The Diagnostic Assessment of Inherited Platelet Function Defects

Part 1: An Overview of the Diagnostic Approach and Laboratory Methods

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Abstract

In this article, our goal is to offer an introduction and overview of the diagnostic approach to inherited platelet function defects (iPFDs) for clinicians and laboratory personnel who are beginning to engage in the field. We describe the most commonly used laboratory methods and propose a diagnostic four-step approach, wherein each stage requires a higher level of expertise and more specialized methods. It should be noted that our proposed approach differs from the ISTH Guidance on this topic in some points. The first step in the diagnostic approach of iPFD should be a thorough medical history and clinical examination. We strongly advocate for the use of a validated bleeding score like the ISTH-BAT (International Society on Thrombosis and Haemostasis Bleeding Assessment Tool). External factors like diet and medication have to be considered. The second step should rule out plasmatic bleeding disorders and von Willebrand disease. Once this has been accomplished, the third step consists of a thorough platelet investigation of platelet phenotype and function. Established methods consist of blood smear analysis by light microscopy, light transmission

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aggregometry, and flow cytometry. Additional techniques such as lumiaggregometry, immune fluorescence microscopy, and platelet-dependent thrombin generation help confirm and specify the diagnosis of iPFD. In the fourth and last step, genetic testing can confirm a diagnosis, reveal novel mutations, and allow to compare unclear genetics with lab results. If diagnosis cannot be established through this process, experimental methods such as electron microscopy can give insight into the underlying disease.

Introduction

Platelet function defects (PFDs) affect various platelet responses, ranging from glycoprotein (GP)-mediated adhesion/activation over granule secretion to phospholipid exposure and thrombin generation. Typical inherited PFDs (iPFD) include the Bernard-Soulier syndrome (BSS), Glanzmann thrombasthenia (GT), and various forms of storage pool disease (SPD). Acquired PFDs (aPFD) are more common, often induced by drug intake or medical/disease conditions. In general, PFDs appear heterogeneous in terms of their characteristics and pathogenesis, making a definitive (differential) diagnosis often challenging.[1]

Regarding laboratory diagnostics, a wide range of analyzing methods is available for investigating a suspected PFD. These methods vary from point-of-care (POC) procedures to more specialized investigations like Born aggregometry (still recognized as the gold standard today), as well as flow cytometric and molecular genetic methods.[2]

The aim of this article is to provide an overview of current methodologies and their combined use in the differential diagnosis of PFDs. It provides an expert opinion on the diagnostic approach on iPFDs, which differs from the ISTH Guidance in certain points.[3]

This article accompanies a second review that focuses on the pathogenesis and clinical manifestations of these PFDs. Together, the two articles aim to provide a concise yet comprehensive overview of PFDs, covering both pathomechanisms and diagnostic approaches, essential for clinical and laboratory practice. It complements the German AWMF S2k guidelines for the diagnosis and management of inherited platelet disorders in which some of the authors of this overview were also involved.

Diagnostic Step Approach to Inherited Platelet Function Defects

As proposed by different guidelines, diagnostic workup of patients with suspected iPFDs should be a multiple-step process, as each subsequent phase of the diagnostic approach necessitates an increased level of personal expertise and more sophisticated laboratory methods.[3–5] ►Fig. 1 presents a proposed diagnostic approach.

Step 1: Medical History and Clinical Examination

The medical history and clinical examination represent the first steps for the diagnosis of iPFDs. In particular, the following issues should be considered:

- *Previous bleeding episodes*: Including timing, provoked versus spontaneous occurrence, severity, and efficacy of local or pharmacologic treatments are critical for the determination of the next assessment steps. Red flags include spontaneous bleeding or transfusion of blood products or coagulation factors during/after surgical procedures that are usually associated with very low bleeding risk.
- *Family history*: A positive family history of increased bleeding may indicate a hereditary platelet dysfunction. However, among the dominant inherited thrombocytopenias such as the MYH9-related disease, *de novo* mutations are common and, therefore, the family history is negative.[6]
- *Medication/nutritional history*: Exclusion of acquired platelet dysfunction (intake of antiplatelet drugs, pain medication, psychoactive drugs, dietary supplements, vitamin K deficiency, etc.). The occurrence of substance intake and bleeding episodes should be carefully assessed.
- *Current symptoms and physical examination*: Petechiae, epistaxis, hematoma, hematuria, menorrhagia, and other forms of unprovoked bleeding may be symptoms of platelet dysfunction. The clinical examination should include a detailed inspection of the skin and mucous. Features of syndromal character are of particular interest, especially in the case of combined low platelet count.
- *International Society on Thrombosis and Haemostasis—bleeding assessment tool (ISTH-BAT)*: Values $\geq$2 in children, $\geq$4 in men, and $\geq$6 in women are considered pathological and require further laboratory investigation.

Deciding on the pathological significance of bleeding symptoms can be challenging. Typical for iPFDs are petechiae and mucocutaneous bleeding. In severe defects, other symptoms such as joint hemorrhages are possible.[7] The ISTH-BAT bleeding score is a useful tool to objectify bleeding tendency. A higher ISTH bleeding score can serve as an indicator for the presence of platelet dysfunction upon exclusion of a von Willebrand disease (VWD) or plasmatic coagulation disorders and should be considered as an indication for further diagnostic analyses in parallel. Furthermore, it has been shown that the ISTH-BAT bleeding score correlates with future bleeding events.[8,9]

If the ISTH-BAT score is normal, a further investigation for iPFD is usually not necessary.[10] But it should be noted that although the ISTH-BAT is a well-validated tool to assess the bleeding risk, it has limitations. Some items, such as syndromic

