# Diagnosis of Platelet Function Disorders: A Challenge for Laboratories

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## Abstract

In patients with normal plasmatic coagulation and bleeding tendency, platelet function defect can be assumed. Congenital platelet function defects are rare. Much more commonly they are acquired. The clinical bleeding tendency of platelet function defects is heterogeneous, which makes diagnostic approaches difficult. During the years, a large variety of tests for morphological phenotyping and functional analysis have been developed. The diagnosis of platelet function defects is based on standardized bleeding assessment tools followed by a profound morphological evaluation of the platelets. Platelet function assays like light transmission aggregation, luminoaggregometry, and impedance aggregometry followed by flow cytometry are commonly used to establish the diagnosis in these patients. Nevertheless, despite great efforts, standardization of these tests is poor and in most cases, quality control is lacking. In addition, these tests are still limited to specialized laboratories. This review summarizes the approaches to morphologic phenotyping and platelet testing in patients with suspected platelet dysfunction, beginning with a standardized bleeding score and ending with flow cytometry testing. The diagnosis of a functional defect requires a good collaboration between the laboratory and the clinician.

**Keywords**

- platelet function defect
- light transmission aggregometry
- inherited platelet disorder
- granule defect

## Introduction

Platelets play the key role in primary hemostasis. They promote blood vessel repair and maintain the integrity of the vascular system. Well-balanced complex inhibitory and activating pathways are responsible for platelet activation, granule release, platelet aggregation, and thrombus formation. The perioperative bleeding risk in patients with a platelet function defect is considerably higher than in patients with thrombocytopenia (24.8 vs. 13.4%, respectively).[1] Therefore, the diagnosis of impaired hereditary and acquired platelet function defects is important to stratify perioperative risk correctly and guide perioperative management. Perioperative intake of drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) affects platelet function and may lead to a clinically relevant bleeding disorder. In addition, other drugs such as antibiotics, cyclic adenosine monophosphate stimulators, phosphodiesterase inhibitors, calcium channel blockers, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers can induce platelet dysfunction and increase bleeding risk.[2]

The assessment of the platelet function is too complex for routine coagulation laboratories. Platelet function tests are time consuming, poorly standardized, and require lots of experience in the field. External quality control testing is rare and not available for most of the methods. National and international surveys revealed interlaboratory differences, and thus methodical standardization is required.[3–6]

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## Etiology of Platelet Function Defects

Platelet function defects are a common cause for bleeding tendency. They are often acquired due to drug treatment or procedures, medical conditions, or hematologic diseases.[7] Also herbal remedies, nutritional supplements, food, and beverages can influence platelet function.[8] Congenital platelet defects are rare and often misdiagnosed. For example, in patients with thrombocytopenia, the misdiagnosis of immune thrombocytopenia is described in up to 61%.[9] Functional defects can affect platelet adhesion, activation, or aggregation. Among others, there are receptor defects, granule defects, transcription factor defects, signal transduction defects, cytoskeleton defects, and defects of the phospholipid membrane (►Table 1).

## Diagnostic Approaches

### Bleeding Scores

Standardized bleeding risk assessment with bleeding scores is a recommended strategy for the evaluation of patients' bleeding risk before surgery. The most commonly used bleeding score is the International Society of Thrombosis and Hemostasis Bleeding Assessment Tool (ISTH-BAT) bleeding score.[10] This bleeding score was primarily validated for inherited bleeding disorders with von Willebrand disease platelet function defects and has since also been used for the assessment of bleeding tendency in different patient groups.[10,11] Nevertheless, its diagnostic value in the perioperative prediction of bleeding risk in patients with only a mild bleeding tendency was low.[12,13] Koscielny et al introduced a standardized questionnaire concerning bleeding history in combination with laboratory testing and was able to verify impaired hemostasis in 40.8% of the patients with positive bleeding history.[14]

### Blood Count

Measurement of platelet count in automated cell counters enables a first evaluation. Furthermore, mean platelet volume can discriminate between small and large platelets. Some cell counters also determine the immature platelet fraction, which is a sign of an increased platelet turnover.

### Morphological Evaluation of Platelets: Microscopy

In a May–Grünwald–Giemsa-stained blood smear of EDTA-anticoagulated blood, platelet size and morphology can be assessed. Patients suffering from Wiskott-Aldrich syndrome, for example, have small size platelets, whereas patients with Bernard–Soulier syndrome or a myosin-9 (MYH9)-related disease show a low platelet count and giant platelets (►Fig. 1A–D). MYH9-related diseases also present with inclusion bodies in neutrophils (Döhle-like bodies). Platelet aggregates can be found in a pseudothrombocytopenia as a preanalytic artifact when using EDTA tubes. In α granule diseases, large pale platelets can be observed.

