

# The Diagnostic Assessment of Platelet Function Defects

## Part 2: Update on Platelet Disorders

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### Abstract

Congenital platelet disorders are rare and targeted treatment is usually not possible. Inherited platelet function disorders (iPFDs) can affect surface receptors and multiple platelet responses such as defects of platelet granules, signal transduction, and procoagulant activity. If iPFDs are also associated with a reduced platelet count (thrombocytopenia), it is not uncommon to be misdiagnosed as immune thrombocytopenia. Because the bleeding tendency of the different platelet disorders is variable, a correct diagnosis of the platelet defect based on phenotyping, function analysis, and genotyping is essential, especially in the perioperative setting. In the case of a platelet receptor deficiency, such as Bernard–Soulier syndrome or Glanzmann thrombasthenia, not only the bleeding tendency but also the risk of isoimmunization after platelet transfusions or pregnancy has to be considered. Platelet granule disorders are commonly associated with either intrinsically quantitative or qualitative granule defects due to impaired granulopoiesis, or granule release defects, which can also affect additional signaling pathways. Functional platelet defects require expertise in the clinical bleeding tendency in terms of the disorder when using antiplatelet agents or other medications that affect platelet function. Platelet defects associated with

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hematological-oncological diseases require comprehensive information about the patient including the clinical implication of the genetic testing. This review focuses on genetics, clinical presentation, and laboratory platelet function analysis of iPFs with or without reduced platelet number. As platelet defects affecting the cytoskeleton usually show thrombocytopenia, but less impaired or normal platelet functional responses, they are not specifically addressed.

## Introduction

Platelets play a crucial role in primary hemostasis and in the amplification of thrombin generation during secondary hemostasis. They are produced by megakaryocytes in the bone marrow and lungs<sup>1</sup> and circulate as small, granular, anucleate cytoplasmic fragments. Under physiological conditions, their concentration ranges between 150,000 and 450,000/ $\mu$ L in children and adults. When endothelial damage occurs, subendothelial structures like collagen become exposed, recruiting the plasmatic von Willebrand factor (VWF). Platelets adhere to these structures and form a primary hemostatic plug, wherein platelet-to-platelet interaction is achieved through cross-linking by fibrinogen and VWF. Concurrent platelet activation is characterized by a change in shape, exposure of anionic phospholipids, and secretion of granule cargo. These mechanisms lead to the formation of a dense clot pattern, providing a surface for the cell-based coagulation cascade to proceed, allowing for fibrin formation and further clot stabilization.<sup>2,3</sup>

Inherited platelet function disorders (iPFs) can affect platelet production, morphology, and function, with clinical presentations varying in severity.<sup>4</sup> Typical clinical manifestations are mucocutaneous and gastrointestinal bleeding, menorrhagia, epistaxis, easy bruising, and bleeding from the oral cavity.<sup>5</sup> The bleeding type and severity of functional platelet defects can be assessed using the ISTH-BAT *bleeding* score.<sup>6</sup> In general, it is difficult to predict the bleeding tendency from genetic findings only<sup>7</sup> since most mutations are private.<sup>8</sup> The application of next-generation sequencing, including whole exome sequencing, will be able to identify rare defects and benefit the diagnostic procedures but national and international networks with bioinformatics will be important to analyze the results.<sup>9</sup>

This review article summarizes the current knowledge on iPFs ([~Supplementary Table S1](#) (available in the online version only) with a focus on genetics, clinical presentation, and laboratory platelet function analysis to support physicians and laboratory staff in the differential diagnosis of platelet function defects.

## Glanzmann Thrombasthenia

Glanzmann thrombasthenia (GT) is a rare autosomal recessive inherited platelet function disorder caused by deficiency or dysfunction of the integrin  $\alpha$ IIb $\beta$ 3 (glycoprotein [GP] IIb/IIIa).<sup>10</sup>  $\alpha$ IIb $\beta$ 3 allows binding of fibrin(ogen) and VWF

upon platelet activation, which is essential for aggregation.<sup>11,12</sup> Lack of functional  $\alpha$ IIb $\beta$ 3 in GT becomes clinically relevant in homozygous or compound heterozygous states. While there are also rare cases of acquired GT, due to autoantibody formation, most cases of GT are inherited and common, seeking medical attention for the first time during childhood.<sup>13</sup> Clinical presentations of GT range in severity, and life-threatening or fatal bleedings can also occur spontaneously and as early as during birth. Clinically relevant bleeding is treated with antifibrinolytics, platelet concentrates, and/or recombinant-activated factor VII concentrates.<sup>12,13</sup>

As in other bleeding disorders, the bleeding history is the starting point for the diagnostic workup, preferably using a standardized bleeding assessment tool.<sup>14,15</sup> However, GT may also remain undiagnosed until invasive procedures are performed.<sup>10,13,16</sup>

