount of thrombin transforms soluble fibrinogen to a fibrin clot and induces hemostasis. In GT, due to the lack of αIIbβ3 receptor for fibrinogen binding, initial thrombin generation is not sufficient to induce platelet aggregate formation at the wound surface and to provide adequate activated platelet surfaces for clotting factors interaction to generate a thrombin burst at the vascular injury site. Therefore, GT platelets have reduced thrombin generation capacity. High-dose rFVIIa improves hemostasis via a TF-independent mechanism. In pharmacological dose, rFVIIa binds to the negatively charged phospholipid surface exposed on activated platelets and can activate FX to FXa, resulting in a burst of thrombin generation, enhancing hemostasis, and improving GT platelet adhesion and aggregation. Increased thrombin generation by high-dose rFVIIa has also been shown to improve fibrin clot structure in GT plasma. It is also suggested that rFVIIa activates platelets resulting in an increase in phosphatidyl-serine exposure on the platelet surface and an increase in homeostatically active platelet-derived circulating microparticles [84–87].

15.4.4.3 Surgical Management

Surgery is an important hemostatic challenge in patients with IPFDs, including GT, and can be accompanied by life-threatening hemorrhage. Currently, the standard therapeutic option for surgical interventions is also the transfusion of platelet concentrate. Prior to a surgical procedure, patients with IPFD should be screened for platelet antibodies. In those patients with a current or history of platelet antibodies and/or refractoriness, rFVIIa, together with (or without) antifibrinolytic agents, is preferred over platelet concentrate. As in the treatment of bleeding, if platelets are to be used, HLA-matched and leukocyte-depleted platelet concentrate is the best therapeutic choice whether HLA antibodies are present or not. If HLA-matched platelet concentrate is not available, random donor platelet concentrate can be used in patients without HLA antibodies or platelet refractoriness [78, 88].

Minor Surgery

Minor surgeries including dental extraction in patients with GT and platelet refractoriness or antiplatelet antibodies can be successfully managed using rFVIIa. rFVIIa is usually given preoperatively and depending on the type of procedure and severity of bleeding may continue for the first 12–24 h.

rFVIIa use in platelet-refractory patients is recommended by the United Kingdom Haemophilia Centre Doctors' Organization for minor surgical prophylaxis including dental extractions. If platelet transfusion is required, HLA-matched leukocyte-depleted platelets are preferred. A rFVIIa dose of 90–140 μg/kg at ≤2.5-h intervals for two or more doses can successfully be used for the prevention of bleeding in minor surgeries. Antifibrinolytics should be started preoperatively and may be continued for 1–2 weeks [8, 78, 88].

Major Surgery

Successful management of major surgeries in patients with GT requires suitable communication between the surgeons, the hemophilia center, and the transfusion laboratory. Antifibrinolytics may be needed in some cases and should be started preoperatively and continued for 7–14 days. Platelet transfusion remains the standard of care in nonimmune GT patients. rFVIIa is recommended for allo-immunized patients (particularly with platelet refractoriness) or in GT patients irrespective of alloimmunization and refractoriness status when platelet is not readily available during major procedures. rFVIIa at ≥90 μg/kg in ≤2.5-h intervals should be used, at least at the beginning. The clinical conditions of the patient will determine the number and duration of subsequent doses [8, 78, 88].

15.4.4.4 Hematopoietic Stem Cell Transplantation

Effective treatments are available for patients with GT that include the use of platelet concentrates and rFVIIa. Nonetheless, some patients continue to have severe and recurrent bleeding episodes despite adequate management and have a very poor quality of life. For these patients, hemopoietic stem cell transplantation (HSCT) may represent a necessary curative therapy. The first successful HSCT was reported in the literature in 1985 on a 4-year-old boy with anti-αIIbβ3 [89]. So far, 47 patients with GT who received HSCT between 1981 and 2016 have been registered with the Center for International Blood and Marrow Transplantation Research (CIBMTR®). As of December 2016, 83% of these patients were alive (9% dead and 9% missing for follow-up) (preliminary data obtained from the Statistical Center of the CIBMTR®; the analysis has not been reviewed or approved by the Advisory or Scientific Committees of the CIBMTR®). Sources of stem cells include bone marrow, cord, blood, and peripheral blood. The conditioning regimens include both myeloablative and non-myeloablative ones [8, 90, 91].

15.4.4.5 Gene Therapy

Gene therapy for GT is in the experimental phase, but there is important preclinical progress in animal models. Hodivala-Dilke et al. reported the first animal model

(mice) of GT with *ITGB3* gene knock-out. These GT mice had prolonged BT and absence of clot retraction compared with heterozygous and normal animals. Platelet function in these mice is corrected after transplantation of hematopoietic cells with a lentivirus vector encoding integrin β3. In a dog model of GT (Great Pyrenees dogs with a genetic defect in *ITGA2B* gene include insertion of 14 nucleotides in *ITGA2B* exon 13), gene therapy resulted in expression of integrin αIIbβ3 that stabilized to ~10% in three dogs after 2, 4, and 5 years. Although this level of integrin αIIbβ3 (GPIIb/IIIa) is similar to that in human GT type II, the dog had decreased cutaneous bruising and shortened BT. Induced pluripotent stem cell (iPSC) was generated from 2 patients with type 1 GT. Insertion of human αIIb cDNA together with the GP1ba promoter led to a high level of αIIb mRNA expression in the iPSC-derived megakaryocytes (MK), and these MK cells responded to agonist stimulation, suggesting a recovery in the expression and activation of integrin αIIbβ3. All these studies can be considered as a very important advance in gene therapy that may eventually apply to human GT [8, 92, 93].

References

1. Tarawah A, Owaidah T, Al-Mulla N, Khanani MF, Elhazmi J, Albagshi M, et al. Management of Glanzmann's thrombasthenia–guidelines based on an expert panel consensus from gulf cooperation council countries. J Appl Hematol. 2019;10(1):1.
2. Nurden AT, Pillois X, Nurden P. Understanding the genetic basis of Glanzmann thrombasthenia: implications for treatment. Expert Rev Hematol. 2012;5(5):487–503.
3. Nurden AT, Fiore M, Nurden P, Pillois X. Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on variants, phenotypic variability, and mouse models. Blood. 2011;118(23):5996–6005.
4. Dorgalaleh A, Poon M-C, Shiravand Y. Glanzmann thrombasthenia. Congenital bleeding disorders. Springer; 2018. p. 327–55.
5. Nurden AT, Pillois X, Fiore M, Heilig R, Nurden P, editors. Glanzmann thrombasthenia-like syndromes associated with macrothrombocytopenias and mutations in the genes encoding the αIIbβ3 integrin. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2011.
6. Srivastava A, Usher S, Nelson EJ, Jayandharan G, Shaji RV, Chandy M, et al. Prenatal diagnosis of Glanzmann thrombasthenia. Natl Med J India. 2003;16(4):207–8.
7. Wang ZY, Ruan CG. [Current state and prospect in treatment of Glanzmann's thrombasthenia]. Zhonghua Xue Ye Xue Za Zhi. 2016;37(5):430–431.
8. Poon MC, Di Minno G, d'Oiron R, Zotz R. New insights into the treatment of Glanzmann Thrombasthenia. Transfus Med Rev. 2016;30(2):92–9.
9. Fang J, Hodivala-Dilke K, Johnson BD, Du LM, Hynes RO, White GC 2nd, et al. Therapeutic expression of the platelet-specific integrin, alphaIIbbeta3, in a murine model for Glanzmann thrombasthenia. Blood. 2005;106(8):2671–9.
10. Seligsohn U. Glanzmann thrombasthenia: a model disease which paved the way to powerful therapeutic agents. Pathophysiol Haemost Thromb. 2002;32(5–6):216–7.
11. Poon M-C. The use of recombinant activated factor VII in patients with Glanzmann's thrombasthenia. Thromb Haemost. 2021;121(03):332–40.
12. Kato A. The biologic and clinical spectrum of Glanzmann's thrombasthenia: implications of integrin alpha IIb beta 3 for its pathogenesis. Crit Rev Oncol Hematol. 1997;26(1):1–23.
13. Bennett JS. Structure and function of the platelet integrin alphaIIbbeta3. J Clin Invest. 2005;115(12):3363–9.

14. Calvete JJ. Platelet integrin GPIIb/IIIa: structure-function correlations. An update and lessons from other integrins. Proc Soc Exp Biol Med. 1999;222(1):29–38.
15. Suzuki H. [Structure and function of platelet alpha IIb beta 3 integrin]. Rinsho Byori. 1997;Suppl 104:60–72.
16. Cierniewski CS. [Structure and function of integrin receptors based on platelet receptor for fibrinogen]. Postepy Biochem. 1994;40(1):45–54.
17. Estevez B, Du X. New concepts and mechanisms of platelet activation signaling. Physiology (Bethesda). 2017;32(2):162–77.
18. Liu J, Das M, Yang J, Ithychanda SS, Yakubenko VP, Plow EF, et al. Structural mechanism of integrin inactivation by filamin. Nat Struct Mol Biol. 2015;22(5):383–9.
19. Nieswandt B, Varga-Szabo D, Elvers M. Integrins in platelet activation. J Thromb Haemost. 2009;7(Suppl 1):206–9.
20. Ma YQ, Qin J, Plow EF. Platelet integrin alpha(IIb)beta(3): activation mechanisms. J Thromb Haemost. 2007;5(7):1345–52.
21. Xu Z, Chen X, Zhi H, Gao J, Bialkowska K, Byzova TV, et al. Direct interaction of kindlin-3 with integrin alphaIIbbeta3 in platelets is required for supporting arterial thrombosis in mice. Arterioscler Thromb Vasc Biol. 2014;34(9):1961–7.
22. Mandava P, Thiagarajan P, Kent TA. Glycoprotein IIb/IIIa antagonists in acute ischaemic stroke: current status and future directions. Drugs. 2008;68(8):1019–28.
23. Goto S, Tamura N, Ishida H. Ability of anti-glycoprotein IIb/IIIa agents to dissolve platelet thrombi formed on a collagen surface under blood flow conditions. J Am Coll Cardiol. 2004;44(2):316–23.
24. Fiore M, Firah N, Pillois X, Nurden P, Heilig R, Nurden AT. Natural history of platelet antibody formation against alphaIIbbeta3 in a French cohort of Glanzmann thrombasthenia patients. Haemophilia. 2012;18(3):e201–9.
25. Kashiwagi H, Kunishima S, Kiyomizu K, Amano Y, Shimada H, Morishita M, et al. Demonstration of novel gain-of-function mutations of alphaIIbbeta3: association with macrothrombocytopenia and glanzmann thrombasthenia-like phenotype. Mol Genet Genomic Med. 2013;1(2):77–86.
26. Toogeh G, Sharifian R, Lak M, Safaee R, Artoni A, Peyvandi F. Presentation and pattern of symptoms in 382 patients with Glanzmann thrombasthenia in Iran. Am J Hematol. 2004;77(2):198–9.
27. George JN, Caen JP, Nurden AT. Glanzmann's thrombasthenia: the spectrum of clinical disease. Blood. 1990;75(7):1383–95.
28. Borhany M, Fatima H, Naz A, Patel H, Shamsi T. Pattern of bleeding and response to therapy in Glanzmann thrombasthenia. Haemophilia. 2012;18(6):e423–5.
29. Simou M, Kouskouni E, Vitoratos N, Economou E, Creatsas G. Polymorphisms of platelet glycoprotein receptors and cell adhesion molecules in fetuses with fetal growth restriction and their mothers as detected with pyrosequencing. In Vivo. 2017;31(2):243–9.
30. Nurden AT, Pillois X, Wilcox DA. Glanzmann thrombasthenia: state of the art and future directions. Semin Thromb Hemost. 2013;39(6):642–55.
31. Franchini M, Favaloro EJ, Lippi G. Glanzmann thrombasthenia: an update. Clin Chim Acta. 2010;411(1–2):1–6.
32. Rosenberg N, Hauschner H, Peretz H, Mor-Cohen R, Landau M, Shenkman B, et al. A 13-bp deletion in alpha(IIb) gene is a founder mutation that predominates in Palestinian-Arab patients with Glanzmann thrombasthenia. J Thromb Haemost. 2005;3(12):2764–72.
33. Newman PJ, Seligsohn U, Lyman S, Coller BS. The molecular genetic basis of Glanzmann thrombasthenia in the Iraqi-Jewish and Arab populations in Israel. Proc Natl Acad Sci U S A. 1991;88(8):3160–4.
34. Negahdari S, Zamani M, Seifi T, Sedighzadeh S, Mazaheri N, Zeighami J, et al. Identification of three novel mutations in the FANCA, FANCC, and ITGA2B genes by whole exome sequencing. Int J Prev Med. 2020;11:117.

35. Kazemi A, Abolghasemi H, Kazemzadeh S, Vahidi R, Faranoush M, Farsinejad A, et al. Molecular characterization of Glanzmann's thrombasthenia in Iran: identification of three novel mutations. Blood Coagul Fibrinolysis. 2017;28(8):681–6.
36. Aloui C, Chakroun T, Granados V, Jemni-Yacoub S, Fagan J, Khelif A, et al. Molecular genetic diagnosis of Tunisian Glanzmann thrombasthenia patients reveals a common nonsense mutation in the ITGA2B gene that seems to be specific for the studied population. Blood Coagul Fibrinolysis. 2018;29(8):689–96.
37. Tokgoz H, Torun Ozkan D, Caliskan U, Akar N. Novel mutations of integrin αIIb and β3 genes in Turkish children with Glanzmann's thrombasthenia. Platelets. 2015;26(8):779–82.
38. Sandrock-Lang K, Oldenburg J, Wiegering V, Halimeh S, Santoso S, Kurnik K, et al. Characterisation of patients with Glanzmann thrombasthenia and identification of 17 novel mutations. Thromb Haemost. 2015;113(04):782–91.
39. Albanyan A, Al-Musa A, AlNounou R, Al Zahrani H, Nasr R, AlJefri A, et al. Diagnosis of Glanzmann thrombasthenia by whole blood impedance analyzer (MEA) vs. light transmission aggregometry. Int J Lab Hematol. 2015;37(4):503–8.
40. Fiore M, Nurden AT, Vinciguerra C, Nurden P, Pillois X. Rapid diagnosis of the French gypsy mutation in Glanzmann thrombasthenia using high-resolution melting analysis. Thromb Haemost. 2010;104(5):1076–7.
41. French DL, Coller BS, Usher S, Berkowitz R, Eng C, Seligsohn U, et al. Prenatal diagnosis of Glanzmann thrombasthenia using the polymorphic markers BRCA1 and THRA1 on chromosome 17. Br J Haematol. 1998;102(2):582–7.
42. Nurden AT, Pillois X, Wilcox DA, editors. Glanzmann thrombasthenia: state of the art and future directions. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2013.
43. Kashiwagi H, Kunishima S, Kiyomizu K, Amano Y, Shimada H, Morishita M, et al. Demonstration of novel gain-of-function mutations of αIIbβ3: association with macrothrombocytopenia and glanzmann thrombasthenia-like phenotype. Mol Genet Genomic Med. 2013;1(2):77–86.
44. Kobayashi Y, Matsui H, Kanai A, Tsumura M, Okada S, Miki M, et al. Identification of the integrin β3 L718P mutation in a pedigree with autosomal dominant thrombocytopenia with anisocytosis. Br J Haematol. 2013;160(4):521–9.
45. Hauschner H, Mor-Cohen R, Messineo S, Mansour W, Seligsohn U, Savoia A, et al. Abnormal cytoplasmic extensions associated with active αIIbβ3 are probably the cause for macrothrombocytopenia in Glanzmann thrombasthenia-like syndrome. Blood Coagul Fibrinolysis. 2015;26(3):302–8.
46. Bury L, Falcinelli E, Chiasserini D, Springer TA, Italiano JE Jr, Gresele P. Cytoskeletal perturbation leads to platelet dysfunction and thrombocytopenia in variant forms of Glanzmann thrombasthenia. Haematologica. 2016;101(1):46.
47. Bury L, Zetterberg E, Leinøe EB, Falcinelli E, Marturano A, Manni G, et al. A novel variant Glanzmann thrombasthenia due to co-inheritance of a loss-and a gain-of-function mutation of ITGB3: evidence of a dominant effect of gain-of-function mutations. Haematologica. 2018;103(6):e259.
48. Bury L, Malara A, Gresele P, Balduini A. Outside-in signalling generated by a constitutively activated integrin αIIbβ3 impairs proplatelet formation in human megakaryocytes. PLoS One. 2012;7(4):e34449.
49. Harrison P, Mackie I, Mumford A, Briggs C, Liesner R, Winter M, et al. Guidelines for the laboratory investigation of heritable disorders of platelet function. Br J Haematol. 2011;155(1):30–44.
50. Favaloro EJ, Bonar R. An update on quality control for the PFA-100/PFA-200. Platelets. 2018;1–6:622.
51. Cho Y-U, Jang S, Park C-J, Chi H-S. Variables that affect platelet function analyzer-100 (PFA-100) closure times and establishment of reference intervals in Korean adults. Ann Clin Lab Sci. 2008;38(3):247–53.