Immunofluorescence microscopy offers the possibility of phenotyping platelets via monoclonal antibodies against receptors, granule markers, and cytoskeletal substances. The major advantage is the necessity of a very small amount of blood for this method (3.5 mL per blood smear). Intravenous blood and capillary blood can be used for the preparation of blood smears. Air-dried blood smears are stained after permeabilization and fixation (►Fig. 2A–D). This is an effective method to recognize receptor defects such as Glanzmann thrombasthenia or Bernard–Soulier syndrome. In patients with MYH9-related diseases, staining the nonmuscular myosin heavy chain IIA (NMMHCII2A), a cytoskeletal contractile protein, cytoplasmic inclusion bodies within the granulocytes show typical clusters. With this finding, genetic testing is no longer crucial for the diagnosis of MYH9-related disease.[15] Also alterations in granule content can be visualized. Good morphologic characterization makes an important contribution to the reassessment of the likely pathogenic variants in platelet genes that cause the bleeding tendency in these patients.[16] A combination of light microscopy and immunofluorescence microscopy allows the diagnosis of the underlying cause of inherited platelet disorders in up to 30% of patients with a suspected inherited platelet disorder.[17]

### In Vivo Bleeding Time

The bleeding time (BT) was first described by Duke and coworkers and later standardized by Ivy et al to assess the platelet function as the first step of primary hemostasis. After a defined skin puncturing on the forearm with a back pressure of 40 mm Hg, bleeding was noted every 30 seconds with filter paper strips, without touching the wound edges until no more blood stained the filter paper.[18] In previous studies, BT showed a low predictive value for perioperative bleeding in unselected patients, but in another study, it seemed useful to predict bleeding in patients with positive bleeding history.[19,20] In patients with recent intake of NSAIDs such as aspirin, BT was not recommended as a reliable method to identify the corresponding defects.[19] Due to poor standardization, BT cannot be recommended in routine assessment.

### In Vitro Bleeding Time

The in vitro equivalent to the in vivo BT is the platelet function analyzer-200 (PFA-200 [Siemens Healthcare GmbH, Erlangen Germany]). It allows for a quantitative measurement of the primary platelet-derived hemostasis at higher shear stress.[21] Briefly, citrated whole blood is led through a capillary toward the aperture of a collagen adenosine diphosphate (ADP) or collagen epinephrine-coated membrane system. As it is a von Willebrand factor–dependent method, it is suitable for the detection of mild to severe von Willebrand syndrome, but it has a low sensitivity for mild platelet function defects and storage pool defects.[22,23] In addition, this method is not reliable in patients with a low platelet count ($<100 \times 10^9$/L) and/or a low hematocrit (<30%). Due to low sensitivity, it is not recommended by the ISTH for the diagnosis of platelet function defects.[24]

### Viscoelastometry

The viscoelastometry is a well-established method for the detection of trauma-induced coagulopathy. The thromboelastography

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**Table 1** Summary of the main platelet defects and diagnostic strategies (adapted from Althaus et al)[56]