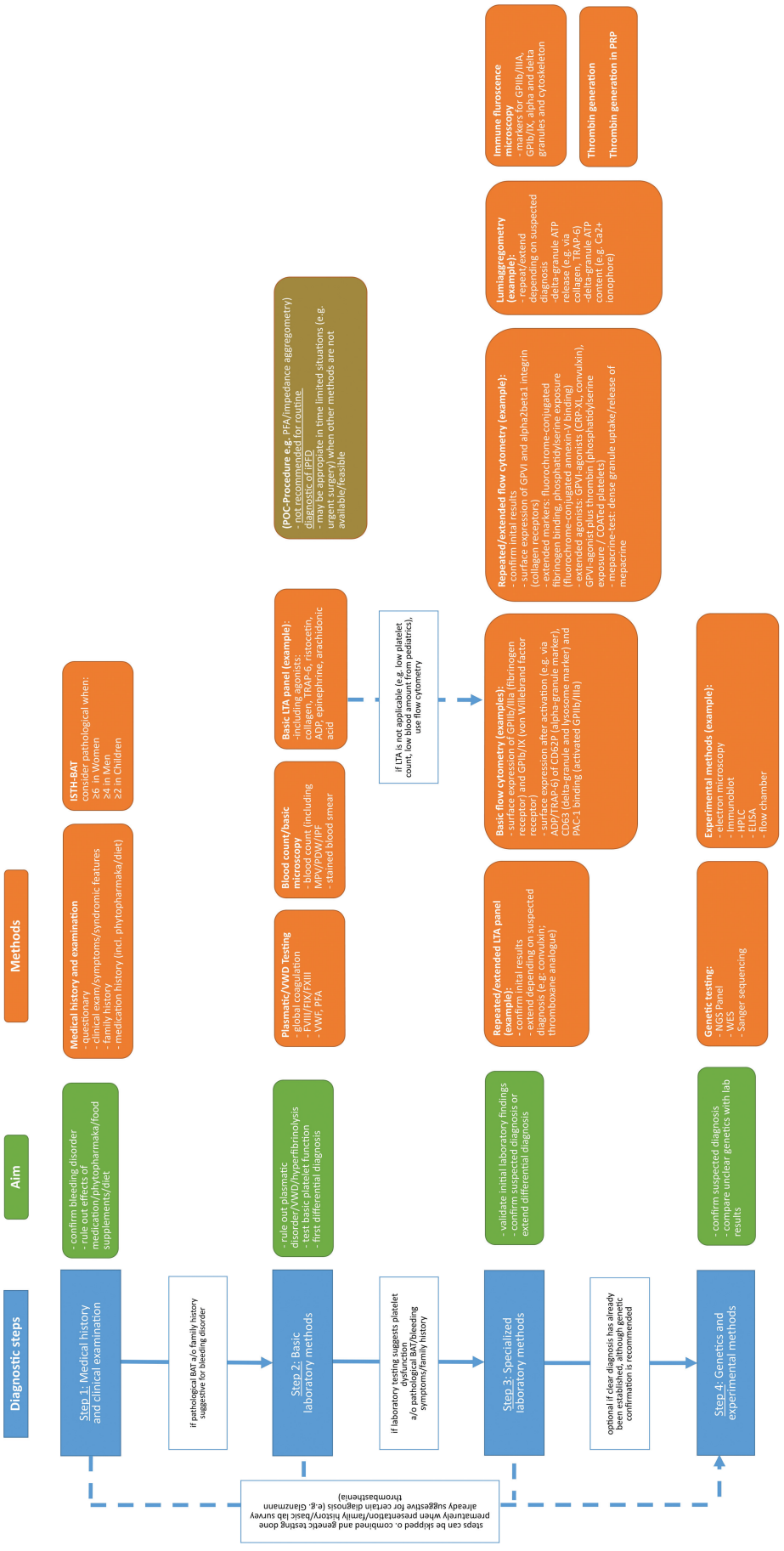

Fig. 1 Proposed diagnostic step workup for inherited platelet function defects.

features and family bleeding history, are not covered by the score. The bleeding score may not be sensitive in patients without previous surgery or trauma. Therefore, further investigations might be indicated despite a low score. In addition, severe bleeding events from other causes (e.g., surgery, uterus atony) may account for pathologic scores despite the absence of platelet disfunction.

Step 2: Basic Laboratory Testing

First routine laboratory investigations should focus on ruling out VWD and plasmatic disorders:

- *Complete blood count*: In addition to the platelet count, the mean platelet volume (MPV), including size distribution and the immature platelet fraction (IPF), should be determined. It should be considered that the anticoagulant (EDTA or citrate) used for blood sampling can affect the MPV value.
- *Stained blood smear examination*: Morphological assessment of platelets.
- *Measurement of international normalized ratio, activated partial thromboplastin time, fibrinogen, von Willebrand factor (VWF) parameters, and factor XIII*: Exclusion of major defects of plasmatic coagulation, including the most common inherited disorders (i.e., hemophilia, VWD).
- *Light transmission aggregometry (LTA) according to Born*: Sensitive for severe-moderate iPFD; specific impact on the diagnosis of GT, GT-like disorders, and BSS. For all other suspected iPFD, LTA should be applied in combination with other methods to specify the platelet dysfunctional responses. *Limitations*: moderate-severe thrombocytopenia, hemolytic and lipidemic plasma samples, sensitive to sample handling and intra-analytical variables.
- *Flow cytometry (basic panel)*: Can be a substitute for LTA, when LTA is not applicable (e.g., low sample volume, moderate-severe thrombocytopenia).
- *POC methods*: Platelet function analyzer (PFA)/whole blood impedance aggregometry: Both methods lack sensitivity and specificity and should not be used as a screening tool.[3] They may provide utility in time-critical situations like urgent surgery when there is suspicion of bleeding disorder. Impedance aggregometry is useful to exclude some drug-induced effects. However, a negative finding cannot exclude an iPFD.[11] PFA results should be considered to be frequently false positive for young children.

Blood count and blood smear analysis can be used to consider initial differential diagnostics. If available, LTA should be performed as it is widely considered the gold standard in the platelet diagnostic workup.[8] Clinically relevant iPFDs are unlikely if the LTA yields normal results. In certain instances of platelet disorders, such as mild δ-storage pool disease and Scott syndrome, LTA results may appear within the normal range. When there is a strong clinical suspicion for an iPFD, additional diagnostic tests should be performed for a comprehensive exclusion.

Step 3: Specific Platelet Laboratory Tests

Laboratory tests for platelet function have to be performed within a few hours after blood donation, which can be a logistic challenge.

- Light-transmission aggregometry (repeated, extended).
- Lumiaggregometry or measurement of ATP release by high-performance liquid chromatography (HPLC).
- Flow cytometry (repeated, extended).
- Immunofluorescence microscopy.
- Thrombin generation capacity in platelet-rich plasma (PRP) versus platelet-poor plasma.

The methodological variability and varying availability across different facilities contribute to heterogeneity in diagnostic protocols, thereby reducing the inter-laboratory standardization in diagnostic approaches.[12] The workup should include the assessment of granule content and release; recommended techniques include lumiaggregometry, flow cytometry, HPLC, and electron microscopy.[13]

Step 4: Genetics and Experimental Methods

- Next-generation sequencing (NGS); targeted for a gene panel or whole-genome/exome sequencing.
- Electron microscopy or high-resolution microscopy techniques.
- Immunoblot.
- HPLC.
- ELISA for soluble proteins and mediators released from activated platelets (sCD62P, sGPIIb/IIIa, sGPVI, thromboxane B_2, and serotonin).
- Ex vivo microfluidic models of thrombus formation using different extracellular matrix proteins (e.g., collagen, fibrinogen, VWF).

A genetic examination can corroborate a suspected diagnosis, especially when the clinical diagnosis has previously been established within the family. Experimental methods may advance knowledge of an underlying pathology if prior tests yield no or inconclusive results.