52. Cattaneo M, Cerletti C, Harrison P, Hayward C, Kenny D, Nugent D, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. J Thromb Haemost. 2013;11(6):1183–9.
53. Hvas A-M, Favaloro EJ. Platelet function analyzed by light transmission aggregometry. Hemostasis and thrombosis. Springer; 2017. p. 321–31.
54. Paniccia R, Priora R, Liotta AA, Abbate R. Platelet function tests: a comparative review. Vasc Health Risk Manag. 2015;11:133.
55. Rand ML, Reddy EC, Israels SJ. Laboratory diagnosis of inherited platelet function disorders. Transfus Apher Sci. 2018;57:485.
56. Frontroth JP. Light transmission aggregometry. Haemostasis. Springer; 2013. p. 227–40.
57. Chan MV, Warner TD. Standardised optical multichannel (optimul) platelet aggregometry using high-speed shaking and fixed time point readings. Platelets. 2012;23(5):404–8.
58. McGlasson DL, Fritsma GA. Whole blood platelet aggregometry and platelet function testing. Semin Thromb Hemost. 2009;35(2):168–80.
59. Awidi A, Maqablah A, Dweik M, Bsoul N, Abu-Khader A. Comparison of platelet aggregation using light transmission and multiple electrode aggregometry in Glanzmann thrombasthenia. Platelets. 2009;20(5):297–301.
60. Chan MV, Armstrong PC, Warner TD. 96-well plate-based aggregometry. Platelets. 2018;1–6:650.
61. Bednar B, Condra C, Gould RJ, Connolly TM. Platelet aggregation monitored in a 96 well microplate reader is useful for evaluation of platelet agonists and antagonists. Thromb Res. 1995;77(5):453–63.
62. Lordkipanidzé M, Lowe GC, Kirkby NS, Chan MV, Lundberg MH, Morgan NV, et al. Characterization of multiple platelet activation pathways in patients with bleeding as a high-throughput screening option: use of 96-well Optimul assay. Blood. 2014;123:e11.
63. Rand ML, Leung R, Packham MA. Platelet function assays. Transfus Apher Sci. 2003;28(3):307–17.
64. Michelson AD. Evaluation of platelet function by flow cytometry. Pathophysiol Haemost Thromb. 2006;35(1–2):67–82.
65. Nagy B Jr, Debreceni IB, Kappelmayer J. Flow cytometric investigation of classical and alternative platelet activation markers. EJIFCC. 2013;23(4):124.
66. Michelson AD. Flow cytometry: a clinical test of platelet function. Open Access Articles. 1996;87:290.
67. Givan AL. Flow cytometry: first principles. Wiley; 2013.
68. Shattil SJ, Cunningham M, Hoxie JA. Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. Blood. 1987;70(1):307–15.
69. Santos M, Valles J, Marcus A, Safier L, Broekman M, Islam N, et al. Enhancement of platelet reactivity and modulation of eicosanoid production by intact erythrocytes. A new approach to platelet activation and recruitment. J Clin Invest. 1991;87(2):571–80.
70. Shattil SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane glycoprotein IIb. IIIa complex during platelet activation. J Biol Chem. 1985;260(20):11107–14.
71. Kitchen S, Olson JD, Preston FE. Quality in laboratory hemostasis and thrombosis. Wiley; 2013.
72. Podda G, Femia E, Cattaneo M. Current and emerging approaches for evaluating platelet disorders. Int J Lab Hematol. 2016;38:50–8.
73. Macfarlane R. A simple method for measuring clot-retraction. Lancet. 1939;233(6039):1199–201.
74. Gresele P, Falcinelli E, Bury L, Pecci A, Alessi MC, Borhany M, et al. The ISTH bleeding assessment tool as predictor of bleeding events in inherited platelet disorders: communication from the ISTH SSC subcommittee on platelet physiology. J Thromb Haemost. 2021;19(5):1364–71.
75. Rodeghiero F, Tosetto A, Abshire T, Arnold D, Coller B, James P, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. J Thromb Haemost. 2010;8(9):2063–5.

76. Elbatarny M, Mollah S, Grabell J, Bae S, Deforest M, Tuttle A, et al. Normal range of bleeding scores for the ISTH-BAT: adult and pediatric data from the merging project. Haemophilia. 2014;20(6):831–5.
77. Yazici ZM, Celik M, Yegin Y, Gunes S, Kayhan FT. Glanzmann's thrombasthenia: a rare case of spontaneous bilateral hemotympanum. Braz J Otorhinolaryngol. 2015;81(2):224–5.
78. Solh T, Botsford A, Solh M. Glanzmann's thrombasthenia: pathogenesis, diagnosis, and current and emerging treatment options. J Blood Med. 2015;6:219–27.
79. Srivastava A, Brewer AK, Mauser-Bunschoten EP, Key NS, Kitchen S, Llinas A, et al. Guidelines for the management of hemophilia. Haemophilia. 2013;19(1):e1–47.
80. Poon M-C, Di Minno G, Zotz R, d'Oiron R. Glanzmann's thrombasthenia: strategies for identification and management. Expert Opin Orphan Drugs. 2017;5(8):641–53.
81. Al-Battat S, Rand ML, Bouskill V, Lau W, Blanchette VS, Kahr WHA, et al. Glanzmann thrombasthenia platelets compete with transfused platelets, reducing the haemostatic impact of platelet transfusions. Br J Haematol. 2017;181:410.
82. Jennings LK, Wang WC, Jackson CW, Fox CF, Bell A. Hemostasis in Glanzmann's thrombasthenia (GT): GT platelets interfere with the aggregation of normal platelets. Am J Pediatr Hematol Oncol. 1991;13(1):84–90.
83. Chitlur M, Rajpurkar M, Recht M, Tarantino MD, Yee DL, Cooper DL, et al. Recognition and management of platelet-refractory bleeding in patients with Glanzmann's thrombasthenia and other severe platelet function disorders. Int J Gen Med. 2017;10:95–9.
84. Rajpurkar M, Chitlur M, Recht M, Cooper DL. Use of recombinant activated factor VII in patients with Glanzmann's thrombasthenia: a review of the literature. Haemophilia. 2014;20(4):464–71.
85. Franchini M. The use of recombinant activated factor VII in platelet disorders: a critical review of the literature. Blood Transfus. 2009;7(1):24–8.
86. Poon MC, Zotz R, Di Minno G, Abrams ZS, Knudsen JB, Laurian Y. Glanzmann's thrombasthenia treatment: a prospective observational registry on the use of recombinant human activated factor VII and other hemostatic agents. Semin Hematol. 2006;43(1 Suppl 1):S33–6.
87. Allen GA, Hoffman M, Roberts HR, Monroe DM 3rd. Recombinant activated factor VII: its mechanism of action and role in the control of hemorrhage. Can J Anaesth. 2002;49(10):S7–14.
88. Gopalakrishnan A, Veeraraghavan R, Panicker P. Hematological and surgical management in Glanzmann's thrombasthenia: a case report. J Indian Soc Pedod Prev Dent. 2014;32(2):181–4.
89. Bellucci S, Devergie A, Gluckman E, Tobelem G, Lethielleux P, Benbunan M, et al. Complete correction of Glanzmann's thrombasthenia by allogeneic bone-marrow transplantation. Br J Haematol. 1985;59(4):635–41.
90. Ramzi M, Dehghani M, Haghighat S, Nejad HH. Stem cell transplant in severe Glanzmann thrombasthenia in an adult patient. Exp Clin Transplant. 2016;14(6):688–90.
91. Wiegering V, Winkler B, Langhammer F, Wolfl M, Wirbelauer J, Sauer K, et al. Allogeneic hematopoietic stem cell transplantation in Glanzmann thrombasthenia complicated by platelet alloimmunization. Klin Padiatr. 2011;223(3):173–5.
92. Fang J, Jensen ES, Boudreaux MK, Du LM, Hawkins TB, Koukouritaki SB, et al. Platelet gene therapy improves hemostatic function for integrin alphaIIbbeta3-deficient dogs. Proc Natl Acad Sci U S A. 2011;108(23):9583–8.
93. Wilcox DA, White GC 2nd. Gene therapy for platelet disorders: studies with Glanzmann's thrombasthenia. J Thromb Haemost. 2003;1(11):2300–11.

Bernard-Soulier Syndrome: Diagnosis and Management

16

Bahare Ghasemi and Akbar Dorgalaleh

16.1 Introduction

Bernard-Soulier syndrome (BSS) is a rare inherited platelet function disorder characterized by thrombocytopenia, giant platelets (macrothrombocytopenia), and abnormal bleeding tendency. The hallmark of BSS is a deficiency or dysfunction of the von Willebrand factor (VWF) receptor, also known as glycoprotein (GP) Ib-IX-V complex (CD42a–d), that is required for normal primary hemostasis [1]. The GPIb-IX-V complex is a crucial receptor for normal hemostasis, and it is necessary for adherence of platelets to the exposed vascular subendothelium via binding to VWF at the site of vascular injury, especially at high shear flow rates [2–4]. BSS is caused by variants in the *GPIBA*, *GPIBB*, or *GP9* genes coding GPIbα, GPIbβ, and GPIX, respectively [5, 6].

BSS is most often inherited in an autosomal recessive manner (biallelic form), and therefore, this disorder is more frequent in areas with a high rate of consanguinity. However, an autosomal dominant pattern of inheritance was also reported for this disorder (monoallelic form) [3, 7].

Although BSS usually presents early in life with various bleeding symptoms, it is not uncommon to receive a late diagnosis in adulthood. Patients with BSS commonly experience mucocutaneous bleeds such as easy bruising, purpura, epistaxis, gingival bleeding, and menorrhagia. In addition, severe postdental hemorrhage, postsurgical bleeding, and post-traumatic hemorrhage can occur among these patients. The bleeding tendency among these patients varies, even among members of the family [1, 3, 6, 7]. Routine and specific assessments can be used for the timely diagnosis of BSS. In a complete blood count (CBC), thrombocytopenia presents,

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_16

and in a peripheral blood smear (PBS) examination, large and giant platelets are seen. Bleeding time (BT) and closure time in the platelet function analyzer-100/200 (PFA-100/200) are prolonged, and ristocetin-induced platelet agglutination is absent [8, 9]. In BSS, ristocetin-induced platelet aggregation (RIPA) is not corrected with the addition of normal plasma as a source of VWF. Flow cytometric analysis of GPIb-IX-V can be used for precise diagnosis, and molecular analysis can be used for confirmation of the disorder [2, 7, 10, 11].

Treatment for BSS involves both general supportive care and specific therapy for bleeding. Patients with BSS should be guided to avoid activities carrying bleeding risk and intake antiplatelet medication. Patients with serious bleeding may require platelet and red blood cell (RBC) transfusion. Administration of antifibrinolytic agents, recombinant activated factor VII (rFVIIa), and hormones (in female patients) may also be effective in the management of bleeding symptoms. In patients with life-threatening bleeding, stem cell transplantation may be considered [1, 3, 12–17].

16.2 Glycoprotein Ib-IX-V Complex

16.2.1 Structure and Function

The GPIb-IX-V complex is a critical platelet receptor that is involved in thrombopoiesis and platelet adhesion and activation [18]. The GPIb-IX-V complex is well-known for its role in primary hemostasis, where platelets adhere to exposed subendothelium at the site of vascular injury [18]. This complex exclusively expressed on the platelet/megakaryocyte lineage, and it is the second most abundant GP on platelet surface. Approximately 25,000 copies of the GPIb-IX-V complex are present on the platelet surface [2, 3, 19–21].

GPIb-IX-V is a heteropentameric complex consisting of four subunits including GPIbα (CD42b) that binds covalently with disulfide bonds to two GPIbβ (CD42c) subunits and noncovalently associated with GPIX (CD42a) and GPV (CD42d). The four distinct genes responsible for coding of four subunits of the GPIb-IX-V complex including *GPIBA* (chromosome 17p13), *GPIBB* (chromosome 22q11.21), *GP9* (chromosome 3q21), and *GP5* (chromosome 3q29) that encode GPIbα, GPIbβ, GPIX, and GPV, respectively. Concomitant and proper expression of GPIbα, GPIbβ, and GPIX subunits is required for appropriate and efficient expression of the GPIb-IX-V complex on the cell surface [1, 3, 11, 12, 19, 22–24].

All four subunits of the GPIb-IX-V complex are type I transmembrane proteins. These subunits belong to the leucine-rich repeat (LRR) superfamily of proteins. Each subunit of the complex has one large N-terminal extracellular domain, a transmembrane helix, and a relatively short C-terminal cytoplasmic domain. The LRR sequences are approximately 24 amino acids in length. These sequences are located on the N-terminal domain of each subunit of the GPIb-IX-V complex. The LRR sequences are required for the stability of each subunit and correct assembly of the GPIb-IX-V complex (Fig. 16.1) [2, 11, 14, 19, 20, 25, 26].

GPIbα is the most important and largest subunit of the GPIb-IX-V complex, mediating binding to almost all known ligands [18, 27]. The extracellular N-terminal

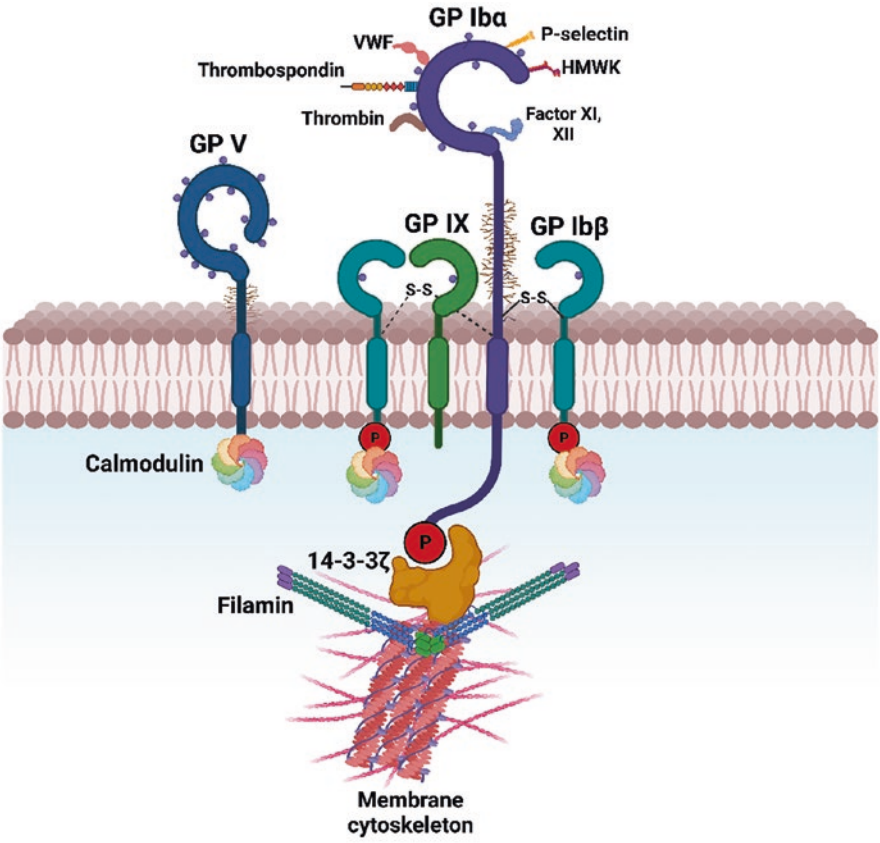

Fig. 16.1 The GPIb-IX-V complex is composed of four distinct subunits: GPIbα, GPIbβ, GPIX, and GPV. GPIbα is linked with disulfide bonds to two GPIbβ subunits, and is noncovalently associated with GPIX and GPV. Each subunit belongs to the leucine-rich repeat (LRR) glycoprotein superfamily and contains a single transmembrane domain separating an extracellular N-terminal from an intracellular C-terminal. GPIbα is the major subunit and contains a ligand-binding site on its extracellular domain for adhesive ligands such as VWF, thrombospondin, and selectin. The GPIb-IX-V complex, via its cytoplasmic domain is associated with cytoskeletal elements such as filamin A and adapter and signaling proteins such as 14-3-3ζ and calmodulin

domain of the GPIbα is made up of seven LRR, also known as the ligand-binding domain, a negatively charged portion involved in thrombin binding, a highly glycosylated region (sialomucin), and a mechanosensitive domain (MSD) close to the platelet membrane [18, 27]. The N-terminal region of GPIbα contains the binding site for adhesive ligands such as VWF, thrombospondin, β2 glycoprotein I (β2 GPI), coagulation factors (factor XI, factor XII, high-molecular-weight kininogen (HMWK), and thrombin, as well as the counter-receptor on activated endothelial cells and platelets (P-selectin) or white blood (macrophage-1 antigen (Mac-1) or integrin $\alpha_M\beta 2$). In addition, the cytoplasmic domain of GPIbα contains binding sites

for 14-3-3ζ, a signaling protein, and filamin A, a cytoskeletal protein [1–3, 11, 19, 22, 28, 29].

GPIbβ and GPIX have smaller extracellular domains than GPIbα; each contains one LRR sequence. The stability of the complex is enhanced by interactions between the extracellular domains of GPIbβ and GPIX and the MSD of GPIbα. In addition, the expression and assembly of the GPIb-IX complex are mediated by the transmembrane domains of GPIbα, GPIbβ, and GPIX rather than their cytoplasmic regions. The absence of each subunit, with the exception of GPV, which has a weak association with the complex, results in impaired complex expression [18, 27].

The main function of the GPIb-IX-V complex is binding via GPIbα to VWF A1-domain that its attachment to exposed subendothelial collagen results in the adhesion of platelets at the site of vascular damage (refer to Chap. 1). Interaction between GPIb-IX-V complex and VWF induces signal transduction. Direct or indirect interaction of cytoplasmic tail of GPIb-IX-V subunits with signaling proteins such as calmodulin, 14-3-3ζ, Phosphoinositide (PI) 3-kinase, TNF receptor-associated factor 4 (TRAF4) and Src family kinase (Lyn and Syc) leads to degranulation, calcium flux and rearrangement of cytoskeletal proteins, activation of integrin αIIbβ3 (GPIIb/IIIa) and platelet aggregation. The GPIb-IX-V complex is an important receptor on platelet surface for coagulation activity due to its binding site for coagulation factors (F) such as thrombin, FXI, FXII, and HMWK that is located on N-terminal region of GPIbα [2, 11, 12]. Interestingly, the GPIb-IX-V complex promotes full platelet activation at low concentrations of thrombin. Studies suggested that GPIbα may act as a coreceptor for protease-activated receptor-1 (PAR-1), which is cleaved by thrombin and induces platelet activation [9, 27, 30].

A further function of the GPIb-IX-V complex is to maintain platelet normal shape through interaction between the GPIbα cytoplasmic tail and the actin-binding protein filamin A; this association connects the GPIb-IX-V complex to the platelet membrane skeleton. Through its interactions with counter receptors such as P-selectin on activated endothelial cells and platelets and Mac-1 on leukocytes, the GPIb-IX-V complex plays a role in inflammation and thrombosis [1–3, 11, 12, 14, 20, 22].

Recent findings revealed roles for the GPIb-IX-V complex in platelet production, thrombopoietin generation, and platelet turnover [24, 27] (Table 16.1).

Table 16.1 Characteristic of the glycoprotein Ib-IX-V subunits

Subunit	Number of amino acids	Molecular weight	Number of leucine-rich repeat	Gene location	Function
GPIbα	610	135 kDa	7	17p13	Major ligand-binding subunit/ receptor for VWF, Thrombin, Thrombospondin, β2 GPI, Factor XI, XII, HMWK, Mac-1, P-selectin. Interaction with filamin A and 14-3-3ζ via cytoplasmic tail
GPIbβ	181	26 kDa	1	22q11.21	Structurally important for GPIbα efficient expression on the plasma membrane. Interaction with calmodulin via cytoplasmic tail
GPIX	160	20 kDa	1	3q21	Structurally important for GPIbα efficient expression on the plasma membrane
GPV	544	82 kDa	15	3q29	Regulation of platelet–collagen interactions. Substrate for active thrombin. Interaction with calmodulin via cytoplasmic tail

GP glycoprotein; *kDa* kilodalton; *VWF* von Willebrand factor; *HMWK* high molecular weight kininogen; *Mac-1* macrophage-1 antigen; *β2 GPI* β2 glycoprotein I

16.3 Bernard–Soulier Syndrome

BSS is a rare inherited platelet function disorder that presents early in life with moderate to severe bleeding diathesis. The estimated incidence of disorder is one per one million in the general population, but due to mild bleeding phenotypes in a number of patients and also due to misdiagnosis, the expected prevalence of disorder is higher. The incidence is also higher in areas with a high rate of consanguineous marriages. Moreover, the calculated frequency of carriers for BSS based on Hardy–Weinberdg law is 1 per 500 individuals in the general population [1, 3, 6, 14, 16, 31, 32].