| Platelet defect | Molecular mechanism | Light transmission aggregometry | Flow cytometry | Further diagnostic |
|---|---|---|---|---|
| **Receptor defect** | | | | |
| Glanzmann thrombasthenia | Reduced or absent expression or dysfunction of GP IIb–IIIa | Absent aggregation with all agonists, normal agglutination with Ristocetin | GP IIb–IIIa complex absent or reduced | IB, IF, genetic testing |
| Bernard–Soulier syndrome | Reduced or absent expression or dysfunction of GP Ib–IX–V | Normal aggregation with all agonists, absent agglutination with Ristocetin | GP Ib–IX–V complex absent or reduced | IB, IF, genetic testing |
| Platelet-type von Willebrand | Increased affinity of the GP Ib–IX–V receptor to the von Willebrand factor | Increase agglutination with Ristocetin at low concentration | Increased binding of the Willebrand factor to the GP Ib–IX–V complex | Genetic testing |
| Collagen receptor defect | Reduced or absent expression or dysfunction of GP Ia–IIa or GP VI | Absent or reduced aggregation after induction with collagen, CRP or convulxin | GP Ia–IIa complex or GP VI absent or reduced | IB, IF, genetic testing |
| **Granule defect** | | | | |
| Alpha granule defect (gray platelet syndrome, GFIB defect, Paris-Trousseau syndrome | Absent α granule, release defect in gray platelet syndrome and GFI1B defect, or increased α granules with release defect in patients with Paris-Trousseau syndrome | No uniform pattern | Reduced expression of CD62P after activation, variable phenotype | Blood smear, IF, genetic testing |
| Delta granule defect (e.g., Hermansky–Pudlak syndrome, Chediak–Higashi syndrome) | Absent delta granules or release defect | Reduced aggregation with ADP, absent or reduced second wave, disaggregation, no uniform pattern | Reduced expression of CD63 after activation | ATP-release, mepacrine uptake, IF, EM, genetic testing |
| Alpha–delta granule defect | Absent or reduced α and delta granules or release defect | No uniform pattern | Reduced expression of CD63 and CD62P after activation, no uniform pattern | Blood smear, mepacrine uptake, IF, EM, genetic testing, ATP-release |
| **Transcription factor defect** | | | | |
| *Familial* thrombocytopenia with malign association | Mutation in RUNX1 | No uniform pattern | Normal | Presence of MYH10 in platelets, IF, IB, genetic testing |
| FLI1-associated granule defect | Defect in FLI1 | No uniform pattern | Normal | Presence of MYH10 in platelets, IF, IB, genetic testing |
| GATA1-defect | Defect in GATA1 leads to thrombocytopenia | No uniform pattern | Normal | Blood smear (macrothrombocytopenia), genetic testing |
| ETV6 | Defect in ETV6 induces mild thrombocytopenia | No uniform pattern | Normal | Presence of ETV6 in platelets, IF, IB, genetic testing |

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**Table 1** (*Continued*)

| Platelet defect | Molecular mechanism | Light transmission aggregometry | Flow cytometry | Further diagnostic |
|---|---|---|---|---|
| **Signal transduction defect** | | | | |
| G-protein defect | According to the G-protein reduction in thrombin receptor (PAR-1, PAR-4), ADP-receptor (P2Y1 and P2Y12), thromboxane receptor, epinephrine receptor, serotonin receptor) | No uniform pattern | No uniform pattern | Genetic testing |
| Phospholipase defect | Defect in cytosolic phospholipase A2α or phospholipase C | No uniform pattern | No uniform pattern | Genetic testing |
| Aspirin-like defect | Defect in the cyclooxygenase pathway (defect of the COX1 enzyme or thromboxane synthesis) | Absent or reduced aggregation with arachidonic acid, optional absent second wave after ADP induction | Normal | Thromboxane A2 measurement, genetic testing |
| **Defect in cytoskeleton** | | | | |
| Wiskott-Aldrich syndrome | Microthrombocytopenia infect tendency, eczema | Normal | Normal | Blood smear, genetic testing |
| Filamin A defect | Macrothrombocytopenia | Disturbed aggregation after induction with Convulxin | Normal | IF (only some platelets do not have filamin A) |
| MYH9-related disease | Macrothrombocytopenia | Absent shape change after collagen induction, nearly normal platelet aggregation | Normal | IF (inclusion bodies in the neutrophils) |
| **Defect in the phospholipid membrane** | | | | |
| Scott syndrome | Impaired expression of the negatively charged phospholipids in activated platelets | Normal | Impaired Annexin binding | Reduced thrombin generation |
| Stormorken syndrome | Increased expression of negatively charged phospholipids in resting platelets | Normal | Increased Annexin binding | Increased thrombin generation |

Abbreviations: ADP, adenosine diphosphate; EM, electron microscopy; ETV6, erythroblast transformation specific (ETS) variant transcription factor 6; FLI1, Friend leukemia integration 1 transcription factor; GATA1, erythroid transcription factor; GP, glycoprotein; IF, immunofluorescence microscopy; IB, immunoblot; MYH9, myosin-9; MYH10, myosin-10; PAR, protease-activated receptor; P2Y1 and P2Y12, purinergic receptors subtypes Y1 and Y12; RUNX1, runt-related transcription factor 1.