To illustrate the application of the proposed algorithm, we present the following exemplary case:

A 19-year-old male patient presented to a general practitioner following unusual postoperative bleeding after a hernia surgery.
Step 1: To assess the bleeding tendency in a standardized manner and determine the need for referral to a specialized center, the ISTH-BAT was administered, revealing a significantly pathological score of 10 points.
Step 2: The patient was then referred to a nearby laboratory center, where platelet count/morphology, plasma coagulation, and VWF parameters were found to be normal. The only available platelet function test, LTA according to Born, showed abnormal results with all agonists except with ristocetin, similar to ►Fig. 2.
Step 3: For specialized platelet diagnostics, the patient was further referred to a university center. Consistent findings in aggregometry were observed, along with a

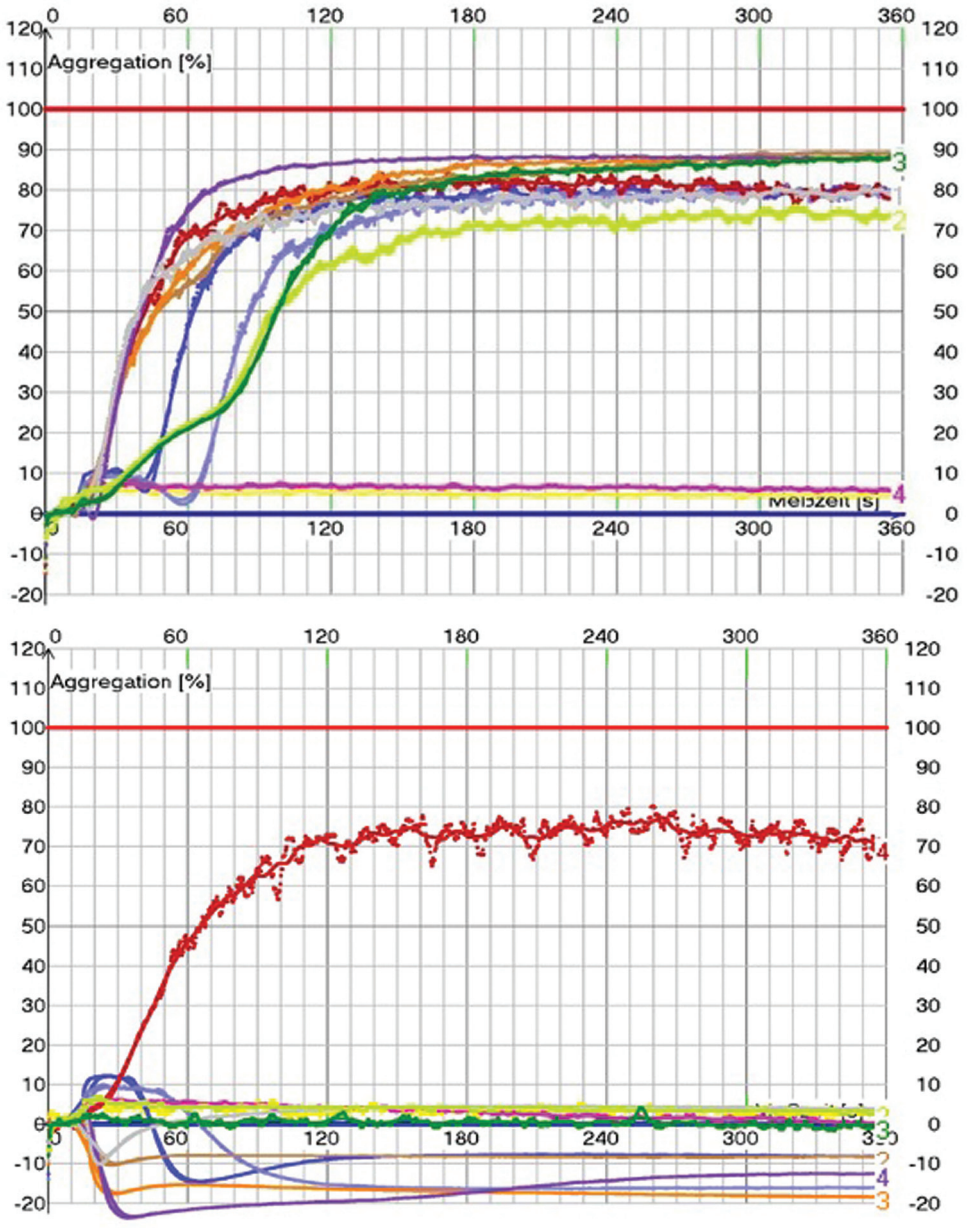

Fig. 2 Example of a normal aggregometry according to Born (top) and from a patient with Glanzmann thrombasthenia (bottom). Agonists include spontaneous (natrium chloride 0.9%, yellow curve); collagen 5 and 1 µg/mL (light and dark blue curve), ristocetin 0.5 and 1.5 mg/mL (pink and red curve); ADP 2 and 5 µM (brown and orange curve); arachidonic acid 1.5 mmol/L (purple curve); TRAP-6 40.0 µmol/L (grey curve); epinephrine 5 and 10 µmol/L(light and dark green).

complete absence of the GPIIb/IIIa receptor assessed by flow cytometry. Analysis of platelet granules by lumiaggregometry and flow cytometry showed no abnormalities. An iPFD of autosomal-recessive GT was diagnosed.
Step 4: Genetic testing confirmed this diagnosis by identifying a homozygous, pathogenic stop mutation in *ITGB3*, encoding the β3-subunit of the receptor.

Point-of-Care Diagnostics

POC devices are established tools in the management of acute bleeding events.[14] Advantages of POC methods include their wide availability and consistent accessibility, simple use with whole blood samples, short turnaround time, and relatively low sample volume requirements. Disadvantages include their low sensitivity and specificity for iPFD and their inapplicability in cases of moderate to severe thrombocytopenia.

Platelet Function Analyzer

The PFA (synonym *in vitro bleeding time*, Fa. Siemens Healthineers) is an automated in vitro bleeding time system. To assess platelet adhesion and aggregation, the PFA draws (buffered) citrated whole blood (preferred anticoagulant: buffered 3.8% citrate solution) under high-shear stress through a capillary and a membrane coated with a platelet agonist, either collagen/epinephrine or collagen/adenosine diphosphate (ADP). A third, less frequently used test cartridge ("P2Y"), is used for the detection of ADP receptor $P2Y_{12}$ inhibition.[15] The time taken

for the capillary aperture to be occluded by the platelet plug is measured as the closure time (CT).[16] Although the manufacturers provide recommendations for normal ranges, it is suggested that each laboratory establishes its own reference ranges upon internal validation.[17]

The PFA is very sensitive to changes in hematocrit (below 30%) and platelet counts (<100.000/µL) making anemia and thrombocytopenia a frequent reason for prolonged CTs in the clinic.[18] Certain diets (e.g., dark chocolate, garlic, fish oil) can also prolong CTs and may lead to false-positive results.[19] Moreover, antiplatelet medications, such as aspirin, and nonsteroidal anti-inflammatory drugs (NSAIDs) are common reasons for prolonged CTs.[20]

PFA has been shown to have good sensitivity in the detection of some types of VWD but poor specificity.[19] Furthermore, it can be useful to monitor treatment with desmopressin in VWD[21] and acquired VWD.[22] While severe platelet defects (like GT) can be detected by PFA, sensitivity and specificity have been reported to be low in mild iPDFs.[11,23] Therefore, it is not recommended to use PFA as a screening tool for iPFD.[24] Pathological results in patients with bleeding symptoms need to be followed by more specific assays like LTA or flow cytometry for comprehensive diagnosis, while normal results are not sufficient to completely rule out iPFD.

