In the diagnostic approach to a patient for possible platelet disorders, evaluation should start with a comprehensive medication and clinical history. Testing to exclude coagulation disorders, especially von Willebrand disease, is important, as the symptoms may be similar to platelet disorders. This should be followed by an assessment of platelet number and morphology, culminating with studies of platelet function.

**Clinical History for Patients with a Bleeding Diathesis**

A thorough clinical and family bleeding history should include an assessment of the duration (i.e., lifelong vs. acute), pattern, and severity of bleeding problems, including whether the bleeding is spontaneous or is associated with trauma or surgery [1, 2]. Microvascular bleeding is typical for platelet-mediated bleeding disorders, which may manifest as a mucocutaneous bleeding pattern. Common symptoms of platelet-mediated bleeding include ecchymosis, petechiae, purpura, epistaxis, and gingival bleeding. This pattern is in distinction to coagulation protein disorders, where deep tissue bleeding such as hemarthroses and intra-cranial hemorrhage are more common [3]. Vascular malformations may give a bleeding pattern similar to platelet disorders, but the pattern is often more focal than diffuse. Acquired purpuras, such as disseminated intravascular coagulation (DIC) or vasculitis, usually can be distinguished from platelet dysfunction [4], as platelet disorders typically cause “wet” purpura with bleeding from mucous membranes, while vascular purpura is usually confined to the skin.

Many drugs, as well as food such as garlic or caffeine, can affect platelet function, so a complete drug and dietary history should be obtained [5]. It is important to remember that aspirin, an irreversible inhibitor of platelet function, is an ingredient of many over-the-counter and prescription medications, such as cold or flu remedies. Platelet dysfunction is associated with many systemic disorders, such as renal disease, hepatic failure, connective tissue disorders, myeloproliferative or myelodysplastic disorders, malignancy, and cardiovascular disease. Additionally, specific clinical features, such as albinism, deafness, nephritis, and susceptibility to infections, may help in the diagnosis of the inherited platelet disorders [6].

**Platelet Count, Platelet Indices, and Morphology**

A first step in the investigation of platelet disorders should be measurement of the platelet count, platelet indices, and review of peripheral smear morphology. This distinguishes thrombocytopenia, thrombocytosis, or normal platelet count. It also helps to exclude other pathologies such as the leukemias, myeloproliferative disorders, myelodysplastic disorders, or consumptive coagulopathies, such as DIC [4].

**Specimen Collection, Handling, and Processing**

Blood specimens collected for platelet counting and morphology should be collected into EDTA (ethylenediaminetetraacetic acid) anticoagulant, typically a purple-capped vacutainer tube [7]. The specimen should be mixed thoroughly and gently after collection to prevent in vitro clotting. A peripherally collected specimen is ideal, but collection from indwelling catheters is acceptable, provided the flushing liquid is removed prior to sampling to avoid dilution. The platelet count is usually stable for up to 24 h after collection, although mean platelet volume decreases after 3 h. An air-dried Wright-stained smear can be made from the EDTA specimen for platelet morphologic analysis.
Test Performance and Interpretation

The platelet count and platelet size distribution are measured by automated hematology analyzers that perform complete blood counts (CBCs), usually by impedance measurement [8]. Some instruments use flow cytometry to count platelets labeled with fluorescent, platelet-specific antibodies; however, the reagent cost for this method is prohibitively high for routine laboratory testing. This technique may improve the accuracy of the platelet count in patients with marked thrombocytopenia.

The platelet count is generally between 150,000 and 400,000/μL of blood in normal individuals. True thrombocytopenia must be distinguished from pseudothrombocytopenia or platelet binding to neutrophils (platelet satellitism). Pseudothrombocytopenia is a spurious in vitro platelet clumping not associated with disease, due to EDTA-dependent, cold-reacting platelet agglutinins that may be observed in patients with high immunoglobulin levels, anticardiolipin antibodies, the glycoprotein IIb/IIIa (GPIIb/IIIa) antagonist drug abciximab, or infections [9]. They typically only bind platelets when calcium is chelated, such as with EDTA. In the setting of pseudothrombocytopenia, a more accurate platelet count can be established by collecting the blood specimen in either citrate or heparin anticoagulants or by collecting blood directly from a finger stick into a diluent.

Giant platelets observed with macrothrombocytopenia syndromes also can give falsely low platelet counts, as the large platelets may be counted as leukocytes by automated cell counters.