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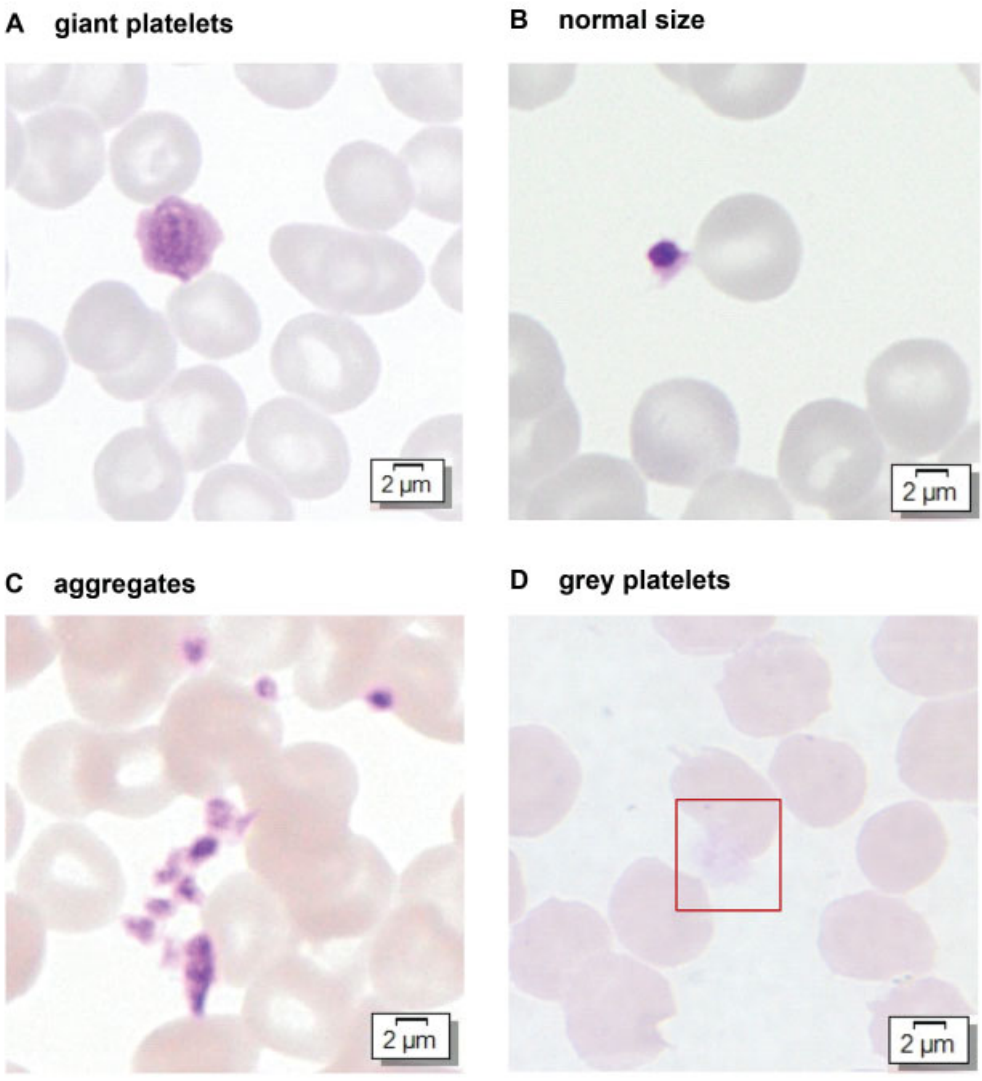


**Fig. 1** Light microscopy as the first step of morphological evaluation. (**A**) Typical light microscopy of a patient with giant platelets. Platelets are nearly as large as a normal red blood cell. (**B**) Normal size platelet from a healthy control. (**C**) An anticoagulant-related false low platelet count as a result of a spontaneous aggregate formation in vitro. (**D**) Patients with gray platelet syndrome have enlarged, but not giant platelets. They show a gray appearance in Giemsa staining.

(TEG; Haemonetics Corp., Boston, Massachusetts, United States), rotational thromboelastometry (ROTEM; Instrumentation Laboratory, Bedford, Massachusetts, United States), and the ClotPro (Haemonetics Corp.) characterize in different parameters the clot initiation (coagulation time, clot formation time), the clot strength and stiffness (maximal clot firmness) and clot resolution/lysis (maximal lysis). Functionally intact platelets and fibrinogen are required for the development of a stable viscoelastic thrombus. While viscoelastometry is not a sensitive method for the detection of platelet disorders, it still plays a role in perioperative and trauma management as an indicator of severe thrombocytopenia and/or extensive platelet function inhibition.[25,26]