Viscoelastic Testing

Viscoelastic assays such as thromboelastographic tests are currently considered an integral part of coagulation diagnostics in emergency room management and during major operations with a high risk of blood loss.[14,25] The method uses a stamp protruding into a vessel containing a whole blood sample. After coagulation activation, the force necessary to rotate either stamp or vessel is measured and increases with continuing coagulation and clot formation.

The methods are useful for measuring bleeding related to function and amount of fibrinogen or hyperfibrinolysis in acute situations. To our knowledge, there are no data about the sensitivity and specificity of viscoelastic tests in iPFDs. From our personal experience, only severe defects such as GT are detected by reduced maximum clot firmness despite normal platelet count (►**Fig. 3**). Thus, viscoelastic tests are unsuitable for both diagnosis and precise characterization of PFD. Especially in pediatric patients, this represents an unnecessary blood sample from which no helpful conclusions can be made regarding platelet function.

Whole Blood Impedance Aggregometry (Multiplate)

The Multiplate (Fa. Roche Diagnostics GmbH) measures platelet aggregation via changes in the electrical impedance in a whole blood sample. Activated platelets attach to the electrodes in the measuring cell, which leads to an increase in electrical resistance. The change in electrical resistance is recorded continuously and the area under the aggregation curve is measured over time (recorded in units [U]). Multiple electrode aggregometry (MEA) is a 5-channel system with double measurements for each test to check the intratest

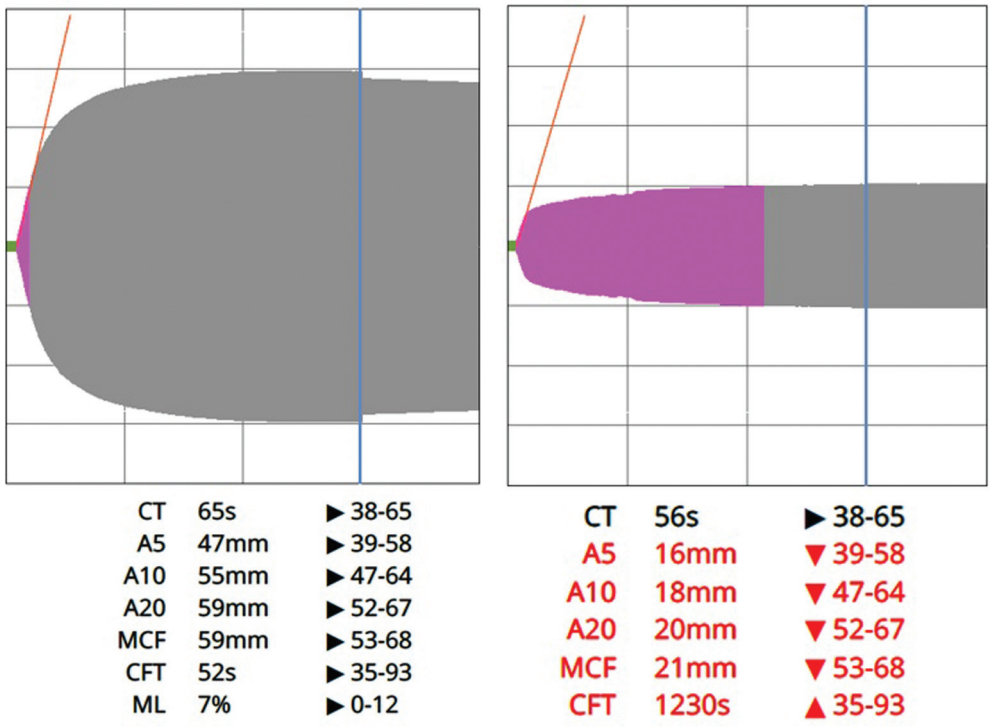

Fig. 3 Comparison of a normal thrombelastography (left) versus Glanzmann thrombasthenia with platelet count 273.000/µL (EX-Test, ClotPro, Fa. Hemonetics), clotting time (CT), clot amplitude after 5/10/20 minutes (A5/A10/A20), maximum clot firmness (MCF), clot formation time (CFT), maximum lysis (ML). In a patient with Glanzmann's thrombasthenia, thrombelastography (EX-Test) cannot distinguish whether it is thrombocytopenia or severe platelet dysfunction. If, as shown here, the reduced maximum clot firmness indicates a reduced platelet count, the finding should be compared with the blood count of the patient. If there is a normal platelet count, the findings may indicate a severe functional disorder, such as Glanzmann thrombasthenia.

variability. For the measurement, 300 µL of hirudinized blood (the use of heparinized or citrated blood is also possible) is mixed with an equal volume of saline at 37 °C, and aggregation is triggered by the addition of specific agonists. The available tests are adapted to the currently available drugs for inhibiting platelet aggregation (ADP for $P2Y_{12}$ antagonists such as clopidogrel, arachidonic acid for aspirin, and thrombin receptor activating peptide [TRAP]-6 for global platelet function). This means that the method is frequently used to detect or exclude treatment with antiplatelet agents. In addition, further diagnostics of platelet defects related to VWF binding can be performed by means of a multiplate using ristocetin. Glycoprotein VI defects can be detected by the use of convulxin as a specific agonist.[26]

Since MEA requires significantly smaller amounts of blood compared to LTA, it represents a suitable option for pediatric patients. However, the main limitation of the method is that MEA is not able to detect moderate and mild platelet dysfunction, which can also have clinical relevance, especially regarding surgery, for example.[27–29] Pathologic findings when using MEA may occur only in severe platelet function defects, such as GT.[30] The use of lower agonist concentrations can help identify mild platelet defects; however, such test conditions are often not standardized or validated.

Light Transmission Aggregometry

A key method in diagnosing platelet functional disorders is LTA. The principle of this method is based on the increasing light transmittance along with platelet aggregation[31–33] in plasma (PRP) or in buffer (washed platelets, or gel-filtered platelets). Platelet aggregation is induced by a variety of relevant agonists in different concentrations (►Fig. 4) including ADP, collagen, epinephrine, ristocetin, TRAPs (e.g., TRAP-6), and arachidonic acid, among others.[34] Commercially available testing devices and solutions are listed in ►Table 1.

