Congenital Bleeding Disorders: Diagnosis and Management

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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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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].

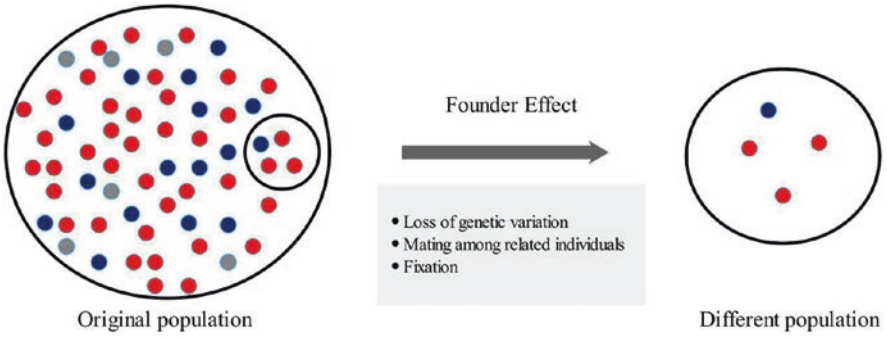

Fig. 2.1 Founder effect. A *founder effect* occurs when a new colony is started by a few members of the original population

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].

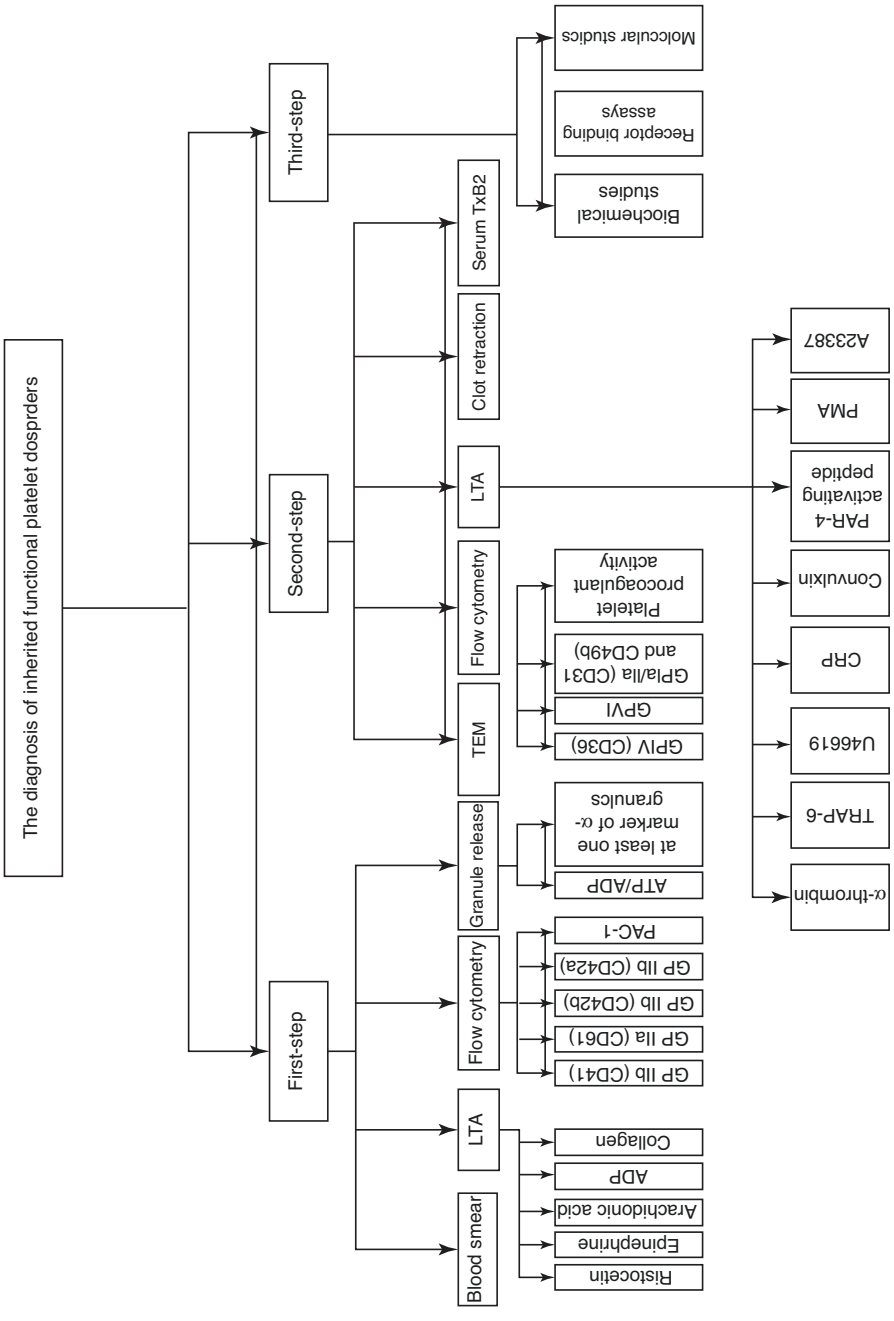

Fig. 2.2 International Society of Thrombosis and Hemostasis (ISTH) suggested algorithm for diagnosis of inherited platelet function [7]

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).