Jean Bernard and Jean Pierre Soulier, two French hematologists, first described BSS in 1948. They described a young man with severe bleeding episodes, prolonged BT, and a low platelet count with extremely large platelets. They named this disorder "hemorrhagiparous thrombotic dystrophy." Weiss and his colleagues in 1974 demonstrated that BSS platelets are unable to adhere to rabbit aortic subendothelium. Nurden and Caen in 1975 observed that one of the important platelet surface GPs was absent in BSS platelets, and finally, they found that this absent GP was the GPIb-IX-V complex [1, 12, 14].

Platelets from BSS patients have quantitative or qualitative abnormalities in the GPIb-IX-V complex, making them unable to adhere to exposed subendothelium at the site of vascular damage (an adhesion defect). At the time of vascular injury, the VWF, via its A3 domain, binds to the exposed subendothelium collagen. Collagen-bound VWF expresses a binding site for platelets. Platelets through GPIbα, a major subunit of the GPIb-IX-V complex, bind to the VWF-A1 domain. This process is the first step in primary hemostasis and leads to platelet adhesion to the subendothelium and subsequent platelet activation and platelet plug formation at the site of vascular damage [12, 20, 33–37].

Several functions are performed by the GPIb-IX-V complex, which makes it a vital platelet receptor. This receptor has different ligands such as VWF, thrombospondin, β2 GPI, and coagulation factors (FXI, FXII, thrombin, and HMWK). Moreover, the GPIb-IX-V complex plays a critical role in the cross-talk between platelets and leukocytes during thrombosis and inflammatory response by interaction with P-selectin and Mac-1. Due to the various functions of this receptor, such as its role in the regulation of platelet size, shape, and formation, as well as its function in hemostasis and interaction with other cells, a defect in the GPIb-IX-V complex can result in several complications [2, 3, 12, 13, 38].

16.4 Molecular Basis

The GPIb-IX-V complex formation requires products of four distinct genes including *GPIBA*, *GPIBB*, *GP9,* and *GP5*. The products of these genes assemble within maturing MK in the bone marrow and then express on the platelet surface [5, 7, 34, 39, 40].

Genes encoding GPIbα, GPIbβ, GPIX, and GPV subunits are located on chromosomes 17p13 (*GPIBA*), 22q11.21 (*GPIBB*), 3q21 (*GP9*), and 3q29 (*GP5*), respectively [1, 6].

These genes share some structural features like other genes belonging to the LRR superfamily. They have simple, compact, and intron-depleted structures. Moreover, promoters of these genes have similar regulatory elements including binding sites for transcription factors such as GATA-1 and Ets. Three of the four genes including *GPIBA*, *GPIBB*, and *GP5* contain two exons and one intron, whereas the *GP9* gene consists of three exons and two introns. In these genes, the coding sequence also resides in one exon except for *GPIBB*. The *GPIBB* gene contains an intron 10 bases after the start codon [1, 3, 11, 14, 39, 41, 42].

Different mutations may occur in GPIbα, GPbβ, and GPIX genes and give rise to impaired biosynthesis and expression of the GPIb-IX-V complex on the platelet surface and subsequently lead to BSS. No BSS-causing mutation has been reported in *GP5* gene so far. This is consistent with the weak association of the GPV subunit with other subunits of the GPIb-IX-V complex as well as the lack of the requirement of GPV for the other subunits expression [1, 2, 19, 34, 43–49].

BSS is most often caused by mutations at both alleles of *GPIBA*, *GPIBB*, and *GP9* genes that cause biallelic forms of BSS (autosomal recessive). There are few

Table 16.2 Main features of two main forms of Bernard–Soulier syndrome

Disorder	Inheritance	Thrombocytopenia	Platelet morphology	Bleeding tendency	Level of the GPIb-IX-V complex on the surface of platelet	RIPA
Biallelic form	AR	Moderate to severe	Giant platelet	Moderate to severe	Very low or absent	Absent
Monoallelic form	AD	Mild	Large platelet	Mild	Normal or slightly reduced	Normal or slightly reduced

RIPA ristocetin-induced platelet agglutination; *AR* autosomal recessive; *AD* autosomal dominant; *GP* glycoprotein

mutations that occur in one allele of *GPIBA* or *GPIBB* genes and result in monoallelic BSS (autosomal dominant) (Table 16.2) [11, 28, 50, 51].

At least 120 variants have been identified as being responsible for BSS to date [52]. On the basis of the last cohort study performed on 211 families with BSS in 2014, 112 different mutations have been described including 45, 39, and 28 variants in *GPIBA*, *GPIBB*, and *GP9* genes, respectively. In this cohort, *GP9* had a higher mutation frequency than the other two genes. Mutations in these genes are heterogeneous, including frameshift insertion/deletion, nonsense mutations, and missense mutations, and they are distributed throughout the genes with no obvious hotspots. These mutations cause GPIb-IX-V complex to be unstable, truncated, or dysfunctional [11–13, 20, 53–55].

Missense mutations are a common genetic defect in BSS that occur in GPIbβ and GPIX subunits more frequently than in GPIbα subunit. Missense mutations usually hamper subunit folding and lead to unstable complex formation with severely decreased expression of the GPIb-IX-V complex on the platelet surface. Missense mutations can also affect functional domains with normal or slightly decreased expression of a dysfunctional GPIb-IX-V complex on the platelet surface [1, 11, 16, 19, 56].

Nonsense mutations lead to truncated subunits with a lack of a transmembrane domain. Frameshift insertion/deletion mutations lead to the production of a novel protein with an altered sequence from the native protein. No splicing defects have been detected, which is due to the simple and compact structure of the genes coding for different subunits of the GPIb-IX (Fig. 16.2).

The majority of mutations affect the extracellular domain more frequently than the transmembrane and cytoplasmic domains. There are some rare mutations that affect the promoter and signal peptide of the genes. For instance, one mutation (g.-160C>G) affects the promoter region of the *GPIBB* gene and disrupts the GATA-1 binding site [3, 11, 34, 47, 49, 57, 58]. In addition, GPIbβ defect can occur in patients with DiGeorge/velocardiofacial syndrome that is caused by microdeletion

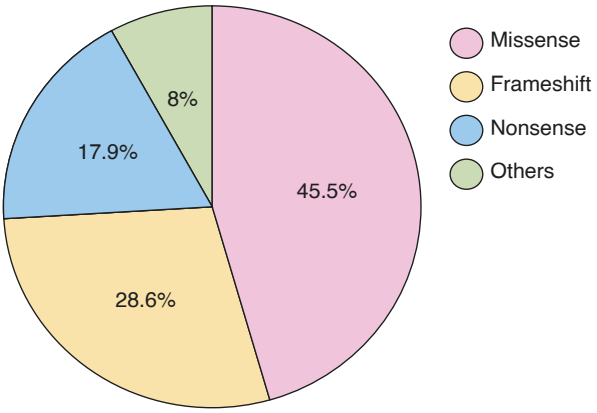

Fig. 16.2 The distribution of different mutations in Bernard–Soulier syndrome (BSS)

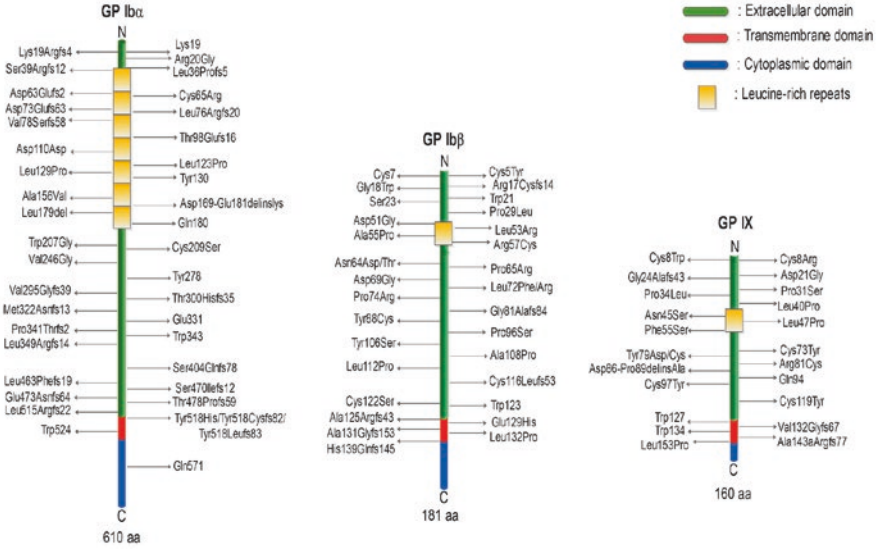

Fig. 16.3 Different mutations throughout GPIbα, GPIbβ, and GPIX subunits associated with Bernard–Soulier syndrome. Most of the mutations hit the extracellular domains of glycoproteins. *GP* glycoprotein; *N* N-terminal; *C* C-terminal

within chromosome 22q11.2, which contains the *GPIBB* gene; thus, these patients are obligatory carriers of BSS (Fig. 16.3) [3, 13, 20, 23, 25, 29, 36, 51, 59, 60].

Most BSS causative mutations are loss-of-function mutations that disrupt complex and trafficking through the endoplasmic reticulum and Golgi apparatus and prevent localization of the complex on the cell surface. There are some rare gain-of-function mutations in the *GPIBA* gene, which cause platelet-type von Willebrand disease (VWD) that is different from BSS and characterized by spontaneously binding of plasma VWF to platelets that give rise to clearance of plasma VWF. This disorder is similar to type 2B VWD (refer to Chap. 3) [1, 3, 7, 34, 40, 53, 59, 61, 62].

Most of BSS-causing mutations are unique and restricted to a single patient or family, but there are some exceptions such as the Asn45Ser mutation in *GP9* gene, which was reported in Caucasian families from northern and central European countries due to founder effect (refer to Chap. 2) [1, 20, 34].

Patients with the classic form of BSS (biallelic form) are homozygous or compound heterozygous for the causative mutations. Since many cases of BSS occur in families with consanguineous marriages, about 85% of them are homozygotes, and only about 13% are compound heterozygotes [55]. BSS carriers (heterozygote individuals in biallelic BSS) who only have one mutant allele are usually asymptomatic, and their platelets express about half the normal levels of the functional GPIb-IX-V complex. However, some BSS carriers may show a mild to moderate bleeding tendency. Most BSS carriers have lower platelet counts than healthy individuals, and they have slightly enlarged platelets. Furthermore, there are a few monoallelic mutations in *GPIBA* and *GPIBB* genes that exert a type of dominant effect and give rise to a milder form of BSS (monoallelic form) [3, 11, 14, 16, 23, 39, 54, 60, 61, 63–66].

The Bolzano variant is a monoallelic form of BSS with autosomal dominant inheritance that is most common in Italian populations. In this variant, the Ala156Val mutation occurs in the *GPIBA* gene (located within the sixth LRR), resulting in macrothrombocytopenia. The Bolzano variant's platelets have a normal or mild reduction of the GPIb-IX-V complex but are unable to bind to VWF. As a result, there is a functional defect in this complex. Furthermore, thrombin binding to GPIbα is normal in the Bolzano variant. [1–3, 12, 14, 39, 61]. In the Bolzano variant, the majority of patients experience only mild thrombocytopenia and infrequent bleeding episodes; however, a few patients with severe bleeding have been reported [38, 67].

Another autosomal dominant variant of BSS is associated with a substitution in the conserved region of GPIbα (Leu57Phe) on the LRR domains. This variant is characterized by macrothrombocytopenia and expression of the dysfunctional GPIbα subunit. The GPIbα shows susceptibility to proteolysis in this variant. In aggregometry studies, response to ristocetin is also slightly reduced [3, 14, 68].

The Copenhagen founder variant is a monoallelic form of BSS that was found in a Danish family. It is caused by a single nucleotide change (c.58T>G) in the *GP1BA* gene [69].

Furthermore, other monoallelic mutations with dominant effects including p.Asn57His and p.Tyr70Asp in the *GPIBA* gene and also p.Arg42Cys and p.Tyr113Cys in the *GPIBB* gene have been reported in a few patients. These mutations are responsible for mild macrothrombocytopenia [5, 51, 54, 61, 70].

16.5 Clinical Manifestations

BSS is a moderate to severe bleeding disorder that usually presents in infancy or early childhood with an abnormal, more profound bleeding tendency. Nevertheless, some patients who have a monoallelic variant of BSS with a milder phenotype may present in adulthood. Consequently, both the severity of the bleeding and the age at which BSS symptoms first appear can vary widely. As a result of platelets' functional defect in BSS, the tendency to bleed is more severe than expected by the platelet

count. Bleeding mostly occurs in mucocutaneous tissues and includes easy bruising, epistaxis, petechiae, purpura, and gingival bleeding. The severity of bleeding worsens with age in BSS, in contrast to Glanzmann thrombasthenia (GT), in which the risk of bleeding decreases with age (see Chap. 15). In female patients during puberty, menorrhagia may become an important issue. Other presentations such as hematuria, gastrointestinal (GI), or genitourinary bleeding may also occur. Severe and life-threatening bleeding can occur during hematological stress situations such as surgical procedures, tooth extraction, trauma, and delivery. Rare manifestations of this disorder include joint bleeding, intracranial hemorrhage (ICH), and deep visceral bleeding. Consistent with the heterogeneous molecular defects in BSS, the severity and frequency of bleeding episodes differ among patients, even in those with the same mutation and from the same family [1, 3, 7, 14, 15, 33, 38, 61, 64, 67, 70–72].

Although BSS carriers (heterozygous individuals in biallelic BSS) are usually asymptomatic, some carriers may show mild to moderate bleeding tendencies [3, 11, 16, 65].

Patients with the autosomal dominant disorder, also known as monoallelic BSS, have a milder phenotype than the biallelic form of the disease. These patients, such as those with the Bolzano variant, usually have no or minor bleeding diathesis (Table 16.3) [40, 53, 61].

Table 16.3 Clinical manifestations of patients with Bernard-Soulier syndrome

Clinical manifestations	Naz et al. (n:7)	Toogeh et al. (n:97)	Afrabiasi et al. (n:7) Presentation/ follow-up	Hadjati et al. (n:15)	Sanchez-Guiu et al. (n:8)
Epistaxis	100%	63.9%	28%/57%	80%	100%
Petechiae and purpura	–	1%	–/29%	6.6%	–
Ecchymosis	–	7.2%	–/29%	–	–
Bleeding from minor wounds	–	–	–	–	13%
Gum bleeding	100%	15.5%	15%/28%	60%	38%
Gastrointestinal bleeding	–	4.1%	–/42%	–	33%
Postdental extraction bleeding	–	–	15%/–	–	57%
Postsurgical bleeding	–	–	–	6.6%	71%
Menorrhagia	–	11.3%	–	–	100%
Postpartum hemorrhage	–	–	–	–	75%
Hematoma	100%	3%	–	6.6%	–
Hemarthrosis	–	–	–	–	–
Postvaccination bleeding	–	–	42%/–	–	–
Bruises	100%	–	–	–	–
Cerebral bleeding	–	1%	–	–	–

N number of patients

16.6 Diagnosis

BSS is initially characterized by macrothrombocytopenia and an abnormal tendency to bleed. These characteristics are not exclusive to BSS; hence, diagnosing BSS might be difficult. Aside from that, the diagnosis of BSS is simple and straightforward in comparison to other mild inherited platelet function disorders. Overall, diagnosing inherited macrothrombocytopenia is challenging and time consuming due to the rarity of these disorders as well as the requirement for specialized laboratory tests. [11, 13, 61, 73–75].

The first step in the diagnosis of an individual suspected of having BSS is obtaining a detailed clinical history, particularly a history of unexpected mucocutaneous bleeding. Initial laboratory assessments should include CBC and a precise examination of PBS. In patients with BSS, these tests revealed a variable degree of thrombocytopenia (30–150 × 10^9/L) and the presence of large or giant platelets in the blood film that can be as large as RBCs. Several patients with platelet counts less than 10 × 10^9/L are also reported. Additionally, platelet counts in the same patient can fluctuate over time [9, 38, 67]. The presence of giant platelets is a significant finding in BSS. Since cell counters usually count cells by their size (the impedance system), giant platelets may count as erythrocytes. Hence, an experienced hematologist must examine a blood smear for the presence of large platelets and visually count the cells to get a reliable platelet count. [1, 3, 11–15, 33, 36, 60, 61, 74, 76].

The precise mechanism of macrothrombocytopenia in BSS is still unclear. Different studies suggested that macrothrombocytopenia could be related to decreased platelet life span, impaired megakaryopoiesis, and defective proplatelet formation, as well as a defect in the interaction between the GPIb-IX-V complex and platelet membrane skeleton. Nevertheless, some studies demonstrated both decreased and normal platelet survival in patients with BSS. Electron microscopy studies determined the aberrant development and vacuolated demarcation membrane system in bone marrow megakaryocytes (MK) from patients with BSS and a murine model of BSS. This abnormality indicates impaired megakaryopoiesis in BSS that may contribute to the formation of giant platelets and thrombocytopenia.

Moreover, in vitro studies suggested that the GPIbα is critical for platelet formation because block of this subunit by monoclonal antibodies or cleavage of its extracellular domain by metalloproteinase mocarhagin inhibited proplatelet formation in human MK. This finding was also confirmed by a study on BSS murine model and patients with BSS. Therefore, defect in GPIbα is responsible for impaired proplatelet formation with the production of platelet macrocytosis and thrombocytopenia. In addition, immunofluorescence studies revealed that α-tubulin distribution within proplatelets was deranged, so alteration of α-tubulin distribution was involved in impaired proplatelet formation. GPIbα was also found to be essential for thrombopoiesis by its role in hepatic thrombopoietin (TPO) synthesis. Hence, thrombocytopenia in BSS may be explained by the low levels of TPO seen in patients with BSS. The GPIb-IX-V complex via cytoplasmic domain of GPIbα interacts with the cytoskeletal protein, filamin A. The GPIbα-filamin A interaction regulates platelet size, shape change, and adhesion to ligands. Hence, a disruption in the interaction between GPIbα and filamin A can result in giant platelet production, and this

disruption is consistent with the enhanced membrane deformability reported in BSS platelets. [2, 3, 12–14, 20, 23, 28, 29, 39, 60, 61, 66, 77–80].