LTA remains the gold standard for platelet function testing. It is particularly sensitive for detecting moderate to severe iPFDs, including GT, GT-like disorders, and BSS. It also demonstrates good sensitivity in assessing the effects of medications on platelet function. Several studies confirm good or excellent inter-method correlation among the different devices.[35]

There are some disadvantages. Manually performed LTA is time-consuming and requires the fresh collection of a relatively large blood volume ranging from 6 to 20 mL, which prevents sample shipping and presents a particular challenge, especially for pediatric patients. Additionally, LTA is technically challenging, as it is influenced by several preanalytical and analytical variables. Moreover, the lowest reliable platelet count ranges from 90.000 to 100.000/µL in PRP,[36,37] which limits the analysis in patients with thrombocytopenia, a condition often found in patients suspected of iPFD. Efforts to address and harmonize these issues have been made in published guidelines.[4,5,38,39]

New coagulation analyzers on the market enable the performing of automated LTA, with reduced sample volume requirements (about 140 µL PRP per tested agonist), reduced

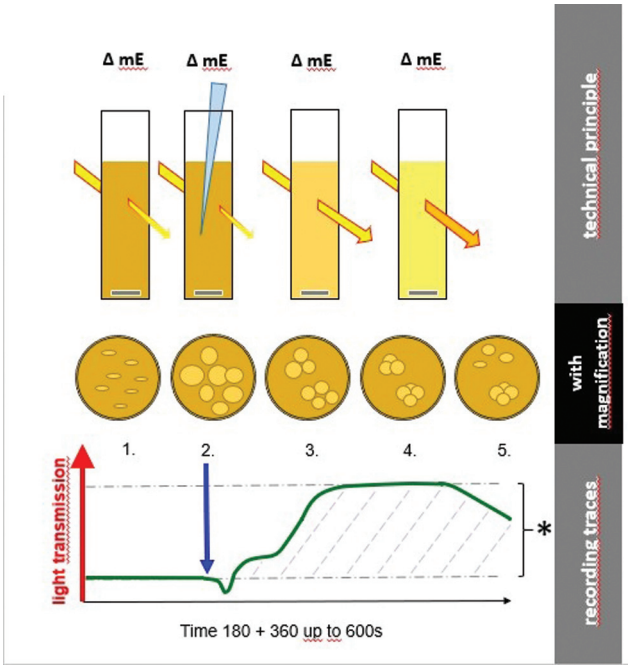

Fig. 4 Principle of light transmission aggregometry. The top row [technical principle] shows the cuvette with platelet-rich plasma (PRP) and a stirring bar, light source, and light detection system (not drawn). The difference in milli extinction (Δ mE) is measured upon the addition of an agonist (indicated by a blue pipette tip). The middle row (with magnification) shows how the platelets react in PRP: resting platelets (1), changing conformation and volume (i.e., "shape change") after adding agonist (2), which is not recorded in WB; formation of in-stable aggregates (1st wave) (3), formation of stable aggregates (2nd wave) (4), and sometimes disaggregation (5) in PRP. The bottom row (recorded tracings) shows the recorded tracings over time in PRP calculating maximum amplitude (%, curly brackets) based on the Δ of the transmitted light (*) or the Δ of electrical resistance and area under the curve (dashed light purple lines), respectively. Adding luciferase reagent to PRP allows recording the release of ATP in an additional channel in selected devices.

turnaround times, and without the need for dedicated, experienced personnel.[40–43] Despite these advantages, the costs of reagents and consumables for automated LTA are usually higher than those for classic LTA, and used agonist concentrations are fixed and not adjustable. Additionally, interpretation still depends on expert examination of aggregation tracings from the patient, in comparison to a healthy control.[44]

Lumiaggregometry

Similar to other methods, a lumiaggregometry measures platelet aggregation in PRP or citrated whole blood. Its unique feature is the simultaneous measurement of adenosine triphosphate (ATP) release from δ-granules via chemiluminescence production of the luciferin–luciferase reaction.[45–47] The integrity of ATP release can be determined upon platelet activation by different agonists such as thrombin or TRAP-6, collagen, arachidonic acid, ADP, and epinephrine. Furthermore, the whole content of ATP in dense bodies of platelets can be measured after incubation with Ca^{2+} ionophore A23187 (►Fig. 5)[48] or by a GPVI agonist plus thrombin receptor PAR-1 activating peptide (e.g., TRAP-6).

Table 1 Commercially available testing devices and solutions for performing light transmission aggregometry

	Platelet-rich plasma–based method			
	Company	**Device**	**Reagents**	**Application**
Manual	Chronolog	490 and 700[a]	User's choice	PD, monitoring
	Helena	AggRAM	User's choice	PD, monitoring
	Biodata	PAP 8	User's choice	PD, monitoring
	Labitec	APACT 4004	User's choice	PD, monitoring
	Stago	TA-V4 and 8	User's choice	PD, monitoring
Automated	Siemens Healthineers	COAG 360[b]	User's choice	PD, monitoring
	Sysmex	CS-2500 CS-5100 CN-3000 CN-6000	User's choice	PD, monitoring
	Behnk Elektronik	Thrombomate XRA	User's choice	PD, monitoring

[a]This device allows for adenosine triphosphate (ATP) secretion testing.
[b]This device will be removed from the market in Q3/2024.

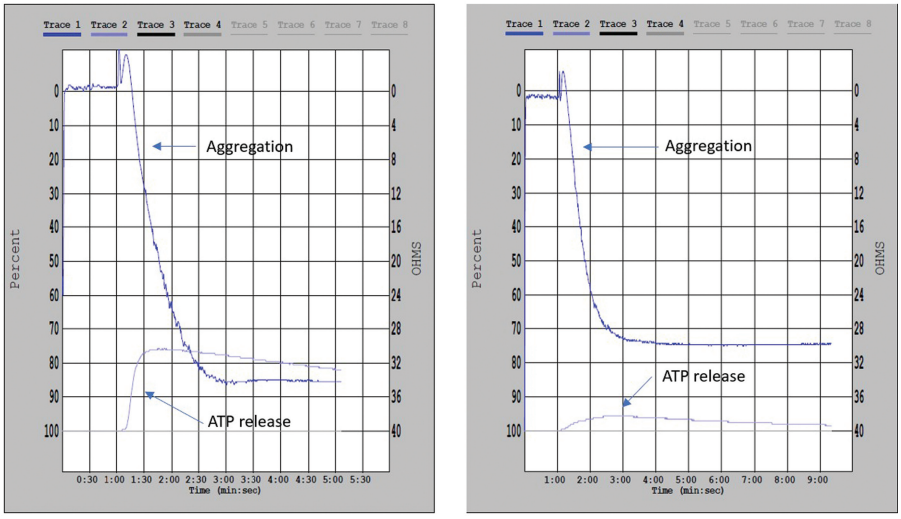

Fig. 5 Example of a normal (left) versus reduced ATP (right) release in lumiaggregometry in PRP, induced by Ca2+ ionophore A23187.

Irreversible damage to the cell membrane results in a maximum influx of Ca^{2+} and thus a complete release of intracellular Ca^{2+} contents. This is particularly useful to detect δ-granule (dense granule) release, further enables differentiating into quantitative or qualitative (release disorders) defects,[49] and provides a viable alternative to δ-granule investigation using scarcely available electron microscopy.

Lumiaggregometry remains one of the most frequently used platelet secretion assays.[13] The method has been found to have high specificity and moderate sensitivity for iPFDs.[50,51] However, it is important to note that lumiaggregometry shows a high intra- and interindividual result variability; so, it is necessary to confirm pathological findings in repeated investigations and establishment of intralaboratory reference ranges.[52] Furthermore, it is not applicable in cases of severe thrombocytopenia and is relatively time-consuming. Additionally, ADP and ATP content can be measured using various other methods, including HPLC.