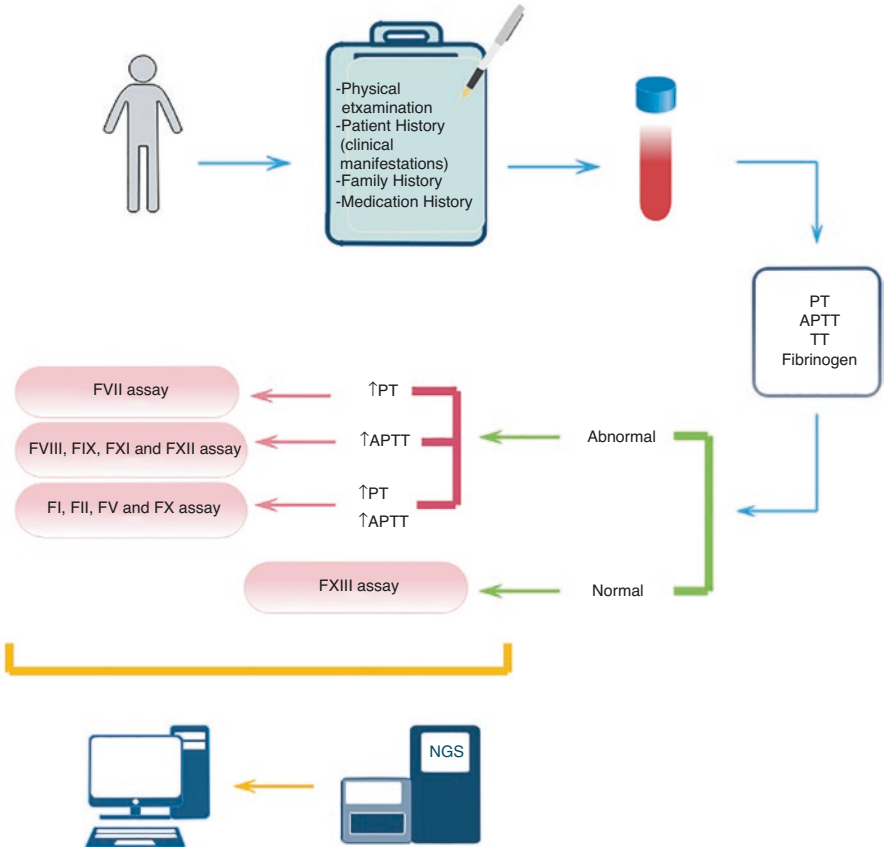

Fig. 2.3 The investigation of patients suspected of having coagulation factors deficiencies [94]. *PT* prothrombin time, *APTT* activated partial thromboplastin time, *TT* thrombin time, *NGS* next-generation sequencing

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].

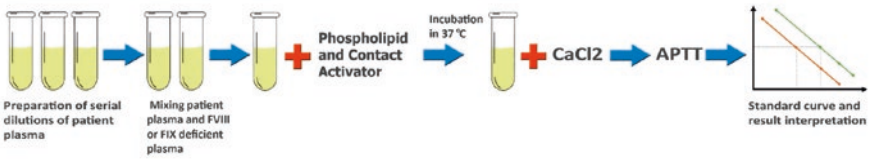

Fig. 2.4 The principles of the one-stage assay based on the activated partial thromboplastin time (APTT)

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).

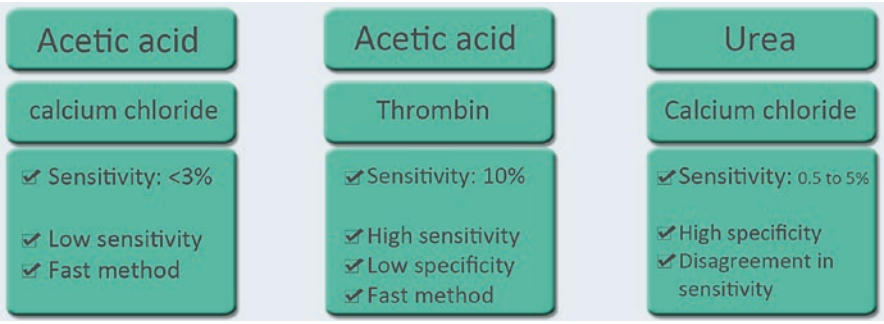

Fig. 2.5 Comparison of different methods of clot solubility testing [4]

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].

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