BT is moderately (5–10 min) to severely (>20 min) prolonged in patients with BSS. BT is a simple and quick in vivo test that evaluates primary hemostasis by assessment of the function of platelets and plasma VWF. BT is less reproducible, invasive, nonspecific, and not sensitive enough to detect many mild platelet disorders. As a result, the use of BT is decreasing today and is largely replaced by PFA-100/200. Although BT is still used for evaluation of primary hemostasis, particularly in laboratories without other platelet function tests [1, 14, 33, 35, 81–83].

Patients with BSS also have a prolonged closure time [8] on the PFA-100/200 instrument when using the collagen/adenosine diphosphate (ADP) and collagen/epinephrine cartridges. This test is an in vitro measure of the BT, in which a small volume (0.8 mL per cartridge) of citrated whole blood is aspirated at a high shear rate through a capillary. The disposable cartridge consists of a microscopic aperture in a membrane coated with platelet agonists, such as collagen/ADP or collagen/epinephrine. These agonists result in platelet activation, adhesion, aggregation, and platelet plug formation, leading to occlusion of the aperture [8]. Closure time (CT) is influenced by platelet count, hematocrit, and VWF level, so in individuals with a hematocrit below 25% and a platelet count below 50×10^9/L, the result should be interpreted carefully. Furthermore, the concentration of sodium citrate that is used as an anticoagulant in this test can affect the CT. The use of sodium citrate (3.8% concentration) leads to more reliable results. Although the PFA-100/200 results are useful for screening for abnormalities in primary hemostasis such as VWD and some platelet function disorders, this test is not very sensitive for the detection of mild platelet function disorders. However, the sensitivity of this test is high for some severe IPFDs such as BSS and GT, but low specificity is a constant issue with the PFA-100/200 [8, 13, 15, 20, 33, 35, 74, 82–85].

Another laboratory hallmark of platelets in BSS is an isolated defect in RIPA that, unlike the defect in VWD, is not corrected by the addition of normal plasma as a source of VWF (refer to Chap. 3) (Fig. 16.4). Platelet aggregation in response to other agonists such as ADP, collagen, arachidonic acid, and epinephrine is normal. Additionally, BSS platelets show decreased aggregation in response to a low dose (but not a high dose) of thrombin [1–3, 12, 15, 23, 29, 31, 33, 86, 87].

Light transmission aggregometry (LTA) is the gold standard and key method for the diagnosis of platelet function disorders. In this method, the ability of platelets to aggregate in response to different agonists such as ADP, collagen, arachidonic acid, epinephrine, and ristocetin is assessed based on the change in optical density of a stirring sample of citrated platelet-rich plasma (PRP) in a cuvette at 37 °C following the addition of agonists. A photometer assesses the change in optical density. If platelets are able to aggregate, an increase in light transmission is observed. In this method, platelet-poor plasma (PPP) can be used to define 100% light transmission. Before testing, PRP should be kept at room temperature (RT) for 15 min. The rate of aggregation (%/min) and maximal percentage of aggregation (%) are parameters that are measured after a fixed period of time (usually 6–10 min). Although, this method is very useful for the diagnosis of platelet function disorders, it has some disadvantages, including manual sample processing, being time consuming,

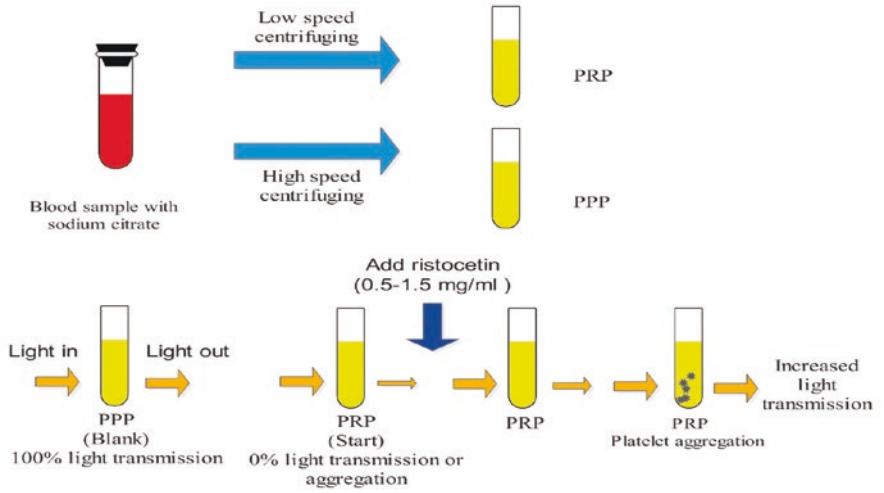

Fig. 16.4 Principle of ristocetin-induced platelet agglutination [9]. RIPA testing is usually performed using platelet-rich plasma (PRP) and a turbidimetric aggregometer. In this assay, a beam of light passes through two cuvettes, one containing platelet-poor plasma (PPP) and the other containing PRP. PPP is used as a blank and determines 100% light transmission. The starting PRP is used as a sample to be analyzed and is determined to have 0% light transmission or 0% aggregation. Adding ristocetin as an agonist induces platelet aggregation by promoting the binding of VWF to glycoprotein Ibα (GPIbα). Platelet aggregation leads to increased light transmission

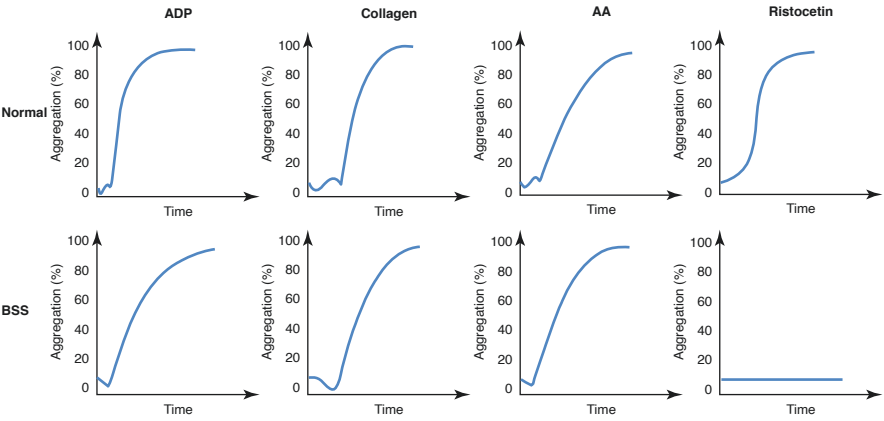

Fig. 16.5 Light transmission aggregometry results in a normal sample and in Bernard–Soulier syndrome (BSS). BSS platelets manifest isolated aggregation defects in response to ristocetin. *BSS* Bernard–Soulier syndrome; *ADP* adenosine diphosphate; *AA* arachidonic acid

requiring a large blood volume, and being difficult to obtain PRP in children and patients with severe thrombocytopenia. Moreover, the results of LTA can be influenced by some pre-analytical and analytical variables, such as blood sampling conditions, platelet count, presence of hemolysis and lipemia, sample processing, and the use of different concentrations of agonists (Fig. 16.5) [13, 74, 81–84, 87–94].

Another platelet function assay is whole blood aggregometry (WBA) or impedance platelet aggregometry. This method monitors changes in electrical impedance between two metal electrodes when platelet aggregation occurs in response to agonists. The adhesion of activated platelets to the surface of electrodes, followed by platelet aggregation in response to agonists, causes an increase in electrical impedance, which is measured in Ohms. WBA is performed on citrated whole blood, and platelet function is assessed with other blood elements and under more physiological conditions than LTA. Moreover, WBA needs a smaller amount of sample than LTA and does not require sample manipulation. The results of WBA can be influenced by platelet count, hematocrit, type of anticoagulant, and delay in testing [74, 81, 82, 91, 93, 94]. In addition, platelet aggregation testing cannot be performed on patients with severe thrombocytopenia or a recent platelet transfusion history [9].

With rare exceptions, the provisional diagnosis of BSS based on aggregometry can be primarily confirmed by flow cytometry analysis. Flow cytometry using a panel of specific monoclonal antibodies that specially recognize the subunits of the GPIb-IX-V complex (CD42a-d) can reveal the absence or severe reduction of the complex on the surface of resting platelets [1, 3, 13–15, 33, 61, 73, 88, 89].

The GPIb-IX-V complex is expressed at normal or slightly lower levels on the surface of platelets in the Bolzano type of BSS. Nevertheless, it functions improperly and is unable to bind to VWF. Moreover, flow cytometry analysis can identify biallelic BSS carriers because they express around half of the functional GPIb-IX-V complex on their platelet surface. [2, 23, 66, 83].

Flow cytometry is a laser-based technology that can be used to diagnose hereditary platelet diseases. This method is extremely useful because it is fast, accurate, and sensitive. This test is a great choice for children because it only needs a small amount of blood. Also, pre-analytical variables rarely affect flow cytometry results. This technique is independent of platelet count and is therefore a suitable test for assessing platelet function in thrombocytopenia patients. Flow cytometry can also assess platelet count and platelet size as well as reticulated platelets. Moreover, another advantage of flow cytometry is that the sample needs less manipulation. On the other hand, this assay is expensive and requires a well-educated operator [13, 15, 81, 83, 84, 94–99].

Flow cytometry analysis could be performed on citrated whole blood, washed platelets, or PRP. Whole blood analysis is better because PRP and washed platelets require additional preparation that may cause platelet activation. Another advantage of using whole blood in this test is the assessment of platelet function with other blood elements and more closely to in vivo conditions. Sodium citrate is used as an anticoagulant in flow cytometry analysis [81, 96].

Coagulation and clot retraction tests should be normal in BSS. A significantly reduced rate of prothrombin consumption and thrombin generation tests is observed in patients with BSS due to defective binding of FXI and thrombin to GPIbα. Some additional tests can be performed in specialized centers for the confirmation of BSS, including sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting for the analysis of the GP of platelets with the use of specific antibodies. Finally, molecular genetic testing for the detection of causative mutations in the

GPIBA, *GPIBB*, and *GP9* genes can be performed for confirmation of the disorder and determination of the underlying mutation(s). [1–3, 8, 13, 14, 20, 60, 68, 100].

Some important issues should be considered in platelet function testing, including blood sampling conditions, blood collection, and sample processing. A platelet study sample should be collected from resting and fasting patients who have refrained from smoking, physical activity, antiplatelet drugs, and caffeine intake because these conditions can affect platelet function. Patients should avoid taking medications that can affect platelet function for 7–10 days before testing. A blood sample should be collected with minimal tourniquet pressure by a 19–21 gauge (G) needle into a tube containing sodium citrate. A blood sample should be properly mixed with anticoagulant by inverting it three to six times. Sodium citrate is a common anticoagulant used for platelet function testing. The ethylenediaminetetraacetic acid (EDTA) can inhibit the binding of natural ligands, and it also makes dissociation of the integrin αIIbβ3 (GPIIb/IIIa) from the platelet surface, so in studies of platelet function, EDTA should be avoided. Samples should be kept at room temperature and avoid shaking and vibration of the tubes, and analysis should be performed within 30 min to 2 h after blood collection [74, 82, 96, 98].

16.7 Differential Diagnosis

Since the primary findings of BSS are not specific, the diagnosis of BSS may be challenging. A considerable number of patients with BSS are not diagnosed early in childhood, and the average age at diagnosis is 16 years. BSS is most often misdiagnosed as ITP because both have macrothrombocytopenia. Differential diagnosis is important for avoiding futile treatments such as steroid therapy and splenectomy. If a patient has recurrent mucocutaneous bleeding, thrombocytopenia, or large platelets on a peripheral smear and their parents are consanguineous, BSS should be suspected. [1, 3, 11, 14, 20, 31, 101].

BSS should also be differentiated from other inherited macrothrombocytopenias such as May–Hegglin, Sebastian, Fechtner, and Epstein's syndrome (myosin heavy chain 9 (MYH9)-related syndromes) and gray platelet syndrome (GPS). Examination of PBS can help distinguish these syndromes. In MYH9-related syndromes, the examination of PBS is very important for the detection of bright-blue cytoplasmic inclusions (Dohle body-like inclusions) in granulocytes. In these syndromes, unlike in BSS, platelet aggregation in response to ristocetin and thrombin is normal. In the blood smear of GPS, large gray-to-gray-blue platelets are present. Platelet aggregometry in patients with GPS shows variable results. GPS platelets in aggregometry studies show a reduced response to collagen as well as thrombin, while the response to ristocetin is normal or slightly reduced (refer to Chap. 17) [3, 20, 31, 33, 35, 60].

Furthermore, BSS and VWD should be distinguished because they can exhibit similar clinical symptoms, such as mucocutaneous bleeding. In both BSS and VWD, no agglutination with ristocetin occurs in the platelet aggregation test. Unlike VWD, BSS cannot be corrected by adding normal plasma as a source of VWF (refer to Chap. 3) [1, 31, 33] (Table 16.4).

Table 16.4 Laboratory features of some inherited macrothrombocytopenias

Disorder	Platelet count	Platelet morphology	Platelet aggregation					
			Arachidonic acid	ADP	Collagen	Thrombin	Epinephrine	Ristocetin
Bernard–Soulier syndrome	Decreased	Large and giant platelets	Normal	Normal	Normal	Normal (but not in a low dose of thrombin)	Normal	Absent
MYH9-related disorders	Decreased	Giant platelets	Normal	Normal	Normal	Normal	Normal or Impaired	Normal
Gray platelet syndrome	Decreased	Large platelets	Impaired	Variable	Impaired	Variable	Variable	Variable

ADP adenosine diphosphate; *MYH9* myosin heavy chain 9

16.8 Management

Since the bleeding diathesis in BSS patients is variable, these patients should be treated on an individual basis. BSS therapeutic approaches include both general and specialized treatments. Patients with BSS should be registered with a specialized center for bleeding disorders that can provide 24-h management services. The majority of patients do not require continual care, and patients should be advised to avoid trauma, contact sports, and the use of antiplatelet medicines such as aspirin. Patients should maintain proper dental hygiene to prevent invasive dental procedures. Minor and local bleeding such as gum bleeding and epistaxis can be managed by local measures including fibrin sealants, gelatin sponges, and compression with gauze soaked with tranexamic acid. Iron deficiency anemia may present due to chronic bleeding episodes or menorrhagia and should be treated. Patients with severe bleeding require medical interventions including RBC and platelet transfusion, but these treatments have several risks such as developing alloantibodies against human leukocyte antigens (HLA) and missing platelet GP and also have the risk of infection transmission. Therefore, these patients should be HLA typed and should receive HLA-compatible platelet units as well as leukocyte-depleted blood products. Moreover, these patients should be vaccinated against hepatitis A and B due to the risk of *transfusion-transmitted infections*. Prophylactic transfusion of platelets and blood before surgery may also be required. Antifibrinolytic drugs such as tranexamic acid and aminocaproic acid, as well as recombinant activated factor VII (rFVIIa), may be used to stop bleeding. In females with menorrhagia, the management of the patient depends on the severity of the bleeding. The first treatment for menorrhagia is the administration of antifibrinolytic agents. Oral contraceptives and hormone supplementation are recommended in the most severe cases of uncontrollable bleeding. Management of pregnancy and childbirth in women with BSS can be challenging, and severe bleeding during delivery and postpartum may occur. In these women, bleeding should be controlled by platelet transfusion, antifibrinolytic drugs, and desmopressin. These women have a possibility of developing antiplatelet antibodies, which can cross the placenta and cause severe thrombocytopenia and bleeding in the fetus or newborn. As a result, pregnancy in patients with BSS requires a multidisciplinary approach and special consideration. In rare patients with life-threatening bleeds, hematopoietic stem cell transplantation may be considered. BSS can also be a candidate for gene therapy in the future [1, 13–17, 20, 36, 62, 67, 68, 83, 102–105] (Table 16.5).

Table 16.5 Management of Patients with Bernard–Soulier syndrome

Treatment modality	Indication	Recommended dose
Desmopressin (DDAVP)	Mild bleeding, before major procedures, after significant trauma, menorrhagia Minor procedure such as dental extractions	0.3 μg/kg (IV) diluted in 20–50 mL saline over 30 min Intranasal spray; 150 μg per dose for a child under 50 kg in weight, 300 μg per dose in an adult
Antifibrinolytic agents (tranexamic acid, aminocaproic acid)	Minor bleedings such as epistaxis and gingival bleeding, menorrhagia, minor surgical procedures, oral/nasal surgery in invasive procedures are used in conjunction with another therapy such as desmopressin and platelet transfusion	Tranexamic acid: 15–25 mg/kg three times per day orally, 10–15 mg/kg (IV) three times per day Aminocaproic acid: 100 mg/kg (IV) over 15 min, 60–80 mg/kg three to four times a day orally
Platelet transfusion	Severe bleedings such as severe menorrhagia and severe epistaxis, surgical procedures, after significant trauma	Depend on patient's clinical condition 10–15 mL/kg in children, one adult therapeutic dose is equivalent to four to six single-donor units
Female hormones	Menorrhagia (when does not respond to initial therapies such as antifibrinolytic agents)	High dose conjugated estrogen for 24–48 h (IV) followed by high doses of oral estrogen—progestin high dose of progesterone such as norethisterone 5 mg every 4 h

DDAVP 1-deamino-8-d-arginine vasopressin; *IV* intravenous

References

1. Lanza F. Bernard-Soulier syndrome (hemorrhagiparous thrombocytic dystrophy). Orphanet J Rare Dis. 2006;1(1):46.
2. Berndt MC, Andrews RK. Bernard-Soulier syndrome. Haematologica. 2011;96(3):355–9.
3. Kunishima S, Kamiya T, Saito H. Genetic abnormalities of Bernard-Soulier syndrome. Int J Hematol. 2002;76(4):319–27.
4. Afrabiasi A, Artoni A, Karimi M, Peyvandi F, Ashouri E, Mannucci P. Glanzmann thrombasthenia and Bernard–Soulier syndrome in south Iran. Int J Lab Hematol. 2005;27(5):324–7.
5. Savoia A, Pastore A, De Rocco D, Civaschi E, Di Stazio M, Bottega R, et al. Clinical and genetic aspects of Bernard-Soulier syndrome: searching for genotype/phenotype correlations. Haematologica. 2011;96(3):417–23.
6. Ali S, Ghosh K, Shetty S. Novel genetic abnormalities in Bernard-Soulier syndrome in India. Ann Hematol. 2014;93(3):381–4.
7. Boeckelmann D, Hengartner H, Greinacher A, Nowak-Göttl U, Sachs U, Peter K, et al. Patients with Bernard-Soulier syndrome and different severity of the bleeding phenotype. Blood Cell Mol Dis. 2017;(67):69–74.
8. Cox K, Price V, Kahr WH. Inherited platelet disorders: a clinical approach to diagnosis and management. Expert Rev Hematol. 2011;4(4):455–72.
9. Tripathi P, Karthika K, Pati H, Tyagi S. Bernard Soulier syndrome; a rare bleeding disorder. 2018.