Flow Cytometry

Originally, flow cytometry was used for research purposes to deeply characterize alterations in platelet responses.[53] Meanwhile, the method is also frequently used in clinical laboratories.[12] However, this technique is often restricted to specialized centers for platelet diagnostics due to high-quality requirements of intralaboratory standardized assessment of preanalytics, assays, and reference values. Flow cytometry provides the analysis of a spectrum of different platelet phenotype and activation parameters (e.g., quantification of surface receptors, activation of the fibrinogen receptor integrin αIIbβ3 [GPIIb/IIIa] granule secretion, procoagulant activity [COATed, coated platelets], signaling targets, platelet–leukocyte interaction, extracellular vesicles, apoptosis, reflecting the platelet activation status in vivo, and the platelet function capacity ex vivo in response to platelet agonists[54,55]; ►**Table 2**, ►**Fig. 6**). In contrast to platelet aggregation assays, flow

Table 2 Flow cytometry tests for the diagnosis of inherited platelet function disorders; Bernard-Soulier syndrome (BSS); Glanzmann thrombasthenia (GT); storage pool deficiency (SPD)

Platelet phenotype/ function test	Anticoagulated whole blood (diluted)	Platelet-rich plasma (diluted)	Platelet agonists	Fluorochrome-conjugated antibodies/dyes	Diagnosis of inherited platelet function disorders
quantification of receptors/ glycoproteins	Yes (preferred)	Yes	No	*von Willebrand receptor:* anti-GPIb/V/IX complex, anti-GPIba (CD42b), anti-GPIX (CD42a); *fibrinogen receptor:* anti-GPIIbIIIa complex, anti-GPIIb (CD41), anti-GPIIIa (CD61); *collagen receptors:* anti-GPVI, anti-CD49b (GPIa), anti-CD29 (GPIIa); CD34	BSS; GT and GT-like disorders; GPVI deficiency; GFI1B macrothrombocytopenia
Activation of fibrinogen receptor GPIIb/IIIa	Yes	Yes	No/yes	PAC-1; fibrinogen-AL488	GT (like disorders) receptor and/or signal transduction defects
α-Granule exocytosis	Yes	Yes	No/yes (strong agonists preferred)	Anti-CD62P; anti-TLT-1	α-SPD; α/δ-SPD; α-granule secretion defect
δ-Granule presence	No	Yes	No	Mepacrine	α-SPD; α/δ-SPD
δ-Granule exocytosis	Yes	Yes	Yes (strong agonists preferred)	Anti-CD63; mepacrine	α-SPD; α/δ-SPD; δ-granule secretion defect
Lysosome exocytosis	Yes	Yes	Yes (strong agonists preferred)	Anti-CD63; anti-LAMP-1 (CD107a); anti-LAMP-2 (CD107b)	δ-SPD with lysosome defects
Procoagulant activity	Yes	Yes	No/yes (strong agonists)	Annexin V-FITC etc., lactadherin-PE, etc., in combination with anti-CD62P (CD62P-positive/ CD62P-negative platelets)	Scott syndrome; Stormorken syndrome

cytometric analysis is based on the detection of fluorescent markers on single platelets within platelet populations but without stirring to avoid platelet aggregation. If impaired platelet aggregation responses do not allow a final diagnosis of a platelet function disorder, a detailed analysis of the platelet dysfunction using flow cytometry is recommended to specify the diagnosis.[3,5] Dependent on the assay specifications, platelet phenotype, and function analysis are performed in buffer-diluted (e.g., Tyrode's buffer 1:10 or 1:20) citrate-anticoagulated (3.2% [109 mM], 3.8% [129 mM], 1:10) whole blood or PRP. Platelets in whole blood are identified after labeling with monoclonal fluorochrome-conjugated antibodies directed against high abundantly expressed platelet-specific surface antigens (vs. corresponding isotype controls) such as CD41/CD61 (fibrinogen receptor integrin aIIbβ3; GPIIb/IIIa) or CD42a (subunit GPIX of von Willebrand factor receptor complex GPIb/V/IX) in combination with gating of single platelets according to size (forward scatter/FSC) and granularity (side scatter/SSC) properties. Specific fluorochrome-conjugated antibodies (vs. corresponding isotype controls) and fluorescent dyes detecting platelet function markers are used to determine potential platelet preactivation in vivo and functional reactivity ex vivo upon stimulation. Platelet agonists (e.g., TRAP-6 10 µM, ADP 10 µM) used for aggregation tests are also suitable for flow cytometric platelet analysis with the exception of fibrillar collagen, which leads to false-positive fluorescence signals by crosslinking several platelets in complexes and thereby preventing single cell analysis. Here, the collagen receptor GPVI agonists cross-linked collagen-related peptide (CRP-XL) or the purified snake venom protein convulxins are used to induce GPVI-dependent platelet activation responses.[56–58]

In comparison to platelet aggregometry, flow cytometry allows platelet diagnostics in pediatric blood samples with small amounts of blood (e.g., 1.8 mL)[59,60] and in samples from patients with thrombocytopenia.[61] As a low platelet count alone often does not explain the bleeding risk, flow cytometry represents a reliable tool to identify combined platelet count and function defects even in severe thrombocytopenia.[62] Disadvantages include the relatively time-