In addition to platelet count, automated cell counters measure indices of platelet size and size distribution [10]. The mean platelet volume (MPV) is an indication of platelet size, with normal MPV ranges 7–11 fl. The platelet distribution width (PDW) is a measure of the dispersion of platelet sizes. True congenital macrothrombocytopenias usually have uniformly large platelets, with a very high MPV and normal PDW; often the platelets are at least twice normal size and may be as large as erythrocytes [11]. The MPV and PDW can detect increased platelet turnover, where MPV will be increased due to the larger size of newly produced platelets, and PDW will be increased due to a mixture of large and small platelets [12, 13]. Alternate techniques based on messenger ribonucleic acid (mRNA) detection in platelets (reticulated platelets) correlate with thrombopoiesis, as mRNA levels are high in newly formed platelets and decline progressively during blood circulation time [14].

Platelet morphologic analysis should accompany evaluation of the platelet count, especially if there is thrombocytopenia. It is best to assess platelet morphology in the thin part of the smear where the erythrocytes have good morphology and are present in a thin monolayer, keeping in mind that the feathered edge or the lateral sides of the smear should be scanned for platelet clumps. On a properly prepared, Wright-stained blood smear, the platelets are approximately 2-μm diameter, with abundant purple-staining granules (Fig. 2.1) [15]. Large platelets, dumbbell-shaped platelets, and megakaryocyte fragments are unusual. The presence of more than a few large platelets suggests increased platelet turnover, myeloproliferative disorder, or a congenital macrothrombocytopenia. Some platelet disorders are associated with unique platelet and/or leukocyte morphology. Giant platelets are seen in Bernard–Soulier disease; other macrothrombocytopenia syndromes associated with myosin heavy chain gene defects (MYH9) additionally may have Dohle body-like neutrophil inclusions [6, 11]. In patients with Wiskott–Aldrich syndrome, the platelets may be small. Platelets in the gray platelet syndrome, an alpha granule deficit, are characteristic for being pale, gray, and hypogranular.

Platelet Function Testing

Platelet function testing is used to detect or characterize a qualitative platelet disorder, such as a hereditary or acquired platelet dysfunction [16, 17]. Platelet function testing also has been utilized to assess and monitor the therapeutic effect of antiplatelet drugs, but the clinical utility of such testing is debated [18]. Caution must be exercised in interpreting platelet function testing in thrombocytopenic patients, as abnormal results are often observed due to the low platelet count; distinction of an abnormal result due to intrinsic platelet dysfunction versus thrombocytopenia alone may not be possible [10].

Platelet function test types can be categorized as (1) screening, (2) aggregation, and (3) specialized testing. Within each category, the various platelet functions measured include
adhesion, aggregation, activation, and granule release. Screening platelet tests measure global platelet function and are widely available in clinical laboratories. Platelet aggregation is the most commonly used platelet function test and is available in dedicated hemostasis laboratories. Specialized platelet testing includes more targeted assays for detailed diagnosis and typically is available only in reference laboratories.

**Screening Assays**

The bleeding time was the original platelet screening assay that was developed by Duke in 1910 [19]. It is fraught with variability and poor correlation with bleeding risk and its use has largely been eliminated [20]. Whole blood platelet function assays have been developed as screening assays for platelet function which utilize small stand-alone devices and can be used in laboratories that otherwise could not perform platelet function studies; some can be utilized in the near-patient setting [16, 21]. However, many of these devices are in the early stages of clinical implementation or are targeted toward monitoring of antiplatelet drugs and will not be further discussed in this chapter. These devices include the VerifyNow™ System (formerly Ultegra, Accumetrics, San Diego, CA), the Plateletworks™ (Helena Laboratories, Beaumont, Texas), and the Impact-R (Matis Medical, Israel). Thromboelastometry measures a combination of coagulation, platelet function, and fibrinolysis and is not covered further in this chapter.

**PFA-100**

The PFA-100 System (Siemens Healthcare Diagnostics Inc., Tarrytown, NY) is a device that measures both platelet adhesion and aggregation in whole blood, using a high-shear testing system (Fig. 2.2) [22].

**Specimen Considerations**

Specimens for PFA-100 testing should be collected using a peripheral venipuncture when possible [23]. The PFA-100 utilizes a whole blood specimen collected into a light blue top vacutainer tube containing 3.2 % buffered trisodium citrate. It is recommended that each laboratory should establish its own reference range using blood specimens from normal individuals. Specimens should be kept at room temperature and transported to the laboratory, with testing completed within 4 h of phlebotomy. Guidelines for performing PFA-100 testing have been developed by the Clinical and Laboratory Standards Institute (CLSI) and are included in the guideline for platelet function testing by aggregometry (H58-A) [24].