### Whole-Blood Impedance Aggregometry

The impedance-based electrode aggregometry allows the assessment of platelet function in a point-of-care setting in hirudin-anticoagulated whole blood without further sample processing. The platelet aggregation is measured by two electronic sensors and the area under the curve is calculated separately by each sensor. It requires a minimal technical knowledge of the operator. It was introduced as a reproducible and sensitive method for the evaluation of antiplatelet drug therapy and assessment of platelet function.[27]

However, whole blood impedance aggregometry was not able to discriminate between patients with and without platelet function defects detected by light transmission aggregometry (LTA) and is therefore not sensitive enough to detect mild platelet function defects.[22]

In addition, large, randomized trials have failed to demonstrate superiority of platelet function monitoring to adjust individually the dual-antiplatelet therapy in cardiovascular disease.[28–30] This led the European Society of Cardiology to recommend that whole blood impedance aggregometry may

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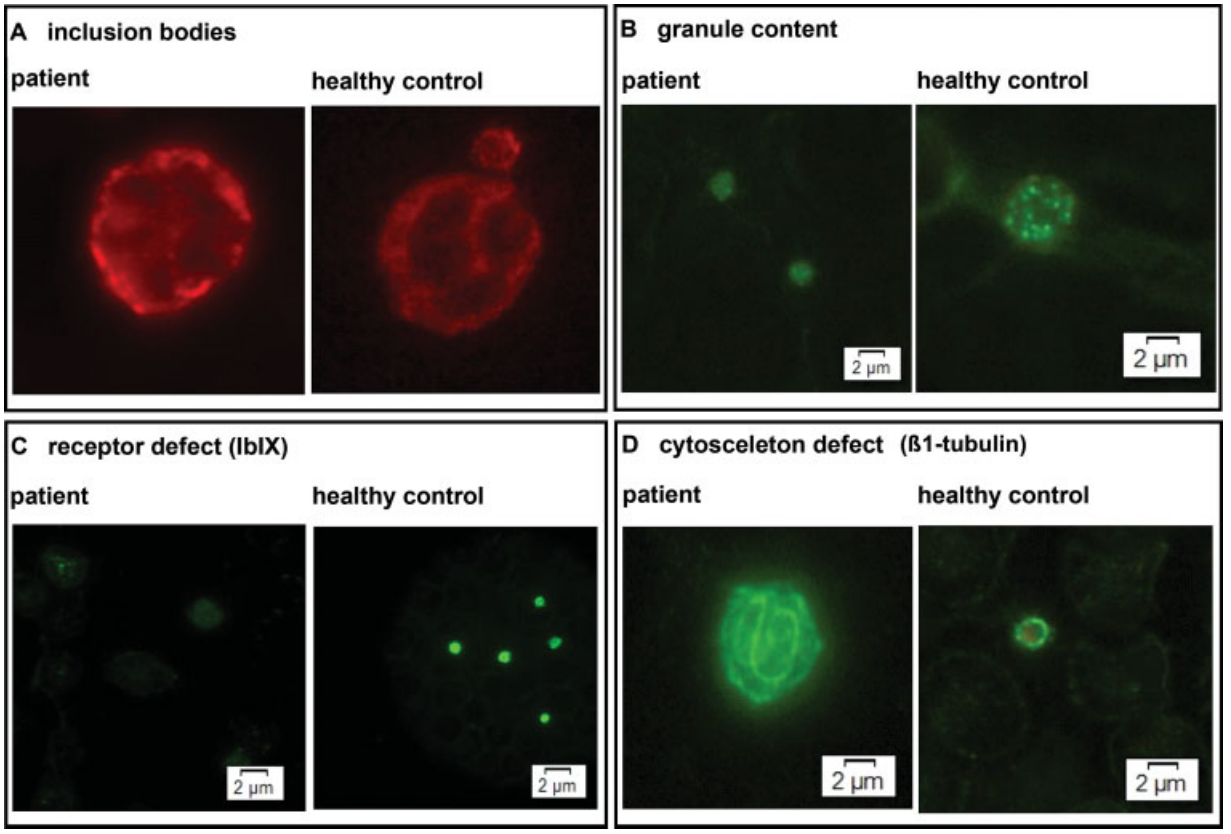


**Fig. 2** Representative immunofluorescence staining of patients and healthy controls with different diseases. (**A**) Immunofluorescence staining of a patient with MYH9-related disease. Nonmuscular myosin heavy chain IIA (NMMHC-IIA) is stained with an anti-monoclonal antibody. Neutrophils show typical clusters. (**B**) Representative immunofluorescence microscopy of a patient with storage pool disease. CD63 is absent in this patient. (**C**) Enlarged platelets of a patient with Bernard–Soulier syndrome. (**D**) β1-Tubulin defects are associated with enlarged platelets and alteration of the β1 tubulin distribution. It resembles a ball of wool. In the healthy control, a typical β1-tubulin ring is visible.