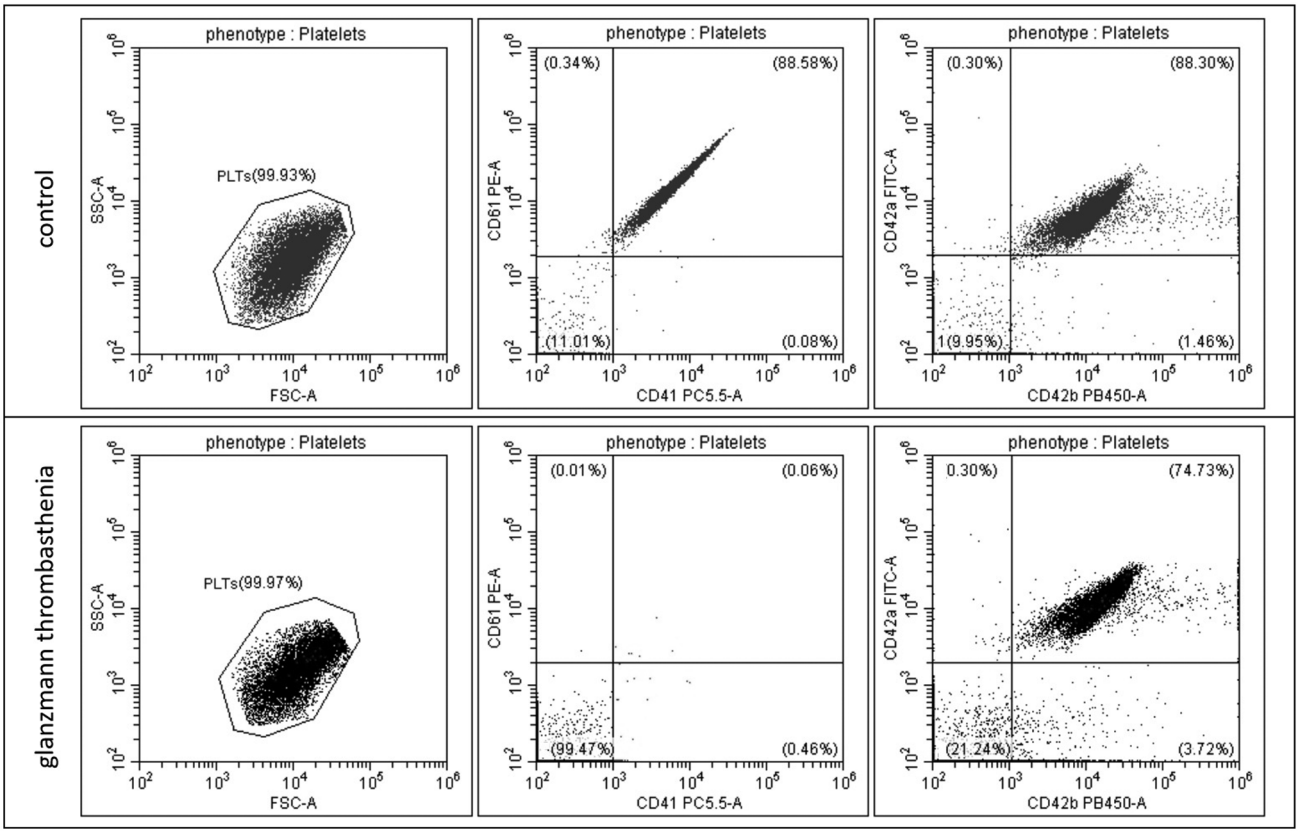

Fig. 6 The image shows flow cytometry results comparing a healthy control (upper row) with a patient diagnosed with Glanzmann thrombasthenia (lower row). Platelets were gated based on their characteristic: forward scatter and sideward scatter properties in flow cytometry (left column). Assessment of fibrinogen receptor expression using anti-GPIIb (CD41) and anti-GPIIIa (CD61) double staining, confirming normal expression in the control and absent fibrinogen receptor in the patient with Glanzmann's thrombasthenia (middle column). Assessment of von Willebrand receptor expression using anti-GPIb (CD42a) and anti-GPIX (CD42b) double staining confirms normal expression in both investigations (right column).

consuming nature of the investigation and moderate susceptibility to sample handling and preanalytical variables. Reliable results are obtained when the tests are performed and analyzed within 4 hours after blood collection.[56] ►**Table 2** depicts recommended flow cytometry tests typically used for the diagnosis of iPFD and aPFD.

Light and Immunofluorescence Microscopy on the Blood Smear

Screening for suspected iPFD or hereditary thrombocytopenia (HT) should always include an assessment of the peripheral blood smear. Although some disorders (e.g., signal transduction defects) cannot diagnosed, changes in platelet structures may indicate a functional defect or identify patterns that point to specific diseases.

Light Microscopy

Light microscopy is the most widely used method. It not only allows pseudothrombocytopenia to be ruled out but also provides initial indications of iPFD.[63] Platelet macrocytosis features the majority of HTs. Absent or markedly reduced azurophilic granules suggest a grey platelet syndrome, single giant granules can be found in Paris-Trousseau syndrome, and small platelets point to Wiskott-Aldrich syndrome (WAS). Red blood cell anisopoikilocytosis hints to platelet disorders with dyserythropoiesis (e.g., *GATA1*- or *GFI1B*-related thrombocytopenia). The basophilic Doehle-like bodies in the granulocytes are pathognomonic for *MYH9*-related disease (*MYH9*-RD).

Immunofluorescence Microscopy

Immunofluorescence microscopy is a less widespread method and enables the specific staining of platelet structures such as surface receptors, cytoskeletal proteins, or granule constituents.[64] In contrast to light microscopy, δ-granules can also be visualized. By using a minimal amount of blood, a comprehensive assessment of platelet phenotype can be made, which enables one to strongly suspect some iPFDs or, at best, to describe the specific platelet defect.

A retrospective study including more than 3,000 suspected patients found that a diagnosis of iPFD or HT could be correctly predicted by immunofluorescence in about 30% of the cases.[65] By comparison with genetic testing, the method was subsequently validated as sensitive and specific for nine disorders: *MYH9*-RD, classical and monoallelic BSS (not for rare functional defects), GT (types I and 2, but not the functional defect form 3), *TUBB1*-related thrombocytopenia (*TUBB1*-RT), *GFI1B*-RT, filamin A-related thrombocytopenia (*FLNA*-RT), δ-storage pool disease (δ-SPD), and WAS/X-linked thrombocytopenia (WAS/XLT).[66]

Electron Microscopy

Conventional electron microscopy is categorized into transmission electron microscopy, transmitting a beam of electrons through a thin section to visualize internal structures, and scanning electron microscopy using a focused beam of electrons to scan the surface of the object. Electron microscopy has aided the study of platelet biology and thrombosis using transmission and scanning techniques to visualize platelet structures and their changes upon activation. It is also used to examine platelet aggregates and thrombi.[67] Due to good sensitivity and specificity, electron microscopy still represents one of the reference investigations for platelet δ-granules and is considered a step 2 examination by the ISTH guidelines.[3] Despite these circumstances, it remains a domain of extremely specialized research institutions, due to its scarce availability and therefore it is not further discussed in this article.

Methodologic Aspects of Immunofluorescence Microscopy

As for light microscopy, immunofluorescence microscopy can be performed using standard air-dried blood slides. However, moisture precipitation or long transport can lead to artifacts. Granule markers appear to be more susceptible to artifacts than receptor or cytoskeletal markers. When using anticoagulated blood, EDTA is advisable with respect to other anticoagulants (e.g., citrate) since it seems to guarantee a better stability of platelet markers. The smears should be fixed and permeabilized before staining using acetone (−20 °C, 2–5 minutes, permeabilizing), methanol (−20 °C, 1 minute, permeabilizing), or 1 to 3% of paraformaldehyde (room temperature, 10–20 minutes) followed by permeabilization with 0.1 to 0.2% triton X-100 for 5 to 10 minutes. The target structure can be optionally visualized using a single fluorescence-labeled antibody or a primary antibody and a fluorescence-labeled secondary antibody. The antibody panel should include receptor structures (e.g., GPIIbIIIa, GPIbIX), cytoskeletal markers (e.g., nonmuscular myosin II A, β1-tubulin), and granule components (e.g., P-selectin, von Willebrand factor, CD63, Lamp1).

Advantages and Disadvantages

The method enables centers with no specialized platelet laboratories to easily send blood smears even long distances, thus gaining access to second-level platelet diagnostics. The low amount (<100 µL) of required blood makes it suitable also for newborns. However, the method provides no hints on platelet functionality in vivo. In addition, platelet activation during blood collection or smear preparation can lead to preanalytical artifacts. Furthermore, the technique is operator-dependent. The establishment of proficiency-testing programs represents an unmet need.