**Test Performance and Interpretation**

The disposable cartridges contain a membrane coated with aggregation agonists (collagen/epinephrine [EPI] or collagen/adenosine diphosphate [ADP]) with a 147-μm diameter central aperture. A blood sample of 800–900 μL is pipetted into a sample cup. The blood is drawn out of the cup and passed through the aperture at a high shear rate (5000–6000 s⁻¹), where the platelets adhere to the membrane, aggregate, and cause aperture occlusion. When the blood flow ceases, the instrument measures the “closure time,” which is a reflection of platelet function.

A normal closure time indicates normal platelet function, while a prolonged closure time indicates platelet dysfunction; a shortened closure time can be seen with an elevated platelet count or increased platelet function [23, 25]. Prolonged closure times can be seen with intrinsic platelet dysfunction as well as <150,000 platelets/μL or hematocrit <35 %; closure times are not affected by heparin or deficiencies of coagulation factors other than fibrinogen [26]. Due to the limitations of the blood volume in the sample cup, the instrument can only measure closure times up to 300 s. Beyond that, the closure time is reported as >300 s. Duplicate testing is only recommended when the initial result shows a prolonged closure time.

Good quality control must be maintained on the instrument, including daily electronic checks and vacuum checks, as well as validation of each new cartridge lot with a fresh normal sample. External proficiency testing for the PFA-100 has recently become available; this challenge utilizes normal donor blood drawn on site into distributed specially formulated sample collection tubes [27].

The collagen/EPI cartridge is the primary screening cartridge; it detects platelet dysfunction induced by intrinsic platelet defects, von Willebrand disease, or platelet-inhibiting agents [23]. Aspirin-like drugs give a prolonged closure time with the collagen/EPI cartridge and a normal closure time with the collagen/ADP cartridge due to a high ADP concentration in the cartridge. See Fig. 2.2 for a PFA-100 interpretation algorithm. Von Willebrand disease, intrinsic platelet dysfunction, and non-aspirin drugs characteristically give an abnormal closure time with both cartridges. The PFA-100 may not be sensitive to all types of von Willebrand disease and platelet dysfunction. For example, in type 2N von Willebrand disease with decrease of only factor VIII, the PFA-100 will give normal results. Additionally, the PFA-100 may not detect platelet storage pool disorders in some patients or some macrothrombocytopenia disorders.

**Platelet Aggregation**

Platelet aggregation measures the ability of agonists to cause in vitro platelet activation and platelet-platelet binding [28]. As such, platelet aggregation is often useful to distinguish intrinsic platelet disorders involving surface glycoproteins, signal transduction, and platelet granules.

Platelet aggregation testing can be performed either in whole blood or using a suspension of platelets in plasma,
termed platelet-rich plasma (PRP). Blood for platelet aggregation studies should be drawn into an anticoagulant solution of 3.2% sodium citrate. Ideally, blood should be obtained from a peripheral venipuncture. Guidelines for performing platelet aggregation testing have been developed by CLSI (H58A) [24].

**Light Transmission Aggregation**

Platelet aggregation studies are most commonly performed using platelet-rich plasma with optical detection of aggregation (turbidimetry).

**Specimen Considerations**

For optical platelet aggregation assays, the whole blood specimen should be kept at room temperature and transported to the laboratory expeditiously, with testing completed within 4 h of phlebotomy [24]. The first step in sample processing requires the production of PRP by differential centrifugation of erythrocytes and leukocytes, resulting in a top suspension of platelets and plasma.

Prior to testing, the platelet count in the PRP is often normalized to 200,000–250,000/μL by mixing appropriate ratios of PRP and PPP, although some recent studies have suggested that this practice may affect platelet aggregation results, and not adjusting or adjusting the platelet count with saline may be more appropriate [29]. With optical aggregation methodologies, PRP platelet counts <100,000/μL may provide insufficiently turbid samples to provide reliable results. For such samples where the functional evaluation of patients with thrombocytopenia is desired, it may be helpful to adjust the platelet count of a normal sample to a similar low platelet count as a normal “thrombocytopenic” control [10]. The optical aggregation assay may suffer from interference from hemolyzed, lipemic, and icteric plasma.

**Test Performance and Interpretation**

In the optical turbidimetric platelet aggregation assay, platelet aggregation is measured spectrophotometrically by the increase in light transmission after addition of an aggregation agonist in a stirred platelet sample [16, 17, 24, 30]. Agonists typically include ADP, collagen, arachidonic acid, epinephrine, and occasionally thrombin receptor-activating peptide (TRAP) (Fig. 2.3). For optical aggregation, the adequacy of the aggregation response is followed by quantifying the maximal percentage of aggregation or the slope of the aggregation curve.