be considered only for specific situations such as in patients with recurrent adverse bleeding events or recurrent thromboembolic events. In these cases, the results may change the treatment strategy.[31]

### Light Transmission Aggregometry According to Born

The LTA is the gold standard in platelet function testing and was first described by Born in 1962 (►Fig. 3A–D).[32] It records changes in light transmission caused by agonist-induced platelet aggregation in platelet-rich plasma (PRP). It is fully dependent on fibrinogen-induced platelet aggregation via glycoprotein (GP) IIb–IIIa or von Willebrand–dependent platelet agglutination via the GP Ib–IX–V receptor. It is recommended as a first-line diagnostic approach by the ISTH and several surveys and recommendations aim to increase standardization of the procedure.[3–5,33,34] Using different agonists in different concentrations allows the differentiation and characterization of specific platelet defects.

According to the ISTH, recommended agonists are epinephrine (α2A receptor) in a concentration of 5 µM and use of higher concentrations in case of impaired platelet function. The diagnostic panel should also include ADP (purinergic receptors subtype Y1 and Y12, P2Y1, and P2Y12 receptor) with the lowest concentration of 2 mM and Horn Collagen (GP Ia–IIa and GP VI receptor, minimum concentration of 2 mg/mL), thrombin receptor activating peptide-6 activating the protease-activated receptor 1 in a concentration of 10 µM. To detect aspirin intake, the additional use of the thromboxane A2-mimetic U46619 (1 µM) and the use of arachidonic acid (1 mM, and higher concentration in case of impaired function) can be considered. Finally, the use of the agglutination agent Ristocetin (1.2 mg/mL, 0.5–0.7 mg/mL) detects the von Willebrand–mediated agglutination of platelets via the GP Ib–IX–V receptor.[5]

The platelet aggregation curve should be evaluated for the presence of shape change, length of the lag phase, slope of aggregation, maximal amplitude or % aggregation at the end of the monitoring period, disaggregation, and the presence of a "secondary wave" induced by epinephrine.[5] For example, for the detection of defects in the granule release, the use of a low agonist concentration is important (missing amplification of the agonist-induced ADP/adenosine triphosphate [ATP] release from the granules as seen in higher agonist concentrations). Defects in the cytoskeleton can cause an absence of shape change with otherwise nearly normal platelet function (missing shape change in giant platelets from patients with MYH9-related diseases). Defects in the GP IIb–IIIa receptor induces the complete absence of platelet aggregation with normal agglutination induced by Ristocetin via the GP Ib–IX receptor (►Fig. 4A).

LTA usually requires a normal platelet count (150–600 × $10^9$/L) in PRP,[5] but optional control samples adjusted