Future Perspectives

A first, international interlaboratory workshop is ongoing. It is expected to facilitate the standardization of the technique and to increase the sensitivity and specificity of the method when used as a screening tool for iPFD and HT. Besides enabling targeted genetic testing, immunofluorescence is predicted to become increasingly complementary to molecular investigations, particularly for interpreting variants of unknown significance (VUS). Furthermore, preliminary data suggest that a semiautomatic immunofluorescence image analysis allowing unbiased and standardized morphologic assessment is becoming a realistic option.

Platelet-Dependent Thrombin Generation

Classically, the calibrated automated thrombography established by Coenraad Hemker at Maastricht University represents the prototype of the fluorogenic platelet-dependent thrombin generation techniques.[68]

Routinely, tissue factor is used to trigger platelet activation responses, including phosphatidylserine (PS) exposure, and platelet-dependent thrombin generation in recalcified PRP. A fluorogenic thrombin substrate is cleaved by thrombin resulting in increasing fluorescence over time, represented by the lag time, velocity index, peak height, and endogenous thrombin potential. Antithrombin and α2-macroglobulin inactivate thrombin, leading to decreased fluorescence generation over time. The endogenous thrombin potential, expressed as the area under the curve of the resulting thrombogram, quantifies the total generated amount if thrombin is given in nM × minute according to standardized thrombin calibrator samples. In addition to intrinsic genetic defects of platelet PS exposure and other mechanisms of coagulation factor binding, distinct iPFDs, well-known to be associated with impaired primary hemostasis, such as GT, BSS, and δ-SPD, also show defective platelet-based thrombin generation.[69] Therefore, the measurement of platelet-dependent thrombin generation is also supportive to determine the influence of inherited and acquired platelet dysfunctions on platelet-based coagulation, which may contribute to explaining bleeding symptoms and severity.

Genetic Basis and Testing of Congenital Platelet Disorders Using Next-Generation Sequencing

The implementation of high-throughput sequencing (HTS) in diagnostic flow allows the testing of a large number of genes in parallel and greatly reduces the workload, turnaround time, and costs. HTS provides an affordable DNA-based analysis to diagnose patients suspected of having a known iPFD.

iPFDs result from genetic variants in genes implicated in megakaryocyte differentiation and/or platelet formation and clearance. The identification of the underlying causative gene defect of iPFDs is challenging given the high degree of heterogeneity. However, the identification of the gene defect is important due to the strong variation in genotype and phenotype correlation and the impact on clinical prognosis, especially, because some defects can lead to hematological malignancies.[70,71]

Three different HTS methods can be used for diagnostics and research: targeted (panel) sequencing (TS), whole-exome

sequencing (WES), and whole-genome sequencing (WGS). While WES and WGS can analyze every gene in the human genome, only a specific set of disease or phenotype-associated genes (typically a group of 10–100 genes) is sequenced using TS. Thus, TS is rapidly becoming a valuable and more suitable tool for clinical diagnosis, while WES and WGS can also identify the underlying gene defect and may be required to improve the knowledge of iPFDs.[70,72]

To facilitate efficient genetic testing of iPFDs, the ISTH established a three-tier system to rank the clinically important genes. The Tier 1 genes are highly associated with underlying iPFDs and are recommended to be the initial focus of genetic testing. Tier 2 includes genes that are deemed relevant for iPFDs diagnostics, but they still require confirmation. This list is reassessed at the yearly ISTH meeting and currently (2022) contains 72 genes (www.isth.org/page/GinTh_GeneLists). These genes can be classified according to the specific pathway disrupted in platelet formation: the thrombopoietin (THPO)/THPO receptor (MPL) signaling pathway, transcriptional regulation, granule formation/trafficking/secretion, cytoskeleton regulation and transmembrane protein-signaling pathway.[73]

Based on this, iPFDs can be classified into three categories including disorders that (1) affect only platelets (thrombocytopenia, abnormal platelet size, shape, ultrastructures, dysfunction), (2) disorders that are associated with syndromic or nonsyndromic phenotypes, and (3) disorders with increased risk of hematologic malignancies.[74,75]

However, distinguishing pathogenic variants from nonpathogenic variants is often challenging and requires complex functional and cell line studies to prove causality. In 2015, the American College of Medical Genetics (ACMG) and the Association for Molecular Pathology (AMP) published a guideline providing a structured approach for sequence variant interpretation.[76]

This guideline specified criteria to assign a pathogenicity assertion for each sequence variant into one of the following five categories: benign, likely benign, VUS, likely pathogenic, and pathogenic. Recommendations have been published for the GT-associated genes *ITGA2B/ITGB3* and for the *RUNX1* gene to classify identified variants.[77,78]

Based on a recent large throughput study of NGS testing of suspected iPFDs, the yield of identifying possible causal genetic variants is about 26 to 48%, with the most common presentation of VUS (around 10%).[79] The interpretation of VUS is a complex challenge due to the absence of sufficient evidence to assign them to the benign or pathogenic category based on limited information.

Local legal regulations (e.g., in Germany, the "*Gendiagnostikgesetz*") must be obeyed. It is important to obtain a patient's consent prior to any genetic testing based on ethical and legal principles, confidentiality, and non-maleficence. To make their own decision, the patient should be fully informed about the risks, benefits, and limitations of the test, as well as the potential impact of the results on their health, career, and future. As an example, predispositions for malignancy may be uncovered.[78,80]

Once an iPFDs is suspected, genetic testing may provide the following benefits:

a. Enables more precise classification of iPFDs confirming clinical and laboratory phenotypes, allowing better prediction of clinical risk of bleeding and syndromic comorbidities, more accurate diagnosis, and subclassification of iPFDs.
b. Can diagnose rare iPFDs when the results of phenotypic laboratory tests are inconclusive.
c. Can be used to detect the carrier status of family members and allows early detection and treatment of other affected family members.
d. Finally, it can provide invaluable insights into the disease and platelet function mechanisms, helping to further advance scientific knowledge in platelet biogenesis and function.

Conclusion

The diagnostic workup of patients with suspected iPFD remains a challenge. Preanalytical considerations, medication intake, and diet, heterogeneity of laboratory methods, and often unclear phenotype–genotype correlations further complicate the path to a diagnosis.

Therefore, the diagnostic approach of iPFD should begin with a thorough medical history and clinical examination. The use of a validated bleeding score like the ISTH-BAT (International Society on Thrombosis and Haemostasis Bleeding Assessment Tool) is strongly recommended. At first, plasmatic bleeding disorder and VWD should be ruled out before starting an in-depth investigation of platelet disorders. Most importantly, well-established methods for platelet examination should be used, including blood smear analysis by light microscopy, LTA, and flow cytometry. In addition, techniques such as lumiaggregometry, immune fluorescence microscopy, and platelet-dependent thrombin generation help confirm and specify the diagnosis of iPFD. Genetic analysis has a very important value in confirming suspected diagnosis and may unravel novel mutations.

Data Availability

Data generated from this study are available from the corresponding author upon reasonable request.

Conflict of Interest

MH: travel support: Sobi; Acommodation/Funded education events: Bayer, NovoNordisk.

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