10. Takata Y, Kanaji T, Moroi M, Seki R, Sano M, Nakazato S, et al. Platelets with a W127X mutation in GPIX express sufficient residual amounts of GPIbα to support adhesion to von Willebrand factor and collagen. Int J Hematol. 2012;96(6):733–42.
11. Savoia A, Kunishima S, De Rocco D, Zieger B, Rand ML, Pujol-Moix N, et al. Spectrum of the mutations in Bernard–Soulier syndrome. Hum Mutat. 2014;35(9):1033–45.
12. Andrews RK, Berndt MC, editors. Bernard–Soulier syndrome: an update. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2013.
13. Diz-Küçükkaya R. Inherited platelet disorders including Glanzmann thrombasthenia and Bernard-Soulier syndrome. ASH Education Program Book. 2013;2013(1):268–75.
14. López JA, Andrews RK, Afshar-Kharghan V, Berndt MC. Bernard-Soulier syndrome. Blood. 1998;91(12):4397–418.
15. Alamelu J, Liesner R. Modern management of severe platelet function disorders. Br J Haematol. 2010;149(6):813–23.
16. Kirchmaier CM, Pillitteri D. Diagnosis and management of inherited platelet disorders. Transfus Med Hemother. 2010;37(5):237–46.
17. Seligsohn U. Treatment of inherited platelet disorders. Haemophilia. 2012;18(s4):161–5.
18. Quach ME, Li R. Structure-function of platelet glycoprotein Ib-IX. J Thromb Haemost. 2020;18(12):3131–41.
19. Li R, Emsley J. The organizing principle of the platelet glycoprotein Ib–IX–V complex. J Thromb Haemost. 2013;11(4):605–14.
20. Diz-Kücükkaya R, López JA. Inherited disorders of platelets. Hematol Oncol Clin. 2013;27(3):613–27.
21. Hayward CP, Rao A, Cattaneo M. Congenital platelet disorders: overview of their mechanisms, diagnostic evaluation and treatment. Haemophilia. 2006;12(s3):128–36.
22. Andrews R, Gardiner E, Shen Y, Whisstock J, Berndt M. Glycoprotein Ib–IX–V. Int J Biochem Cell Biol. 2003;35(8):1170–4.
23. Balduini CL, Iolascon A, Savoia A. Inherited thrombocytopenias: from genes to therapy. Haematologica. 2002;87(8):860–80.
24. Quach ME. GPIb-IX-V and platelet clearance. Platelets. 2022;33(6):817–22.
25. Kunishima S, Imai T, Kobayashi R, Kato M, Ogawa S, Saito H. Bernard–Soulier syndrome caused by a hemizygous GPIbβ mutation and 22q11.2 deletion. Pediatr Int. 2013;55(4):434–7.
26. Gardiner EE, Andrews RK. Platelet receptor expression and shedding: glycoprotein Ib-IX-V and glycoprotein VI. Transfus Med Rev. 2014;28(2):56–60.
27. Bendas G, Schlesinger M. The GPIb-IX complex on platelets: insight into its novel physiological functions affecting immune surveillance, hepatic thrombopoietin generation, platelet clearance and its relevance for cancer development and metastasis. Exp Hematol Oncol. 2022;11(1):19.
28. Favier R, Raslova H. Progress in understanding the diagnosis and molecular genetics of macrothrombocytopenias. Br J Haematol. 2015;170(5):626–39.
29. Rabbolini DJ, Morel-Kopp MC, Stevenson W, Ward CM, editors. Inherited macrothrombocytopenias. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2014.
30. Sharma S, Chak RK, Khanna R. Management of haemostasis during dental extraction in a Bernard-Soulier syndrome child. BMJ Case Rep CP. 2019;12(7):e229082.
31. Pham A, Wang J. Bernard-Soulier syndrome: an inherited platelet disorder. Arch Pathol Lab Med. 2007;131(12):1834–6.
32. Naz A, Jamal MY, Amanat S, Najmuddin A, Patel H, Raziq F, et al. Autosomal recessive inherited bleeding disorders in Pakistan: a cross-sectional study from selected regions. Orphanet J Rare Dis. 2017;12(1):66.
33. Carubbi C, Masselli E, Nouvenne A, Russo D, Galli D, Mirandola P, et al. Laboratory diagnostics of inherited platelet disorders. Clin Chem Lab Med. 2014;52(8):1091–106.
34. Shlebak A, Poles A, Manning R, Almuhareb S, De La Funte J, Mitchell M, et al. A novel homozygous c. 800C> G substitution in GP1BA exon 2 in a Kuwaiti family with Bernard-Soulier syndrome. Acta Haematol. 2015;134(3):193–8.
35. Matthews DC. Inherited disorders of platelet function. Pediatr Clin. 2013;60(6):1475–88.

36. Simon D, Kunicki T, Nugent D. Platelet function defects. Haemophilia. 2008;14(6):1240–9.
37. Andrews RK, Berndt MC. Platelet adhesion: a game of catch and release. J Clin Invest. 2008;118(9):3009.
38. Almomani MH, Mangla A. Bernard Soulier syndrome. 2020.
39. Nurden AT, Nurden P. Inherited defects of platelet function. Rev Clin Exp Hematol. 2001;5(4):314–34.
40. Gothwal M, Sandrock-Lang K, Zieger B. Genetics of inherited platelet disorders. Hamostaseologie. 2014;34(2):133–41.
41. Roth GJ, Yagi M, Bastian LS. The platelet glycoprotein Ib-V-IX system: regulation of gene expression. Stem Cells. 1996;14(S1):188–93.
42. Yagi M, Edelhoff S, Disteche CM, Roth GJ. Human platelet glycoproteins V and IX: mapping of two leucine-rich glycoprotein genes to chromosome 3 and analysis of structures. Biochemistry. 1995;34(49):16132–7.
43. Clemetson KJ. Platelet glycoproteins and their role in diseases. Transfus Clin Biol. 2001;8(3):155–62.
44. Okoli S, Madan B, Mwirigi A, Moore G, Drew A, Mitchell M, et al. A diagnostic dilemma: variant Bernard-Soulier syndrome, a difficult clinical and genetic diagnosis. Haemophilia. 2015;21(6):e510–3.
45. Freson K, Wijgaerts A, Van Geet C. Update on the causes of platelet disorders and functional consequences. Int J Lab Hematol. 2014;36(3):313–25.
46. Zieger B, Jenny A, Tsakiris D, Bartsch I, Sandrock K, Schubart C, et al. A large Swiss family with Bernard-Soulier syndrome. Hamostaseologie. 2009;29(2):161–7.
47. Salles II, Feys HB, Iserbyt BF, De Meyer SF, Vanhoorelbeke K, Deckmyn H. Inherited traits affecting platelet function. Blood Rev. 2008;22(3):155–72.
48. Kenny D, Morateck PA, Gill JC, Montgomery RR. The critical interaction of glycoprotein (GP) Ibβ with GPIX—a genetic cause of Bernard-Soulier syndrome. Blood. 1999;93(9):2968–75.
49. McEwan PA, Yang W, Carr KH, Mo X, Zheng X, Li R, et al. Quaternary organization of GPIb-IX complex and insights into Bernard-Soulier syndrome revealed by the structures of GPIbβ and a GPIbβ/GPIX chimera. Blood. 2011;118(19):5292–301.
50. Miller JL, editor. Glycoprotein analysis for the diagnostic evaluation of platelet disorders. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2009.
51. Noris P, Perrotta S, Bottega R, Pecci A, Melazzini F, Civaschi E, et al. Clinical and laboratory features of 103 patients from 42 Italian families with inherited thrombocytopenia derived from the monoallelic Ala156Val mutation of GPIbα (Bolzano mutation). Haematologica. 2012;97(1):82–8.
52. Barozzi S, Bozzi V, De Rocco D, Giangregorio T, Noris P, Savoia A, et al. A novel mutation in GP1BB reveals the role of the cytoplasmic domain of GPIbβ in the pathophysiology of Bernard-Soulier syndrome and GPIb-IX complex assembly. Int J Mol Sci. 2021;22(19):10190.
53. Rao AK, Songdej N. Parsing the repertoire of GPIb-IX-V disorders. Blood. 2017;129(4):403–4.
54. Sivapalaratnam S, Westbury SK, Stephens JC, Greene D, Downes K, Kelly AM, et al. Rare variants in GP1BB are responsible for autosomal dominant macrothrombocytopenia. Blood. 2017;129(4):520–4.
55. Minkov M, Zeitlhofer P, Zoubek A, Kager L, Panzer S, Haas OA. Novel compound heterozygous mutations in two families with Bernard–Soulier syndrome. Front Pediatr. 2021;8:589812.
56. Sánchez-Guiu I, Antón AI, Padilla J, Velasco F, Lucia JF, Lozano M, et al. Functional and molecular characterization of inherited platelet disorders in the Iberian Peninsula: results from a collaborative study. Orphanet J Rare Dis. 2014;9(1):213.
57. HadjKacem B, Elleuch H, Trigui R, Gargouri J, Gargouri AF. The same genetic defect in three Tunisian families with Bernard Soulier syndrome: a probable founder stop mutation in GPIbbeta. Ann Hematol. 2010;89(1):75–81.
58. Lanza F, De La Salle C, Baas MJ, Schwartz A, Boval B, Cazenave JP, et al. A Leu7Pro mutation in the signal peptide of platelet glycoprotein (GP) IX in a case of Bernard–

Soulier syndrome abolishes surface expression of the GPIb–V–IX complex. Br J Haematol. 2002;118(1):260–6.
59. Nurden A, Nurden P. Advances in our understanding of the molecular basis of disorders of platelet function. J Thromb Haemost. 2011;9(s1):76–91.
60. Kunishima S, Saito H. Congenital macrothrombocytopenias. Blood Rev. 2006;20(2):111–21.
61. Balduini C, Savoia A, Seri M. Inherited thrombocytopenias frequently diagnosed in adults. J Thromb Haemost. 2013;11(6):1006–19.
62. Nurden A, Freson K, Seligsohn U. Inherited platelet disorders. Haemophilia. 2012;18(s4):154–60.
63. Ali S, Shetty S, Ghosh K. A novel mutation in GP1BA gene leads to mono-allelic Bernard Soulier syndrome form of macrothrombocytopenia. Blood Coagul Fibrinolysis. 2017;28(1):94–5.
64. Bragadottir G, Birgisdottir ER, Gudmundsdottir BR, Hilmarsdottir B, Vidarsson B, Magnusson MK, et al. Clinical phenotype in heterozygote and biallelic Bernard-Soulier syndrome—a case control study. Am J Hematol. 2015;90(2):149–55.
65. Savoia A, Balduini CL, Savino M, Noris P, Del Vecchio M, Perrotta S, et al. Autosomal dominant macrothrombocytopenia in Italy is most frequently a type of heterozygous Bernard-Soulier syndrome. Blood. 2001;97(5):1330–5.
66. Balduini A, Malara A, Pecci A, Badalucco S, Bozzi V, Pallotta I, et al. Proplatelet formation in heterozygous Bernard-Soulier syndrome type Bolzano. J Thromb Haemost. 2009;7(3):478–84.
67. Grainger JD, Thachil J, Will AM. How we treat the platelet glycoprotein defects; Glanzmann thrombasthenia and Bernard Soulier syndrome in children and adults. Br J Haematol. 2018;182(5):621–32.
68. Nurden A. Qualitative disorders of platelets and megakaryocytes. J Thromb Haemost. 2005;3(8):1773–82.
69. Leinøe E, Brøns N, Rasmussen AØ, Gabrielaite M, Zaninetti C, Palankar R, et al. The Copenhagen founder variant GP1BA c. 58T> G is the most frequent cause of inherited thrombocytopenia in Denmark. J Thromb Haemost. 2021;19(11):2884–92.
70. Pecci A, Balduini CL. Lessons in platelet production from inherited thrombocytopenias. Br J Haematol. 2014;165(2):179–92.
71. Toogeh G, Keyhani M, Sharifian R, Safaee R, Emami A, Dalili H. A study of Bernard-Soulier syndrome in Tehran, Iran. Arch Iran Med. 2010;13(6):549.
72. Gh T. Quantitative immunophemotyping of platelet surface glycoproteins among Iranian patients with Bernard-Soulier syndrome. Iran J Blood Cancer. 2014;7(1):3–9.
73. Gresele P. Diagnosis of inherited platelet function disorders: guidance from the SSC of the ISTH. J Thromb Haemost. 2015;13(2):314–22.
74. Israels S. Laboratory testing for platelet function disorders. Int J Lab Hematol. 2015;37(S1):18–24.
75. Gresele P, Harrison P, Bury L, Falcinelli E, Gachet C, Hayward C, et al. Diagnosis of suspected inherited platelet function disorders: results of a worldwide survey. J Thromb Haemost. 2014;12(9):1562–9.
76. Krishnegowda M, Rajashekaraiah V. Platelet disorders: an overview. Blood Coagul Fibrinolysis. 2015;26(5):479–91.
77. Li J, Dai K, Wang Z, Cao L, Bai X, Ruan C. Platelet functional alterations in a Bernard-Soulier syndrome patient with filamin a mutation. J Hematol Oncol. 2015;8(1):79.
78. Balduini A, Malara A, Balduini CL, Noris P. Megakaryocytes derived from patients with the classical form of Bernard-Soulier syndrome show no ability to extend proplatelets in vitro. Platelets. 2011;22(4):308–11.
79. Strassel C, Eckly A, Léon C, Petitjean C, Freund M, Cazenave J-P, et al. Intrinsic impaired proplatelet formation and microtubule coil assembly of megakaryocytes in a mouse model of Bernard-Soulier syndrome. Haematologica. 2009;94(6):800–10.
80. Xu M, Li J, Neves MAD, Zhu G, Carrim N, Yu R, et al. GPIbα is required for platelet-mediated hepatic thrombopoietin generation. Blood. 2018;132(6):622–34.

81. Paniccia R, Priora R, Liotta AA, Abbate R. Platelet function tests: a comparative review. Vasc Health Risk Manag. 2015;11:133.
82. Lordkipanidzé M, editor. Platelet function tests. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2016.
83. Bolton-Maggs PH, Chalmers EA, Collins PW, Harrison P, Kitchen S, Liesner RJ, et al. A review of inherited platelet disorders with guidelines for their management on behalf of the UKHCDO. Br J Haematol. 2006;135(5):603–33.
84. Choi J-L, Li S, Han J-Y. Platelet function tests: a review of progresses in clinical application. Biomed Res Int. 2014;2014.
85. Hayward C, Harrison P, Cattaneo M, Ortel T, Rao A. Platelet function analyzer (PFA)-100® closure time in the evaluation of platelet disorders and platelet function. J Thromb Haemost. 2006;4(2):312–9.
86. Hartman MJ, Caccamese JF, Bergman SA. Perioperative management of a patient with Bernard-Soulier syndrome for third molar surgery. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2007;103(5):626–9.
87. Frontroth JP, Favaloro EJ. Ristocetin-induced platelet aggregation (RIPA) and RIPA mixing studies. Hemostasis and thrombosis. Springer; 2017. p. 473–94.
88. Gresele P, Bury L, Falcinelli E, editors. Inherited platelet function disorders: algorithms for phenotypic and genetic investigation. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2016.
89. Podda G, Femia EA, Pugliano M, Cattaneo M. Congenital defects of platelet function. Platelets. 2012;23(7):552–63.
90. Knöfler R, Streif W. Strategies in clinical and laboratory diagnosis of inherited platelet function disorders in children. Transfus Med Hemother. 2010;37(5):231–5.
91. Koltai K, Kesmarky G, Feher G, Tibold A, Toth K. Platelet Aggregometry testing: molecular mechanisms, techniques and clinical implications. Int J Mol Sci. 2017;18(8):1803.
92. Frontroth JP. Light transmission aggregometry. Haemostasis. 2013;992:227–40.
93. McGlasson DL, Fritsma GA, editors. Whole blood platelet aggregometry and platelet function testing. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2009.
94. Rechner A. Platelet function testing in clinical diagnostics. Hamostaseologie. 2011;31(2):79.
95. Rubak P, Nissen PH, Kristensen SD, Hvas A-M. Investigation of platelet function and platelet disorders using flow cytometry. Platelets. 2016;27(1):66–74.
96. Linden MD. Platelet flow cytometry. Haemostasis. 2013;992:241–62.
97. Michelson AD, Barnard MR, Krueger LA, Frelinger A, Furman MI. Evaluation of platelet function by flow cytometry. Methods. 2000;21(3):259–70.
98. Saboor M, Moinuddin M, Ilyas S. New horizons in platelets flow cytometry. Malays J Med Sci. 2013;20(2):62.
99. Özdemir ZC, Kar YD, Ceylaner S, Bör Ö. A novel mutation in the GP1BA gene in Bernard–Soulier syndrome. Blood Coagul Fibrinolysis. 2020;31(1):83–6.
100. Macêdo MB, Brito JMM, da Silva MP, Brito JA. Primigravida with Bernard-Soulier syndrome: a case report. BMC Res Notes. 2015;8(1):178.
101. Li X, Wang S, Wu J, Wang H, Wang J, Dong X, et al. A case of Bernard-Soulier syndrome due to a novel homozygous missense mutation in an exon of the GP1BA gene. Acta Haematol. 2020;143(1):60–4.
102. Prabu P, Parapia L. Bernard–Soulier syndrome in pregnancy. Int J Lab Hematol. 2006;28(3):198–201.
103. Uotila J, Tammela O, Mäkipernaa A. Fetomaternal platelet immunization associated with maternal Bernard-Soulier syndrome. Am J Perinatol. 2008;25(04):219–23.
104. Valera MC, Kemoun P, Cousty S, Sie P, Payrastre B. Inherited platelet disorders and oral health. J Oral Pathol Med. 2013;42(2):115–24.
105. Perez AV, Oliveira Filho CM, Pazinato TC, Silva MS, Valério EG, Amaral SN, et al. Bernard-Soulier syndrome in pregnancy: a case report. Open J Obstet Gynecol Irvine. 2019;9(6):838–44.