With use of a subthreshold concentration of agonist, there is typically a primary wave of aggregation, with subsequent disaggregation due to lack of granule release. Optimal platelet aggregation shows a biphasic pattern for the agonists ADP and epinephrine; the initial increase in aggregation is due to primary aggregation in response to activation of the glycoprotein IIb/IIIa platelet membrane receptor, while the second wave of aggregation is the result of platelet degranulation with recruitment of additional platelet aggregates. Lack of a secondary wave suggests a platelet storage pool disorder caused either by reduced numbers of granules or defective release. Other agonists, such as arachidonic acid, thrombin receptor agonists, and collagen, usually show only a single wave of aggregation. Collagen characteristically shows an initial shape change prior to the wave of aggregation. Normal aggregation characteristically results in greater
than 70 or 80% aggregation, but all laboratories should establish their own reference ranges for each agonist.

Another important reagent used in the evaluation of platelet function by aggregation is the antibiotic ristocetin, which facilitates the binding of vWF to the glycoprotein Ib/IX/V complex. A normal ristocetin-induced platelet aggregation (RIPA) result requires the presence of both functional vWF and normal GPIb/IX/V, so RIPA can detect both von Willebrand disease and some platelet dysfunctions, such as Bernard Soulier syndrome (Fig. 2.4).

Many factors can affect platelet aggregation results, such as thrombocytopenia, thrombocytosis, processing technique, processing temperature, stirring rate, and processing time [24]. In addition, clinicians ordering the tests should advise patients to discontinue, if possible, any medication, such as aspirin or nonsteroidal anti-inflammatory agents, which may interfere with the assessment of the test results [4, 5].

A survey of platelet function testing techniques among North American Specialized Coagulation Laboratory Association (NASCOLA) member laboratories revealed that there is a wide variety in practice in the performance of platelet aggregation testing [31]. The NASCOLA study revealed that the majority of laboratories reported results containing quantitative values (% aggregation and/or slope) and a qualitative interpretation. The majority of laboratories utilized the agonists collagen, arachidonic acid, epinephrine, and ristocetin; however, the final test concentrations of these agonists varied widely, sometimes by several orders of magnitude.

There is little data in the literature to define the sensitivity and specificity of agonist concentration for the diagnosis of platelet disorders. However, the median concentrations of the agonists from this survey give some guidance for typical concentrations used in practice. These findings were incorporated into the CLSI guideline for platelet aggregation testing in an attempt to standardize laboratory practice [24, 32]. The standard agonists used include: ADP, 0.5–10 μM, typically 5 μM; collagen, 1.0–5.0 μg/mL, typically 2 μg/mL; epinephrine, 0.5–10 μM, typically 5 μM; ristocetin low dose, ≤0.6 mg/mL; and ristocetin high dose, 0.8–1.5 mg/mL. Arachidonic acid, 0.5–1.6 mM can be added to the panel to interrogate prostaglandin pathways/aspirin effect.

**Whole Blood Aggregometry**

Platelet aggregometry studies can also be performed in whole blood by an impedance technique. [33] Agonists tested include those used in light transmission aggregation (LTA), with the exception of epinephrine since response is only seen in roughly half of patients. The extent of aggregation is determined by submerging an electrode probe assembly in blood. Impedance between two wires in the probe changes as platelets aggregate on their surfaces. Results are typically reported by maximum response with each agonist, measured in ohms. Whole blood aggregation has less specimen-handling required and is performed in a more physiologic milieu than optical aggregation, with inclusion of erythrocytes and leukocytes.
Specialized Platelet Function Assays

Platelet Release Studies

Studies of granule release may be helpful to discern alpha and dense granule storage pool disorders from platelet release disorders. Platelet dense release can be measured by a lumiggregation technique whereby a luciferin-luciferase enzyme reagent that is extracted from fireflies measures dense granule ATP release during aggregation, resulting in luminescence (Fig. 2.3) [34]. Alpha granule release can be measured by the platelet-specific proteins platelet factor 4 (PF4), β-thromboglobulin (βTG) and P-selectin, but these have not been widely used clinically due to stringent sample collection and processing requirements.