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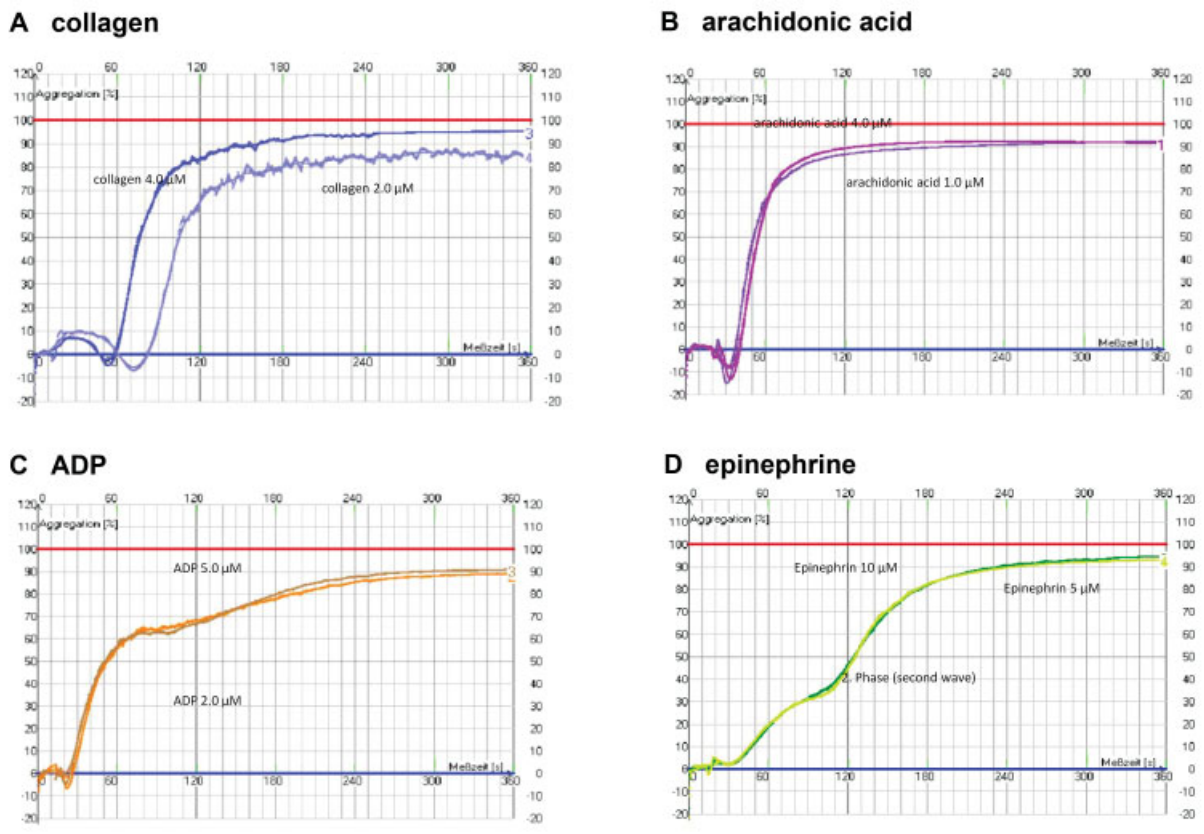


**Fig. 3** Classical light transmission aggregometry in a healthy control. (**A**) Representative normal tracings of collagen with typical shape change. (**B**) Arachidonic acid is converted into thromboxane A2 in platelets. In patients with aspirin intake or with defects in the cyclooxygenase pathway, aggregation response is absent. (**C**) ADP can bind to the ADP receptor on the surface of platelets. The binding initiates an intracellular calcium release which reflects the first wave of aggregation. The second wave is induced by the release of ADP from delta granules. (**D**) Epinephrine binds to the α2-adrenergic receptors on the surface of platelets. This induces an inhibition of the adenylyl cyclase and the release of calcium ions. It is also inhibited by aspirin and NSAIDs and the wave is similar to ADP.

with buffer with comparable platelet count may serve as a laboratory reference control to assess platelet function in patients with low platelet count.[35] Sometimes, especially in patients with enlarged platelets, it is difficult to enrich platelets. In these patients, it can be feasible to generate PRP with lower centrifugation force or spontaneous sedimentation.

A modification from the LTA is the additional bioluminescent determination of the ATP release via luciferin–luciferase reaction. Oxidative decarboxylation of luciferin in the