Gray Platelet Syndrome: Diagnosis and Management

17

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17.1 Introduction

The gray platelet syndrome (GPS) is a rare inherited disorder of α-granule formation that is characterized by mild to moderate or, infrequently, severe bleeding manifestations, thrombocytopenia, large gray platelets, marrow fibrosis, spleen enlargement, and markedly increased serum vitamin B12 levels [1]. Moreover, a low neutrophil count, reduced neutrophil granulation, atypical manifestations of autoimmune disease, and recurrent infection or occasionally low-grade inflammation have been reported in a small number of cases of GPS [2–4]. In animal models, reduced count and impaired degranulation of natural killer (NK) cells have been demonstrated as well [5]. GPS was first described by Raccuglia in 1979 and is included in the category of platelet storage pool disease. Due to the lack of α-granules, GPS is associated with decreased concentrations of several α-granule constituents including fibrinogen, factor V, von Willebrand factor (vWF), thrombospondin, platelet factor 4 (PF4), and platelet-derived growth factor (PDGF). Platelet aggregation studies may be normal in response to most agonists, but they are often abnormal for collagen and thrombin [6]. Blood film examination shows large pale platelets. Electron microscopy analysis shows large platelets with few or no

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A. Dorgalaleh (ed.), *Congenital Bleeding Disorders*,
https://doi.org/10.1007/978-3-031-43156-2_17

α-granules but dense bodies, mitochondria, lysosomes, and peroxisomes appear normal [7, 8]. The disease is caused by multiple mechanisms and inheritance patterns. For example, most GPS patients identified so far have had recessive mutations in the neurobeachin-like 2 (*NBEAL2*) gene at 3p21.31 [9]. In patients with autosomal dominant and X-linked GPS, growth factor independence 1b (*GFI1B*) and *GATA1* genes are affected, respectively [8, 10]. Most GPS patients do not need treatment, but, if required, since there is no specific treatment, supportive care includes platelet transfusion, splenectomy, and 1-desamino-8-D-arginine vasopressin (DDAVP) [6, 11–13]. Recently, hematopoietic stem cell transplantation has been reported as a curative treatment for reversing both the platelet defect and the bone marrow (BM) fibrosis [14].

17.2 Clinical Manifestations

GPS is clinically heterogeneous, and most patients have a history of spontaneous mucocutaneous bleeding that seems to vary from mild to moderate in severity. Rare patients experience more severe bleeding such as intracranial hemorrhage. Life-threatening bleeding has also been reported in some patients with GPS in the setting of trauma. Splenomegaly with a frequency of about 80–90% may be found in patients with BM fibrosis, although it may also be observed in patients without fibrosis [6, 8]. BM fibrosis may be present in 58% of patients with a median age of 28.5 years (range, 10–52 years) [4]. It may occur as a consequence of leakage of growth factors and mitogens (such as PDGF, transforming growth factor β [TGF-β], platelet-derived endothelial growth factor [ECGF], and vascular endothelial growth factor [VEGF]) from the hypogranular platelets and megakaryocytes into the extracellular BM space [15–17]. Interestingly, extramedullary hematopoiesis has also been reported in one patient with GPS [18]. Atypical manifestations of autoimmune disease (such as autoimmune lymphoproliferative-like syndrome, Hashimoto's thyroiditis, and rheumatoid arthritis), skin disorder (e.g., alopecia, discoid lupus erythematosus, and vitiligo), and recurrent infection and occasionally low-grade inflammation have been found in a small number of cases of patients with GPS [2–4]. In animal models, reduced count and impaired degranulation of NK cells and neutrophils have been demonstrated, suggesting deranged innate immunity [5]. Proteomic analysis and RNA-sequencing profile have demonstrated overexpression of CD4+ T helper cells at the protein and transcript level in GPS patients [4].

17.3 Molecular Basis

GPS was first described by Raccuglia in 1971, but the causative gene and underlying pathophysiology remained unknown for a long time [19]. The most important molecular analyses in GPS are summarized in Table 17.1. In 2000, using molecular analysis, it was revealed that GPS is mostly inherited in an autosomal recessive pattern. Ten years later, a study on 25 patients with GPS from 14 independent families

Table 17.1 The most important molecular investigations in gray platelet syndrome (GPS) patients

Name	Year	Method	Result	Reference
Mori et al.	1984	TEM and LTA	Determination of autosomal dominant pattern in GPS	[27]
Hyman et al.	2003	DNA microarray	Overexpression of cytoskeletal proteins is the base of the molecular mechanism of myelofibrosis in GPS.	[16]
Benit et al.	2005	Sanger sequencing	No sign of mutation in *HZF* in GPS patients	[28]
De Candia et al.	2006	Sanger sequencing and immunofluorescence microscopy	Decreased platelet aggregation => no response of platelet to thrombin, PAR1-AP or PAR4-AP	[29]
Tubemam et al.	2007	Sanger sequencing and linkage analysis	X-linked GPS due to a *GATA1* (Arg21, Gln) mutation	[26]
Gunay-Aygun et al.	2010	Genome-wide linkage analysis and homozygosity mapping	Determination of GPS causative gene position in a 9.4 Mb interval on chromosome 3p	[8]
Fabro et al.	2011	SNP arrays and homozygosity mapping	Decrease of 9.4 Mb interval to 1.7 Mb	[20]
Alber et al.	2011	NGS (exome sequencing)	Mutations in GPS causative gene "*NBEAL2*"	[22]
Kahr et al.	2011	NGS (exome sequencing)	Mutations in GPS causative gene "*NBEAL2*"	[21]
Gunay-Aygun et al.	2011	Proteomic analysis	Mutations in GPS causative gene "*NBEAL2*"	[10]
Depperman et al.	2013	Fluorescence labeling of α-granule, TEM, and recombination	Knocking out of *NBEAL2* gene in rat and observation of clinical manifestations of GPS	[30]
Monteferraria et al.	2013	Sequencing and linkage analysis	Mutations in GPS causative gene "*GFI1B*"	[31]
Guerrero et al.	2014	TEM, qPCR, and sequencing	Knocking out of *NBEAL2* gene in rat and observation of clinical manifestations of GPS	[32]
Rieux-laucat et al.	2015	Homozygosity mapping and WES	Mimicking of autoimmune lymphoproliferative syndrome by GPS	[3]
Dibuduo et al.	2016	TEM, cloning, and flow cytometry studies	GPS MK with *NBEAL2* mutations cultured for the first time	[33]
Tomberg et al.	2016	WES	8 bp deletion in *NBEAL2* (GPS mice)	[34]

(continued)

Table 17.1 (continued)

Name	Year	Method	Result	Reference
Bottega et al.	2017	Flow cytometry—sequencing	Novel mutation of *NBEAL2*	[35]
Wang et al.	2017	Blood smear examination, TEM, flow cytometry, exome sequencing analyses, and Sanger analysis	A novel nonsense *NBEAL2* gene mutation (g.27713C>A)	[36]
Sims et al.	2020	DNA sequencing, BM biopsy, plasma proteomic, and phenotyping	Novel manifestations of immune dysregulation and granule defects in GPS	[4]
Tariq et al.	2021	Blood smear and BM analysis-electron microscopy, and NGS	Case report with a novel mutation of *NBEAL2* (c.5674C>T, p.Gln1892X)	[37]

cDNA complementary deoxyribonucleic acid; *GP* glycoprotein; *HZF* hematopoietic zinc finger; *LTA* light transmission aggregometry; *MK* megakaryocyte; *NGS* next-generation sequencing; *qPCR* quantitative polymerase chain reaction; *SNP* single-nucleotide polymorphism; *TEM* transmission electron microscopy; *WES* whole-exome sequencing

using genome-wide linkage analysis revealed that the causative gene of GPS was localized to a 9.4-Mb interval on 3p21.1-3p22.1 [8]. Then, in 2011, in another study with the utilization of homozygosity mapping with a single-nucleotide polymorphism (SNP) array, 3p21 as a recessive locus for GPS was confirmed, and this interval narrowed significantly from 9.4 Mb to 1.7 Mb [20]. Shortly thereafter, mutations in *NBEAL2*, encoding neurobeachin-like 2, were identified as the genetic cause of GPS by three different groups using multiple approaches, including next-generation RNA sequence analysis, DNA sequencing, and proteomic analysis [10, 21, 22]. The *NBEAL2* gene has 54 exons and encodes a scaffolding protein containing armadillo-type fold, BEACH (beige and Chédiak–Higashi syndrome), Concavalin A-like lectin, pleckstrin homology, and multiple WD40 domains. These domains are involved in vesicle trafficking, membrane dynamics, and synaptosome formation and are related to LYST and LRBA [9]. *NBEAL2* is expressed in megakaryocytes (MK), platelets, neutrophils, monocytes, and NK cells. To date, 86 different *NBEAL2* variants have been recognized in 69 GPS cases or families described. Genetic abnormalities in GPS include missense, nonsense, frameshift, splicing, and small indel variants, which are distributed along the gene [23]. Causative mutations in *NBEAL2* thus account for autosomal recessive GPS (Fig. 17.1).

Mutations in *GFI1B*, a transcription factor that stimulates megakaryocytic and erythroid proliferation and differentiation, have been described in GPS patients with an autosomal dominant inheritance manner. The genetic defects in *GFI1B* include a frameshift mutation in the fifth zinc finger DNA-binding domain, truncating, and nonsense mutations [24, 25]. Arg216Glu at the location of 759 of *GATA1* gene, an upstream regulator of genes involved in platelet α-granule biogenesis, has been described as a cause of X-linked forms of GPS [26].

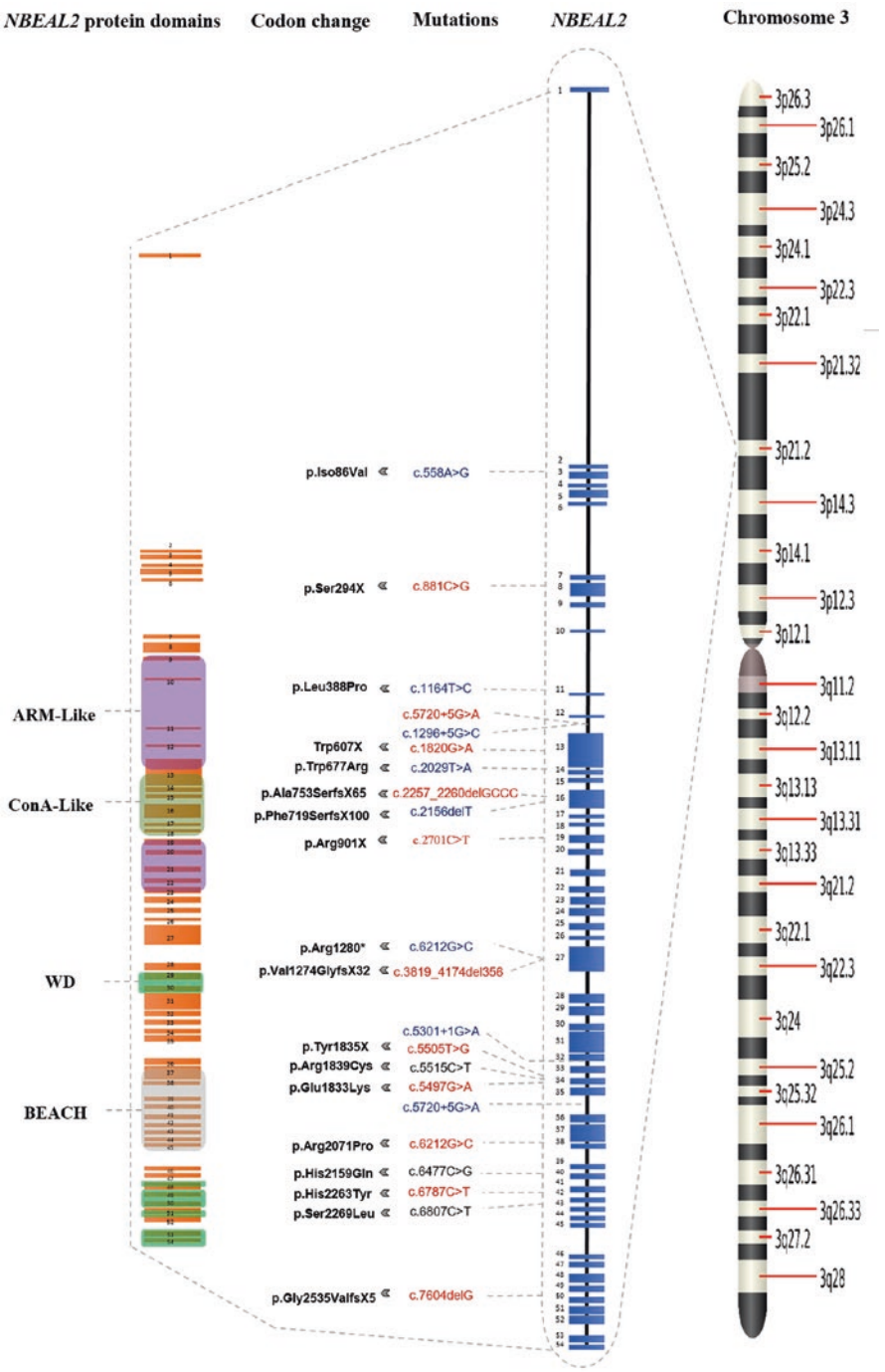

Fig. 17.1 Mutations in the *NBEAL2* gene that have been identified in patients with GPS. *GPS* gray platelet syndrome. Adopted from "Shahraki H, Dorgalaleh A, Bain BJ. Gray platelet Syndrome (GPS). Congenital Bleeding Disorders: Springer; 2018. p. 379–96" with permission

17.4 Laboratory Diagnosis

Haemostasis (ISTH), a diagnostic workup of GPS, like any other inherited platelet function disorder (IPFD), is classified as first-, second-, and third-step laboratory tests (Fig. 17.2). Depending on the available facilities, genetic analysis rather than TEM may be an appropriate third step.

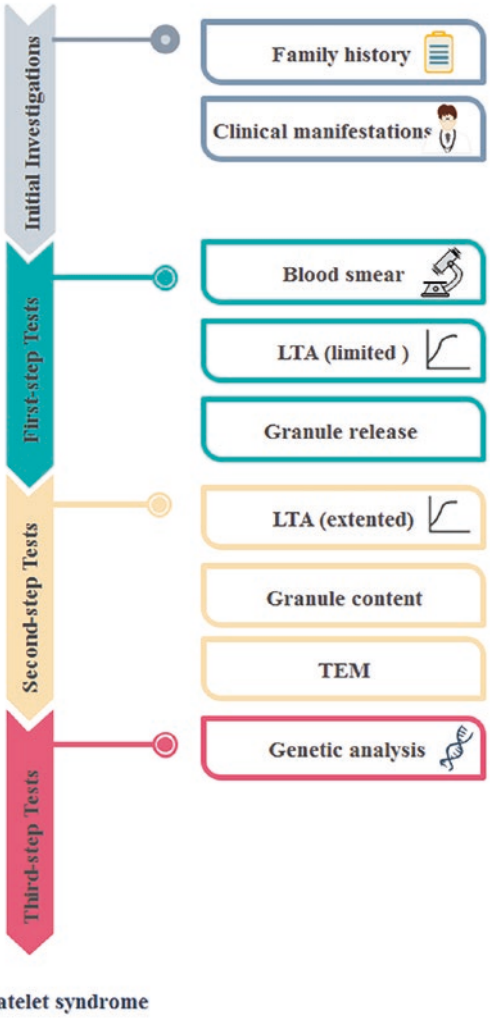

Fig. 17.2 Diagnostic workup of gray platelet syndrome that is classified as first-, second-, and third-step laboratory tests. *LTA* light transmission aggregometry; *TEM* transmission electron microscopy

17.4.1 Primary Tests

17.4.1.1 Platelet Count and Peripheral Blood Smear

A low platelet count can occur in inherited and acquired conditions and should be distinguished from pseudo-thrombocytopenia resulting from ethylene diamine tetra-acetic acid (EDTA)-induced platelet aggregation or from platelet satellitism. In all IPFDs, the count and morphology of the peripheral blood platelets should be evaluated for size, staining characteristics, and granularity using a Wright–Giemsa- or May–Grünwald–Giemsa-stained peripheral blood smear before requesting further diagnostic tests. In the presence of large platelets, the platelet count must be verified from the blood smear. In suspected GPS, it is also necessary to exclude a pseudo-GPS due to in vitro degranulation and swelling of platelets [38]. Mean platelet volume (MPV) and platelet distribution width are important diagnostic parameters that provide further information regarding platelet defects. An increased MPV can indicate increased platelet turnover, but this is not specific. MPV may be pathologically augmented in inherited macrothrombocytopenic disorders such as May–Hegglin anomaly, Bernard–Soulier syndrome, and GPS, as well as in myeloproliferative neoplasms, such as essential thrombocythemia, chronic myeloid leukemia, primary myelofibrosis, and polycythemia vera. Conversely, the MPV may be reduced in some patients with marrow failure and in Wiskott–Aldrich syndrome. In individuals with GPS, the number of platelets is moderately reduced ($30–100 \times 10^9$/L), and the MPV is markedly increased (mean 13 fL). The blood film demonstrates thrombocytopenia with large platelets that seem gray and either are agranular or show reduced granules (Fig. 17.3). Vacuolated platelets and poorly granulated polymorphonuclear neutrophils are found in some patients with GPS [39].

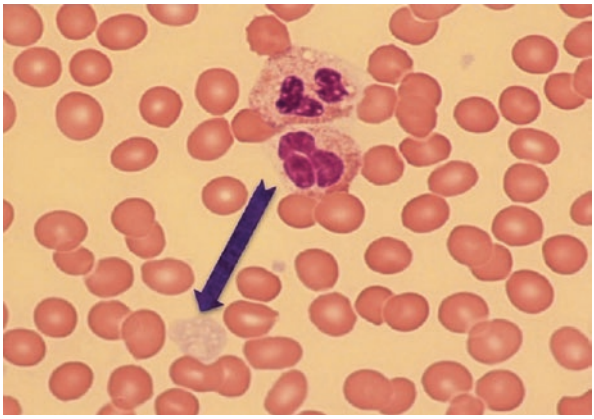

Fig. 17.3 Blood film of a patient with gray platelet syndrome. A giant agranular gray platelet is indicated by the arrow. Adopted from "Shahraki H, Dorgalaleh A, Bain BJ. Gray platelet Syndrome (GPS). Congenital Bleeding Disorders: Springer; 2018. p. 379–96" with permission

17.4.1.2 Routine Coagulation Tests (Activated Partial Thromboplastin Time, Prothrombin Time, Thrombin Time, and Bleeding Time)

Since there is no coagulation factor abnormality in GPS, the prothrombin time, activated partial thromboplastin time, and thrombin time are all normal.