Thromboxane Metabolites

Thromboxane A₂ (TXA₂) is synthesized from arachidonic acid by cyclooxygenase (COX)-1 and thromboxane synthase during platelet activation. While TXA₂ has a short half-life, stable metabolites are formed, including TBX₂ in blood and 11-dehydro-TXB₂, which is excreted in urine. Levels of these metabolites serve as an indication of COX pathway activity and may be useful in monitoring response to inhibitors of this pathway, such as aspirin.

Adhesion Assays

Many specialized experimental devices for studying platelet adhesion have been developed, but these are used largely in the research setting. A device in development is the Impact, a modified cone and plate viscometer [35]. The apparatus induces laminar flow to the sample with uniform shear stress (1800 sec⁻¹) between a disposable coverslip and a rotating polystyrene cone and measures shear-induced platelet adhesion and aggregation.

Flow Cytometry

Flow cytometry has been utilized to study platelet structure and function, but this technique is only employed in specialized centers [36, 37]. Flow cytometric analysis is based on
the detection of cell surface proteins by laser light scatter and fluorescently labeled antibodies. With this technique, the expression of a panel of proteins can be analyzed for each platelet individually. Benefits of platelet flow cytometry include the ability to detect the activation state of circulating platelets, to study the reactivity of platelets to specific agonists, and to study platelet function in a very small sample with a relatively low platelet count.

Platelet flow cytometry can be used to detect the presence of typical platelet surface glycoproteins as well as decreased expression or deficiency of these glycoproteins. It has been used to detect the absence of GPIIb/IIIa receptors in patients with Glanzmann thrombasthenia and has been used to study deficiencies of glycoproteins Ia, Ib, IIb, IV, and IX [11, 16, 36, 37]. Platelet activation leads to a conformational change in some surface receptors, and with the use of appropriate antibodies, the percentage of activated platelets in a specimen can be determined. Measurement of platelet activation by flow cytometry has been utilized to diagnose alpha and dense granule storage pool disorders and release/signaling disorders, where measurement of activation-dependent markers, such as mepacrine and P-selectin, is done before and after addition of a platelet agonist, such as TRAP or ADP [38].

**Platelet Turnover (Platelet Reticulocyte Analysis)**

Platelets with increased RNA content (reticulated platelets) can be measured by flow cytometry using dyes that bind to RNA and DNA, such as thiazole orange, auramine O, and coriphosphine [14]. Reticulated platelet analysis has been studied as a diagnostic tool to evaluate whether thrombocytopenia is due to increased platelet destruction or decreased platelet production, as platelets newly released from bone marrow have increased RNA content. It is anticipated that implementation of reticulated platelet counts may help to avoid bone marrow examination in some individuals with thrombocytopenia [39]. The immature platelet fraction (IPF) on the Sysmex XE and XN-series analyzers (Sysmex Corp, Kobe, Japan), where a nucleic acid-specific fluorescent dye is detected in platelets, has been shown to be useful in the diagnosis of peripheral platelet consumption and as a guide to transfusion after hematopoietic stem cell transplantation [40].

**Electron Microscopy**

Electron microscopy (EM) may be utilized for the ultrastructural evaluation of platelets. Wholemount EM techniques have been developed for assessing storage pool disorders, while thin section EM is utilized for assessing ultrastructural morphology [41]. In patients with suspected dense granule storage pool disorders, whole mount EM shows a decrease or absence of the organelles (cytoplasmic dense bodies) storing adenine nucleotides, serotonin, and calcium.

**Platelet Genetic Testing**

Genetic testing for diagnosis of platelet disorders is not widely available. However, targeted mutation analysis and sequencing of some genes associated with inherited platelet disorders, such as $MYH9$ (May–Hegglin Anomaly, Sebastian, Fechtner and Epstein syndromes, etc.), $HPS1$ and $HPS3$ (Hermansky-Pudlack), $MPL$ (congenital amegakaryocytic thrombocytopenia), and WAS (Wiskott–Aldrich syndrome, X-linked thrombocytopenia), is available at some clinical reference laboratories [42, 43]. Panels are also offered to detect mutations in the genes in the GPIb/IX complex to assay for Bernard-Soulier syndrome or the GP Ib/IIIa complex in Glanzmann thrombasthenia. In patients with $MYH9$ disorders, immunofluorescence analysis of myosin IIa can highlight abnormal protein localization within the neutrophils; the pattern of localization correlates to the site of $MYH9$ mutation.

**Summary**

Laboratory testing for platelet function is more complex than plasma-based assays for coagulation proteins because of the cellular nature of platelets. Platelet testing has mainly been limited to large medical centers, but there has been significant technological development of newer platelet function assays that have brought some platelet function testing capabilities to smaller laboratories and point-of-care settings.

**References**


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