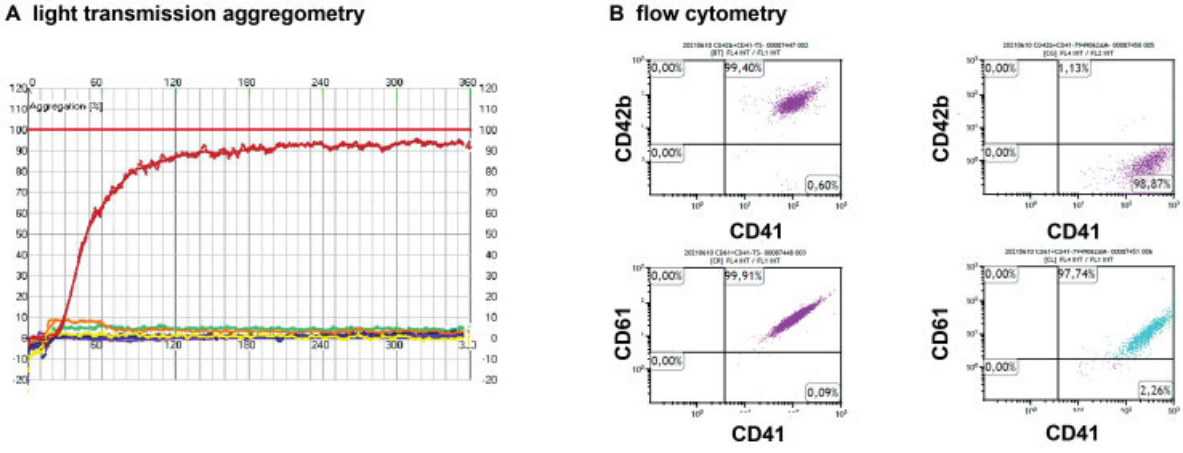


**Fig. 4** Representative light transmission aggregometry and flow cytometry of a patient with Glanzmann thrombasthenia. (**A**) Patients with Glanzmann thrombasthenia show an absent aggregation to all agonists except ristocetin. Ristocetin induces an agglutination through von Willebrand factor and the GP Ib–IX receptor. (**B**) Flow cytometry of platelets of this patient showed a normal binding to CD61 with absent activity with CD41.

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presence of ATP results in a light emission which can be detected. It is evaluated with whole blood and PRP for the detection of delta-granule defects.[36]

### Flow Cytometry

Investigation of platelet function defects via flow cytometry can evaluate GP expression and granule release. Via fluorescence-labeled antibodies, GP receptors are detected like the collagen receptor GP Ia–IIa (α2β1, CD49/CD29), the GP IIb–IIIa receptor (GP IIb–IIIa = αIIbβ3, CD41/61), and the GP Ib–IX–V receptor (GP Ibα [CD42b], GP Ibβ [CD42c], GP IX [CD42a], and GP V [CD42d]; ►Fig. 4 B).

Furthermore, flow cytometry is able to detect several aspects of platelet activation status in response to a variety of platelet agonists. Alpha granule release, for example, can be detected by P-selectin and thrombospondin expression.[37,38] CD40L release can be measured as a marker for activated platelets.[39] Agonist-induced dense granule and lysosome exocytosis enable the detection of mepacrine uptake (dense granule),[40,41] CD63 expression (dense granules, granulolysin),[42,43] Lamp 2 (dense granules and lysosomes),[44] and Lamp 1 (lysosomes).[45]

Changes in intracellular calcium levels via Fluo-3,[46] or measurement of phosphorylation events like vasodilator-stimulated phosphoprotein or serine-threonine kinase signaling, also enable the assessment of platelet activation status even for a small sample size and in case of thrombocytopenia.[47–49]

Flow cytometry is not used solely for the detection of GP expression and platelet activation status. Another approach is the detection of procoagulant platelets, which enhance thromboembolic events via increased phosphatidylserine expression.[50,51]

### Immunoblot

The immunoblot is an immunodetection of blotted proteins by antibodies, after protein extraction from the platelets, electrophoresis, and transfer of the extracted proteins to a gel. It can produce qualitative and semiquantitative data about the protein of interest in platelets. Immunoblot is an important confirmatory step in the classification of new platelet diseases. It is not a routine method and reserved for research purposes.

### Genetic Testing

Genetic testing is an important diagnostic tool in the identification of inherited platelet disorders. Claire Lentaigne described 51 causative genes to be responsible for inherited platelet disorders.[52] Genetic testing can serve as confirmation of the results of morphologic and functional testing and, in some cases, allow for better clinical decision making. For example, in MYH9-related diseases, several mutations have been described as hotspot regions for nonhematologic renal impairment.[53] In these cases, the early administration of angiotensin II receptor type 1 (AT1) antagonists may have beneficial effects.[54]

Some platelet syndromes are also associated with an increased risk of malignancies,[55] and require a detailed genetic counseling for the patient. The patient should be well informed to carefully decide whether she or he wishes to receive genetic information or not and that this test result might include information about the risk of future illness like malignancies.

Despite the advances in functional testing during the years, the diagnosis of platelet function defects remains challenging. It requires a good collaboration between the clinician and the laboratories. Multiple assays are necessary to allow narrowing down the diagnosis in platelet function defects. A good characterization of platelet function defects should include bleeding history in a standardized bleeding assessment tool, platelet morphology by microscopy (Giemsa staining and immunofluorescence), as well as functional testing. An unexpected test result should always be repeated and all consumed drugs and their drug interactions should be taken into consideration in unexpected bleeding situation. This assessment will also improve the genetic testing, as many causative genetic defects are still unknown and good characterization is needed to prove new genes and unknown variants as a causative reason for special defects.

In conclusion, a specialized diagnostic approach is important to identify the platelet function defects in patients with a bleeding tendency. With a better characterization of the defects in platelets, it offers a new opportunity for individualized medicine in patients with acquired defects and to avoid unnecessary treatment in patients with inherited platelet defects.

### Conflicts of Interest

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