The template bleeding time (BT) can be used as a screening test for the assessment of in vivo platelet function. It is based on evaluating the time that is required for platelets to produce a hemostatic plug within vessels in injured skin, leading to the cessation of hemorrhage. In GPS patients, the bleeding time is prolonged [40]. Clot retraction and the FA-100 test are abnormal [6]. Thrombocytopenia may be corrected by splenectomy, but the platelet defect persists and the BT may still be prolonged. DDAVP decreases the BT in GPS patients who have no detectable levels of platelet vWF but have a normal plasma pool.

17.4.2 BM Analysis

BM study in GPS patients demonstrates α-granule deficiency in megakaryocytes, intense reticulin and collagen fibrosis, and emperipolesis. Additionally, the number and maturation of MKs generally appear otherwise normal in marrow biopsies of GPS patients [6].

17.4.3 Myelofibrosis

The observed myelofibrosis probably results from the constant release of MK growth factors and cytokines such as PDGF, TGF-β, ECGF, and VEGF into the extracellular BM space. Moreover, the leakage of PF4, an inhibitor of collagenase, into the BM environment may accelerate collagen accumulation, contributing to myelofibrosis [6, 41].

17.4.3.1 Emperipolesis

Emperipolesis is an unusual biological process in which a cell engulfs another viable cell. Megakaryocytic emperipolesis, particularly the presence of polymorphonuclear neutrophils within the cytoplasm of megakaryocytes, is observed in less than 2% of BM MKs in healthy people. However, it has been shown in about 38–65% of the MKs of GPS patients with both *NBEAL2* and *GFI1B* mutations [42, 43]. Although the exact mechanism of megakaryocytic emperipolesis in GPS patients is still unidentified, a few studies have demonstrated that the mislocalization or increase of P-selectin on the megakaryocyte membrane can promote neutrophil–megakaryocyte interactions through P-selectin glycoprotein ligand 1 (PSGL-1), leading to neutrophil sequestration within the megakaryocyte cytoplasm. Neutrophil sequestration within the MK cytoplasm leads to the release of fibrogenic megakaryocyte cytokines such as PDGF and TGF-β, as well as neutrophil proteases, into the microenvironment with development of myelofibrosis, similar to mechanisms

hypothesized for primary myelofibrosis. Furthermore, in some mouse model studies, it has been demonstrated that the mutations in the *GATA1* gene prompt megakaryocytic emperipolesis and resultant myelofibrosis [6, 44, 45].

17.4.4 Electron Microscopy

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are electron microscopy (EM) techniques that are widely used for imaging the ultrastructure of cellular components (Box 17.1).

Box 17.1 The Principle of TEM

The first TEM was done by Albert Prebus and James Hiller at the University of Toronto in 1938. The global design of a transmission electron microscope is similar to that of a light microscope, except that light is replaced by electrons and the glass lenses are replaced by electromagnetic lenses. The design of a scanning electron microscope is similar to that of a confocal laser scanning microscope. Electron microscopes have a higher resolution than optical microscopes, approximately 0.5 nm in TEM and 1 nm in SEM allowing the imaging of cellular ultrastructure. The resolution of electron microscopes follows the law of the light microscope (Ernst Abbé law) and depends on the wavelength of electrons and the numerical aperture. EM uses a high vacuum (10^{-7} to 10^{-10} mbar) to inhibit oxidation or burning of the warmed tungsten tip [46, 47].

Advantages of electron microscopes: Electron microscopes have a higher resolving power than optical microscopes. Additionally, electron microscopes can create much higher magnification images than light microscopes because of the electromagnetic lenses. Scanning electron microscopes can display a three-dimensional image and show the surface structure of the specimen with atomic-scale resolution [48].

Disadvantages of electron microscopes: Because EM applies a high vacuum to create the image, it is impossible to visualize living specimens. The preparation procedure of the biological specimen and the requirement for skilled interpretation of the images obtained are the most important and challenging parts of electron microscopy. In every step of specimen preparation, artifacts can be introduced into the specimen. Many of these artifacts can be minimized by using cryo techniques for both TEM and SEM. In addition, specimen preparation for electron microscopy is often a very time-consuming process [49, 50].

For platelet transmission electron microscopy (PTEM), a whole blood sample is collected into a tube containing an acid–citrate–dextrose solution with a ratio of 9 parts blood to 1 part anticoagulant and pH 6.5, and then the citrated platelet-rich

plasma (C-PRP) is separated and stored at room temperature (RT). Also, a C-PRP sample for PTEM must be transported at RT because improper transportation can cause a false reduction of dense granule counts. Additionally, storage of C-PRP at a low temperature (2–4 °C) causes discoid shape change in platelets. EDTA is an unsuitable anticoagulant for PTEM because it affects the platelet membranes leading to the loss of the platelet's discoid shape. However, in some conditions, EDTA is added to the plasma to maximally chelate calcium in plasma, and in these conditions, platelets preserve their discoid shape only for about 30 min. Also, it is preferable that the pH of the sample is maintained by placing the C-PRP in an environment of 95% O_2 and 5% CO_2 to prevent the platelet shape change [51, 52]. A frozen specimen is not suitable for platelet TEM. PTEM is a sensitive technique for the identification of the ultrastructure of platelets in genetic disorders such as GPS. In fact, TEM can be regarded as the gold standard method for the determination of the platelet's specific morphology and confirmation of a GPS diagnosis [41, 53]. TEM studies of GPS platelets demonstrate the absence or marked deficiency of the α-granules in megakaryocytes and platelets, although they have primitive α-granule precursors. Thus, the underlying defect in GPS is the inability of megakaryocytes to package their ingeniously synthesized secretory proteins into α-granules, which leads to the loss or reduction of α-granules and thus the appearance of hypogranular and gray platelets [8]. Additionally, the morphology of platelets in GPS patients is very variable, and marked anisocytosis (size variation) is observed in PTEM. Smaller platelets usually are discoid in form, while larger platelets are more spherical and vacuolated. Vacuoles may indicate dilated parts of the open-surface connected canalicular system or empty precursors of α-granules that have released their contents into the BM space during the maturation of megakaryocytes. Platelet-dense bodies, mitochondria, peroxisomes, Golgi apparatus, and lysosomes are usually normal. However, some studies have shown a mild reduction in dense bodies [6, 8, 27].

17.4.5 Light Transmission Aggregometry

Light transmission aggregometry (LTA) is considered to be the gold standard technique for the assessment of platelet function in bleeding diatheses. The patient's platelet-rich plasma sample and a standard panel of agonists, including collagen, adenosine diphosphate (ADP), arachidonic acid, epinephrine, ristocetin, and thrombin receptor-activating peptide (TRAP), are generally utilized for this assay [54]. (For more information about aggregometry, refer to Chap. 15).

In GPS patients, the responses to platelet function testing are highly variable between patients. The platelets aggregate with ADP, arachidonic acid, epinephrine, and ristocetin in most patients but fail to respond to collagen, thrombin (or TRAP), and ADP in some patients [6, 55].

17.4.6 Analysis of Platelet Lysate

Assessment of platelet α-granule contents such as PF4, β thromboglobulin (β-TG), fibronectin, fibrinogen, and growth factors such as PDGF and TGF-β1 by enzyme-linked immunosorbent assay (ELISA) or luminometry (firefly technique) either in whole platelet lysates (PLs) or in platelet releasates can be applied as a part of GPS diagnosis. A PL can be prepared by several methods, such as repeated freeze-thawing, sonication, or the use of the detergent Triton X-100. The levels of all these proteins in PLs are dramatically reduced and in turn increased in plasma [6, 56, 57]. Thrombospondin 1 (TSP) protein is another platelet α-granule content that is secreted at sites of platelet activation and aggregation and is implicated in leukocyte, fibroblast, and endothelial cell differentiation. TSP levels can be assessed by a western blot technique; a reduced level of TSP was observed by western blot analysis in GPS patients [6].

17.4.7 Platelet Secretion Assays

More than 90% of all inherited platelet disorders are attributed to defects of platelet secretion and therefore, in the diagnosis of patients with bleeding manifestations, assessment of platelet granule release is of special importance [58]. Secretion of platelet granules is dependent on a signaling pathway, which is initiated by the interaction of an agonist with its specific receptor on the platelet surface. This pathway leads to alterations in the levels of intracellular second messengers such as Ca^{2+} ions, inositol triphosphate, and diacylglycerol. These alterations result in remodeling of the platelet cytoskeleton and subsequently granules fuse with the platelet surface membrane and release their contents. If any step of this process is impaired, abnormal results from platelet secretion assays will be obtained.

Several laboratory methods have been established for the assessment of platelet granule release. These methods may investigate platelet secretion as part of platelet response to agonists or may be restricted to specific granule components.

17.4.7.1 Laboratory Assays for α-Granule Release

Platelet α-granules contain a wide variety of components, which may be soluble in the granule or be bound to the granule membrane. For evaluating α-granule secretion, soluble components, preferably PF4 and β-TG, which have a higher specificity for platelets, can be measured following platelet activation. The fusion of granule membrane with platelet plasma membrane during granule secretion leads to the exposure of granule membrane-bound proteins such as P-selectin, which can then be assessed as an alternative marker of granule release [58].

Measurement of PF4 and β-TG

PF4 and β-TG belong to the CXC chemokine family and are exclusively produced in megakaryocytes. These proteins are stored in platelet α-granules at concentrations of >0.5 μg/10^8 platelets and are secreted following platelet activation;

therefore, they are considered to be helpful indicators for evaluating platelet α-granule release. Radioimmunoassay is the traditional method for measuring PF4 and β-TG [59], but it is no longer performed. Currently, an ELISA technique is routinely used for this purpose [60]. This assay is a sandwich method that takes advantage of two antigen-specific antibodies. The first antibody is coated on the plates and is called the capture antibody. The second antibody, which is conjugated and known as the detection antibody, is provided as a reagent and will be added to the plates after the addition of the sample. Several ELISA kits are commercially available for quantitation of PF4 or β-TG.

Assessment of Platelet Surface P-Selectin

P-selectin is a membrane protein of platelet α-granules, and if present on the surface of platelets, it can be suggestive of α-granule release. P-selectin is also present in a soluble form, which may be derived from platelet membranes, endothelial cells, or other tissues and is thus not specific for platelets. Assessment of platelet surface P-selectin is performed by flow cytometry using a P-selectin-specific monoclonal antibody. It is usually a semiquantitative method in which the proportion of platelets that are positive for surface P-selectin is measured before and after the addition of an agonist and compared to each other. This assay is a sensitive and cost-effective method but requires trained staff [58].

17.4.8 Serum Vitamin B12 Assessment

Vitamin B12 is a water-soluble vitamin and is necessary for erythropoiesis, DNA replication, and nerve function. B12 deficiency is characteristic of pernicious anemia, while high levels of B12 may be due to liver or renal disease or certain types of leukemia. Also, serum vitamin B12 concentration is increased significantly in patients with GPS. Therefore, the measurement of serum B12 levels is sometimes useful. There are different methods to measure the vitamin B12 concentration. Some of these techniques are utilized in the medical field and others in pharmacological, industrial, and basic science studies. Choosing the suitable technique for B12 measurement depends on the kind of sample, aim of the assay, time limitation, cost, specificity, and sensitivity of the test [8, 61, 62].

17.4.9 Genetic Analysis

Because of the presence of different genetic variants with an identical phenotype, exact diagnosis of bleeding disorders can be challenging and requires suitable molecular and genetic testing. Molecular investigations provide much information regarding the disease inheritance pattern, which in turn permits us to screen the members of a family, including those who are apparently unaffected and tell us which patients require nonhematological evaluations. It can also influence the patient's clinical management and may provide some information about the

prognosis of a disorder. There are many molecular and genetic tests, such as proteomic studies, autozygosity mapping, SNP array, genome-wide linkage analysis, Sanger sequencing analysis, and next-generation sequencing (NGS) technology, for definitive diagnosis of genetic disorders, but choosing an appropriate, fast, and cost-effective test with high specificity and sensitivity is essential [63, 64]. Autozygosity mapping is utilized for the recognition of the disorder-causing mutations with an autosomal recessive inheritance in relatives, while SNP analysis is the assessment of a single base-pair variation in the DNA sequence of individuals in a population and, if limited to zones of autozygosity, immediately rules out many of the novel variants and accelerates characterizing causative genes. Sanger sequencing is an expensive and time-consuming technique for DNA sequencing for the determination of the sequence of plentiful, relatively small fragments of human DNA. NGS is a very powerful approach based on the wide-scale, simultaneous identification of novel or known causative mutations associated with disease. This technique significantly diminishes the time and cost required in comparison with Sanger sequencing as well as improving the rate of detection of the underlying genetic cause in consanguineous families. Furthermore, NGS allows us to analyze a candidate panel of genes, the whole-exome/coding regions of the genome (WES), or the whole genome (WGS) in suspected genetic disorders. Sequencing of a desired panel of genes in a condition with a clear phenotype is cheaper than sequencing of the whole exome or genome, but because of the occurrence of most disease-causing mutations within the coding regions of the genome and because of the complexity of the WGS results, WES is generally preferred [64–66].

The advantage of NGS in the diagnosis of GPS was demonstrated in 2011 by sequencing the exome of *NBEAL2* gene, a member of a gene family implicated in platelet granule biogenesis, as the causative gene for autosomal recessive GPS. Currently, NGS is considered an accurate and reliable method to scrutinize the *NBEAL2* gene in suspected GPS patients with an autosomal recessive inheritance [22, 65, 67]. The biological importance of *NBEAL2* gene is verified by silencing both alleles of *NBEAL2* in zebrafish embryos leading to complete abrogation of platelet production. Also, using linkage analysis and homozygosity mapping, it has been demonstrated that the GPS-causing mutations occur within a significant interval of 9.4 megabases on chromosome 3p21.1–3p22.1. Altogether, these findings represent the essential role of the *NBEAL2* protein in thrombocyte production and the etiology of autosomal recessive GPS [22]. *NBEAL2* level is increased during megakaryocyte maturation and is implicated in vesicle trafficking, apoptosis, and platelet α-granule formation [21, 22, 65].

X-linked GPS was first reported by Tubman et al. in a family as a consequence of a G-to-A transition (R216Q mutation) at location 759 within exon 4 of the *GATA1* gene, an upstream regulator of the gene that is implicated in the development of the platelet α-granules [67]. First, the same group with the utilization of the genome-wide linkage analysis detected the GPS-causing gene located in the 63 cM on the X chromosome between markers G1057 and DXS6797 within the *GATA1* gene. Moreover, in 2017, Wijgaerts et al., with comparison of platelet size and the number of α-granules in two *NBEAL2*- and two *GATA1*-deficient patients and mice with a

GATA1 defect, revealed a decreased number of α-granules in all groups and noted that platelets of two patients and the *GATA1*-deficient mice showed almost no expression of the *NBEAL2* gene. Hence, they concluded that a reduced number of α-granules could be due to dysregulation of *NBEAL2*. In addition, chromatin immunoprecipitation sequencing has disclosed that a regulatory region 31 kilobase upstream of *NBEAL2* has five binding sites for *GATA1*. Based on all these findings, Wijgaerts et al. concluded that transcription factor *GATA1* regulates the expression of *NBEAL2* gene via a long-distance enhancer [68].

A nonsense mutation (C859T) in the growth factor independent 1B (*GFI1B*) gene, the gene encoding the transcription factor, accounted for an autosomal dominant form of GPS in one family [25]. Subsequently, other families have been described. For example, a heterozygous 1 bp insertion in exon 7 of the *GFI1B* identified by Stevenson et al. in affected members of a large family with a bleeding disorder and reduced levels of the α-granule-related proteins revealed by EM, and a nonsense mutation (A793T) at zinc finger domain 4 of the *GFI1B* gene characterized by Ferreira et al. in a 13-year boy with αδ storage pool deficiency [24, 69, 70]. The *GFI1B* gene, located on chromosome 9q34.13, distal to *ABL1*, encodes a transcriptional repressor that has been involved in megakaryopoiesis and platelet production [25]. In 2017, Wijgaerts et al. suggested that the term GPS only be used for patients with an *NBEAL2* mutation and not for *GATA1* and *GFI1B* defects. They suggested that in spite of the existence of significant similarities (e.g., large platelets with a lack of α-granules, platelet dysfunction, and marrow fibrosis) between *NBEAL2*, *GATA1*, and *GFI1B* patients, there are important differences such as erythrocyte abnormalities that are only observed in patients with *GATA1* and *GFI1B* defects and the presence of CD34-positive platelets in *GFI1B* patients [68].

Alpha granule deficiency and gray platelets can also be observed in some other conditions such as arthrogryposis, renal dysfunction, and cholestasis syndrome (ARCS). ARCS is an unusual autosomal recessive disease frequently characterized by various and rare manifestations such as hypotonia-related arthrogryposis, renal tubular acidosis, and neonatal cholestatic jaundice. Additional clinical presentations of ARCS include nephrocalcinosis, ichthyosis, lower limb congenital anomaly, hypothyroidism, liver failure, bleeding tendency, and platelet dysfunction. ARCS is caused by mutations in *VPS33B* (ARCS1) at 15q26.1 and *VIPAS39* (encoding *VPS16B*) at 14q24.3 (ARCS2). *VSP33B* and *VPS16B* genes encode proteins that are involved in intracellular vesicle trafficking and platelet α-granule formation [71–74]. Electron microscope images from patients with ARCS show a lack of platelet α-granules and platelets appear pale and large in a peripheral blood smear [74].

Turro et al. identified a dominant gain-of-function mutation in universal tyrosine SRC in nine cases. This mutation leads to thrombocytopenia, a bleeding tendency, platelet dysfunction with abnormal granules, BM fibrosis, and bone pathologies. Using genome sequencing and Human Phenotype Ontology patient coding, they demonstrated that the E527K substitution in SRC's kinase domain accounts for this new syndrome. The blood film from these patients showed a decreased number of platelets and the presence of platelets with heterogeneous size and GPS-like phenotype (about 10–30%), and BM biopsy showed an increase in MKs, trilineage

Table 17.2 Conditions that can be associated with gray platelets

Disease	Inheritance	Mutation	Platelet features	Additional clinical signs	Reference
Arthrogryphosis, renal dysfunction, and cholestasis (ARC) syndrome	AR	*VPS33B* at 15q26.1 or *VIPAS39* at 14q24.3	Normal platelet count or mild thrombocytopenia, large pale (gray) platelets, decreased α-granule proteins	Joint contractures, renal tubular acidosis, cholestasis, developmental retardation	[71–73]
Thrombocytopenia myelofibrosis and bone abnormalities	AD	Gain-of-function mutation in *SRC* at 20q11.23	10–30% of platelets gray, platelets vary in size from smaller to larger than normal	Facial dysmorphism, abnormal bones, loss of teeth, myelofibrosis and splenomegaly	[75, 76]

dysplasia, and myelofibrosis. In addition, the MPL levels were normal in SRC-mutant cases with myelofibrosis, but their plasma thrombopoietin levels were elevated [75] (Table 17.2).

17.4.10 Management of Patients with GPS

There is no specific treatment for GPS patients, but the best management is medical supportive care and on-demand management. Patients should be evaluated regularly for thrombocytopenia and should avoid drugs interfering with platelet function, particularly aspirin and nonsteroidal anti-inflammatory medications. Platelet transfusion can be beneficial preoperatively or to treat active bleeding but is associated with the risk of alloimmunization. In this condition, if possible, HLA-matched donor platelets should be used. DDAVP, a synthetic analog of the antidiuretic hormone vasopressin, which improves bleeding time and clotting, is an appropriate choice in preparation for surgery or invasive procedures. Except in patients with marked splenomegaly, splenectomy does not appear to be useful in GPS [6, 11–13]. Hematopoietic stem cell transplantation can be a curative treatment for GPS patients with pancytopenia and BM fibrosis [14].

17.5 Concluding Remarks

GPS is an IPFD associated with α-granule deficiency, which is due to mutations in the *NBEAL2*, *GFI1B*, or *GATA1* genes. Diagnostic characteristics are macrothrombocytopenia and the presence of gray platelets, BM fibrosis, and MK emperipolesis of neutrophils on BM examination, splenomegaly, and reduced platelet aggregation with a range of agonists including collagen and thrombin, decreased concentration

of platelet α-granule-related proteins, and increased serum vitamin B12. In addition, TEM studies display large platelets with few or no α granules, and the usual number of dense bodies, mitochondria, lysosomes, and peroxisomes. Finally, molecular analysis of the underlying genes is also useful.

Acknowledgment None declared.

Conflict of Interest None declared.

References

1. Flaumenhaft R. Platelet secretion. Platelets (third edition). Elsevier; 2013. p. 343–66.
2. Steinberg-Shemer O, Tamary H. Gray platelet syndrome mimicking atypical autoimmune lymphoproliferative syndrome: the key is in the blood smear. Blood. 2018;131(24):2737.
3. Rensing-Ehl A, Pannicke U, Zimmermann S-Y, Lorenz MR, Neven B, Fuchs I, et al. Gray platelet syndrome can mimic autoimmune lymphoproliferative syndrome. Blood. 2015;126(16):1967–9.
4. Sims MC, Mayer L, Collins JH, Bariana TK, Megy K, Lavenu-Bombled C, et al. Novel manifestations of immune dysregulation and granule defects in gray platelet syndrome. Blood. 2020;136(17):1956–67.
5. Glembotsky AC, De Luca G, Heller PG. A deep dive into the pathology of gray platelet syndrome: new insights on immune dysregulation. J Blood Med. 2021;12:719.
6. Nurden AT, Nurden P. The gray platelet syndrome: clinical spectrum of the disease. Blood Rev. 2007;21(1):21–36.
7. Ma AD, Key NS. Molecular basis of hemostatic and thrombotic diseases. Elsevier; 2009. p. 247–64.
8. Gunay-Aygun M, Zivony-Elboum Y, Gumruk F, Geiger D, Cetin M, Khayat M, et al. Gray platelet syndrome: natural history of a large patient cohort and locus assignment to chromosome 3p. Blood. 2010;116:4990–5001.
9. Cullinane AR, Schäffer AA, Huizing M. The BEACH is hot: a LYST of emerging roles for BEACH-domain containing proteins in human disease. Traffic. 2013;14(7):749–66.
10. Gunay-Aygun M, Falik-Zaccai TC, Vilboux T, Zivony-Elboum Y, Gumruk F, Cetin M, et al. NBEAL2 is mutated in gray platelet syndrome and is required for biogenesis of platelet α-granules. Nat Genet. 2011;43(8):732–4.
11. Pfueller SL, Howard MA, White JG, Menon C, Berry EW. Shortening of bleeding time by 1-deamino-8-arginine vasopressin (DDAVP) in the absence of platelet von Willebrand factor in gray platelet syndrome. Thromb Haemost. 1987;58(08):1060–3.
12. Nurden A, Nurden P. Inherited disorders of platelet function: selected updates. J Thromb Haemost. 2015;13:S2–9.
13. Gresele P, Bury L, Falcinelli E, editors. Inherited platelet function disorders: algorithms for phenotypic and genetic investigation. Seminars in thrombosis and hemostasis. Thieme Medical Publishers; 2016.
14. Favier R, Roussel X, Audia S, Bordet JC, De Maistre E, Hirsch P, et al. Correction of severe myelofibrosis, impaired platelet functions and abnormalities in a patient with gray platelet syndrome successfully treated by stem cell transplantation. Platelets. 2020;31(4):536–40.
15. Caen J, Deschamps J, Bodevin E, Bryckaert M, Dupuy E, Wasteson A. Megakaryocytes and myelofibrosis in gray platelet syndrome. Nouv Rev Fr Hematol (1978). 1987;29(2):109–14.
16. Hyman T, Huizing M, Blumberg PM, Falik-Zaccai TC, Anikster Y, Gahl WA. Use of a cDNA microarray to determine molecular mechanisms involved in grey platelet syndrome. Br J Haematol. 2003;122(1):142–9.

17. Breton-Gorius J, Vainchenker W, Nurden A, Levy-Toledano S, Caen J. Defective alpha-granule production in megakaryocytes from gray platelet syndrome: ultrastructural studies of bone marrow cells and megakaryocytes growing in culture from blood precursors. Am J Pathol. 1981;102(1):10.
18. Jantunen E, Hänninen A, Naukkarinen A, Vornanen M, Lahtinen R. Gray platelet syndrome with splenomegaly and signs of extramedullary hematopoiesis: a case report with review of the literature. Am J Hematol. 1994;46(3):218–24.
19. Raccuglia G. Gray platelet syndrome: a variety of qualitative platelet disorder. Am J Med. 1971;51(6):818–28.
20. Fabbro S, Kahr WH, Hinckley J, Wang K, Moseley J, Ryu G-Y, et al. Homozygosity mapping with SNP arrays confirms 3p21 as a recessive locus for gray platelet syndrome and narrows the interval significantly. Blood. 2011;117(12):3430–4.
21. Kahr WH, Hinckley J, Li L, Schwertz H, Christensen H, Rowley JW, et al. Mutations in NBEAL2, encoding a BEACH protein, cause gray platelet syndrome. Nat Genet. 2011;43(8):738–40.
22. Albers CA, Cvejic A, Favier R, Bouwmans EE, Alessi M-C, Bertone P, et al. Exome sequencing identifies NBEAL2 as the causative gene for gray platelet syndrome. Nat Genet. 2011;43(8):735–7.
23. Pluthero FG, Di Paola J, Carcao MD, Kahr WH. NBEAL2 mutations and bleeding in patients with gray platelet syndrome. Platelets. 2018;29(6):632–5.
24. Stevenson W, Morel-Kopp MC, Chen Q, Liang H, Bromhead C, Wright S, et al. GFI 1B mutation causes a bleeding disorder with abnormal platelet function. J Thromb Haemost. 2013;11(11):2039–47.
25. Monteferrario D, Bolar NA, Marneth AE, Hebeda KM, Bergevoet SM, Veenstra H, et al. A dominant-negative GFI1B mutation in the gray platelet syndrome. N Engl J Med. 2014;370(3):245–53.
26. VeN T, Levine JE, Campagna DR, Monahan-Earley R, Dvorak AM, Neufeld EJ, et al. X-linked gray platelet syndrome due to a GATA1 Arg216Gln mutation. Blood. 2007;109(8):3297–9.
27. Mori K, Suzuki S, Sugai K. Electron microscopic and functional studies on platelets in gray platelet syndrome. Tohoku J Exp Med. 1984;143(3):261–87.
28. Benit L, Cramer E, Masse J, Dusanter-Fourt I, Favier R. Molecular study of the hematopoietic zinc finger gene in three unrelated families with gray platelet syndrome. J Thromb Haemost. 2005;3(9):2077–80.
29. De Candia E, Pecci A, Ciabattoni G, De Cristofaro R, Rutella S, Yao-Wu Z, et al. Defective platelet responsiveness to thrombin and protease-activated receptors agonists in a novel case of gray platelet syndrome: correlation between the platelet defect and the α-granule content in the patient and four relatives. J Thromb Haemost. 2007;5(3):551–9.
30. Deppermann C, Cherpokova D, Nurden P, Schulz J-N, Thielmann I, Kraft P, et al. Gray platelet syndrome and defective thrombo-inflammation in Nbeal2-deficient mice. J Clin Invest. 2013;123(8):3331–42.
31. Van der Reijden BA, Monteferrario D, Bolar N, Marneth A, Hebeda K, Bergevoet S, et al. A dominant-negative GFI1B mutation in gray platelet syndrome. Washington, DC: American Society of Hematology; 2013.
32. Guerrero JA, Bennett C, van der Weyden L, McKinney H, Chin M, Nurden P, et al. Gray platelet syndrome: proinflammatory megakaryocytes and α-granule loss cause myelofibrosis and confer metastasis resistance in mice. Blood. 2014;124(24):3624–35.
33. Di Buduo CA, Alberelli MA, Glembotsky AC, Podda G, Lev PR, Cattaneo M, et al. Abnormal proplatelet formation and emperipolesis in cultured human megakaryocytes from gray platelet syndrome patients. Sci Rep. 2016;6(1):1–11.
34. Tomberg K, Khoriaty R, Westrick RJ, Fairfield HE, Reinholdt LG, Brodsky GL, et al. Spontaneous 8bp deletion in Nbeal2 recapitulates the gray platelet syndrome in mice. PLoS One. 2016;11(3):e0150852.
35. Bottega R, Nicchia E, Alfano C, Glembotsky AC, Pastore A, Bertaggia-Calderara D, et al. Gray platelet syndrome: novel mutations of the NBEAL2 gene. Am J Hematol. 2017;92(2):E20–E2.

36. Cao L, Su J, Li J, Yu Z, Bai X, Wang Z, et al. A novel nonsense NBEAL2 gene mutation causing severe bleeding in a patient with gray platelet syndrome. Platelets. 2018;29(3):288–91.
37. Tariq H, Perez Botero J, Higgins RA, Medina EA. Gray platelet syndrome presenting with pancytopenia, splenomegaly, and bone marrow fibrosis: case report with a novel NBEAL2 mutation. Am J Clin Pathol. 2021;156(2):253–8.
38. Pancione Y, Fumi M, Sale S, Rocco V, Bain BJ. Gray platelets–artifact or real? Am J Hematol. 2016;91(5):538.
39. Jobe SM, Di Paola J. Congenital and acquired disorders of platelet function and number. Consultative hemostasis and thrombosis. Elsevier; 2019. p. 145–66.
40. Paniccia R, Priora R, Liotta AA, Abbate R. Platelet function tests: a comparative review. Vasc Health Risk Manag. 2015;11:133.
41. Martin K, Ma AD, Key NS. Molecular basis of hemostatic and thrombotic diseases. Molecular pathology (second edition). Elsevier; 2018. p. 277–97.
42. Rastogi V, Sharma R, Misra SR, Yadav L, Sharma V. Emperipolesis—a review. J Clin Diagn Res. 2014;8(12):ZM01.
43. Di Buduo CA, Alberelli MA, Glembotsky AC, Podda G, Lev PR, Cattaneo M, et al. Abnormal proplatelet formation and emperipolesis in cultured human megakaryocytes from gray platelet syndrome patients. Sci Rep. 2016;6:23213.
44. Larocca LM, Heller PG, Podda G, Pujol-Moix N, Glembotsky AC, Pecci A, et al. Megakaryocytic emperipolesis and platelet function abnormalities in five patients with gray platelet syndrome. Platelets. 2015;26(8):751–7.
45. Goyal M, Thekkelakayil ST, Gupta A. Megakaryocytic emperipolesis associated with thrombocytopenia: causative or coincidence? Turk J Hematol. 2017;34(4):370.
46. Sigle W. Analytical transmission electron microscopy. Annu Rev Mater Res. 2005;35:239–314.
47. Brydson R, Brown A, Benning LG, Livi K. Analytical transmission electron microscopy. Rev Mineral Geochem. 2014;78(1):219–69.
48. Webb A, Kagadis GC. Introduction to biomedical imaging. Med Phys. 2003;30(8):2267.
49. Raghavendra P, Pullaiah T. Advances in cell and molecular diagnostics. Academic Press; 2018.
50. Ma H, Shieh K-J, Qiao TX, Cherng S. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Nat Sci. 2006:14.
51. White JG. Electron microscopy methods for studying platelet structure and function. Platelets and megakaryocytes. Springer; 2004. p. 47–63.
52. Pretorius E, Oberholzer HM, van der Spuy WJ, Meiring JH. Macrothrombocytopenia: investigating the ultrastructure of platelets and fibrin networks using scanning and transmission electron microscopy. Ultrastruct Pathol. 2009;33(5):216–21.
53. Perez Botero J, Warad DM, He R, Uhl CB, Tian S, Otteson GE, et al. Comprehensive platelet phenotypic laboratory testing and bleeding history scoring for diagnosis of suspected hereditary platelet disorders: a single-institution experience. Am J Clin Pathol. 2017;148(1):23–32.
54. Hvas A-M, Favaloro EJ. Platelet function analyzed by light transmission aggregometry. Hemostasis and thrombosis. Springer; 2017. p. 321–31.
55. Rogers HJ, Nakashima MO, Kottke-Marchant K. Hemostasis and thrombosis. Hematopathology (third edition). Elsevier; 2019. p. 57–105. e4.
56. Kirchmaier CM, Pillitteri D. Diagnosis and management of inherited platelet disorders. Transfus Med Hemother. 2010;37(5):237–46.
57. Kamath S, Blann AD, Caine GJ, Gurney D, Chin BS, Lip GY. Platelet P-selectin levels in relation to plasma soluble P-selectin and β-thromboglobulin levels in atrial fibrillation. Stroke. 2002;33(5):1237–42.
58. Mumford AD, Frelinger AL III, Gachet C, Gresele P, Noris P, Harrison P, et al. A review of platelet secretion assays for the diagnosis of inherited platelet secretion disorders. Thromb Haemost. 2015;114(01):14–25.
59. Kaplan KL, Nossel HL, Drillings M, Lesznik G. Radioimmunoassay of platelet factor 4 and β-thromboglobulin: development and application to studies of platelet release in relation to fibrinopeptide a generation. Br J Haematol. 1978;39(1):129–46.

60. Schraw T, Whiteheart S. The development of a quantitative enzyme-linked immunosorbent assay to detect human platelet factor 4. Transfusion (Paris). 2005;45(5):717–24.
61. Karmi O, Zayed A, Baraghethi S, Qadi M, Ghanem R. Measurement of vitamin B12 concentration: a review on available methods. IIOAB J. 2011;2(2):23–32.
62. Tsiminis G, Schartner EP, Brooks JL, Hutchinson MR. Measuring and tracking vitamin B12: a review of current methods with a focus on optical spectroscopy. Appl Spectrosc Rev. 2017;52(5):439–55.
63. Sivapalaratnam S, Collins J, Gomez K. Diagnosis of inherited bleeding disorders in the genomic era. Br J Haematol. 2017;179(3):363–76.
64. Watson S, Lowe G, Lordkipanidze M, Morgan N, consortium G. Genotyping and phenotyping of platelet function disorders. J Thromb Haemost. 2013;11:351–63.
65. Leo VC. Next-generation sequencing in the study of platelets. In: Platelets in thrombotic and non-thrombotic disorders. Springer; 2017. p. 699–714.
66. Morozova O, Marra MA. Applications of next-generation sequencing technologies in functional genomics. Genomics. 2008;92(5):255–64.
67. Tubman VN, Levine JE, Campagna DR, Monahan-Earley R, Dvorak AM, Neufeld EJ, et al. X-linked gray platelet syndrome due to a GATA1 Arg216Gln mutation. Blood. 2007;109(8):3297–9.
68. Wijgaerts A, Wittevrongel C, Thys C, Devos T, Peerlinck K, Tijssen MR, et al. The transcription factor GATA1 regulates NBEAL2 expression through a long-distance enhancer. Haematologica. 2017;102(4):695–706.
69. Ferreira CR, Chen D, Abraham SM, Adams DR, Simon KL, Malicdan MC, et al. Combined alpha-delta platelet storage pool deficiency is associated with mutations in GFI1B. Mol Genet Metab. 2017;120(3):288–94.
70. Anguita E, Candel FJ, Chaparro A, Roldán-Etcheverry JJ. Transcription factor GFI1B in health and disease. Front Oncol. 2017;7:54.
71. Urban D, Li L, Christensen H, Pluthero FG, Chen SZ, Puhacz M, et al. The VPS33B-binding protein VPS16B is required in megakaryocyte and platelet α-granule biogenesis. Blood. 2012;120(25):5032–40.
72. Diz-Küçükkaya R. Inherited platelet disorders including Glanzmann thrombasthenia and Bernard-Soulier syndrome. Hematology. 2013;2013(1):268–75.
73. Chen CH, Lo RW, Urban D, Pluthero FG, Kahr WH. α-Granule biogenesis: from disease to discovery. Platelets. 2017;28(2):147–54.
74. Satomura Y, Bessho K, Nawa N, Kondo H, Ito S, Togawa T, et al. Novel gene mutations in three Japanese patients with ARC syndrome associated mild phenotypes: a case series. J Med Case Rep. 2022;16(1):1–6.
75. Turro E, Greene D, Wijgaerts A, Thys C, Lentaigne C, Bariana TK, et al. A dominant gain-of-function mutation in universal tyrosine kinase SRC causes thrombocytopenia, myelofibrosis, bleeding, and bone pathologies. Sci Transl Med. 2016;8(328):328ra30.
76. Collins J, Astle WJ, Megy K, Mumford AD, Vuckovic D. Advances in understanding the pathogenesis of hereditary macrothrombocytopenia. Br J Haematol. 2021;195(1):25–45.