Platelets of patients with GT usually appear normal regarding phenotype and number in microscopic blood smears, except for autosomal dominant *ITBG3* variants with thrombocytopenia, enlarged platelets, and anisocytosis.<sup>12,17</sup> The platelet function analyzer (PFA), as a tool for the global assessment of primary hemostasis, is considered to have a high sensitivity for severe iPFs such as GT, but low sensitivity for mild iPFs. Similar findings have been reported for whole-blood impedance aggregometry.<sup>18–20</sup> In contrast, light transmission aggregometry (LTA) remains the gold standard for platelet function analyses, revealing GT by reduced aggregation pattern upon stimulation with all agonists except ristocetin. The latter is because ristocetin-mediated binding of VWF to platelet GPIIb $\alpha$  directly results in platelet clumping due to major passive agglutination and not to fibrinogen-dependent aggregation. In addition, Glanzmann platelets present with disturbed clot retraction and adhesion on fibrinogen due to loss or impaired  $\alpha$ IIb $\beta$ 3 integrin outside-in signaling. Nevertheless, in the case of a typical aggregation pattern of Glanzmann's disease (no/impaired response to all agonists except ristocetin), other diseases (e.g., myeloproliferative disorders with activation defects in  $\alpha$ IIb $\beta$ 3 or antiplatelet  $\alpha$ IIb $\beta$ 3 therapy with abciximab, eptifibatide, tirofiban) and a preanalytical artifact (low temperature during sample transport) should also be considered in the differential diagnosis. For instance, autoantibodies or paraproteins that bind to the receptor can lead to loss of function without platelet destruction. However, this is an acquired Glanzmann, which must be distinguished from the congenital variant.<sup>21</sup> Distinguishing between these two

forms involves several clinical and laboratory investigations. Family history and onset of symptoms can help differentiate between congenital and acquired disease. In addition, Glanzmann platelets present with disturbed adhesion on fibrinogen and clot retraction.

Flow cytometric analysis (FCA) of platelet GP expression should be performed when GT is suspected. In FCA, the binding of an antibody that recognizes the extracellular domain of  $\alpha\text{IIb}\beta 3$  is notably reduced or missing. Furthermore, platelet activation analysis detecting the binding of an antibody specific for activated  $\alpha\text{IIb}\beta 3$  (PAC-1) should be applied.<sup>22</sup> The advantages of FCA include low sample volume, feasibility even in pronounced thrombocytopenia, and the ability to identify heterozygous GT carriers. However, this requires a careful gating strategy and examination of first-degree relatives should be included. It is essential to acknowledge the importance of appropriate reference interval validation of the method employed, especially to classify GT subtypes based on residual expression (I: <5–10%; II: >5–10%; III: normal expression of nonfunctional protein).<sup>13,23</sup> However, the severity of bleeding in patients with GT varies markedly. In addition to FCA, immunofluorescence microscopy can be applied. This involves treating fixed blood smears with antibodies of corresponding specificity to  $\alpha\text{IIb}\beta 3$  integrin or its subunits, then analyzing their binding using fluorochrome-conjugated secondary antibodies.<sup>24</sup> Molecular genetics of targeting mutations in the coding of *ITGA2B* and *ITGB3* genes should be performed to confirm the inherited platelet disorder.<sup>10</sup>

Upon establishment of the diagnosis of GT, all patients should be human leukocyte antigen (HLA)-typed and have a baseline human platelet antibody (HPA) and human leukocyte antigen antibody screen, as the risk of isoantibody formation after platelet transfusions against the missing  $\alpha\text{IIb}\beta 3$  integrin is high. This is of clinical importance especially in pregnant patients, since anti- $\alpha\text{IIb}\beta 3$  antibodies may cross the placenta and cause fetal/neonatal thrombocytopenia.<sup>25,26</sup>

## Bernard–Soulier Syndrome

In 1948, Jean Bernard and Jean-Pierre Soulier published the first description of a patient with inherited thrombocytopenia, prolonged *in vivo* bleeding time, and enlarged platelets.<sup>27</sup> Bernard–Soulier syndrome (BSS) is a rare hereditary, most likely underdiagnosed bleeding disorder with variable macrothrombocytopenia, petechiae, epistaxis, gingival bleeding, menorrhagia, rare gastrointestinal bleedings, and severe bleedings associated with trauma or surgery.<sup>7,28,29</sup> Almost 50% of the patients with BSS have been misdiagnosed and treated as immune thrombocytopenia in the past.<sup>8</sup>

BSS patients may have quantitative and/or qualitative defects of the platelet receptor GPIb/IX/V, which is composed of four subunits: glycosylated GPIb $\alpha$  is attached by a disulfide bond to GPIb $\beta$ , and noncovalently associated with GPIX and GPV.<sup>9,30,31</sup> Each platelet expresses 20,000 to 25,000 copies of GPIb/IX and ~11,000 molecules of GPV.<sup>32</sup>

The cytoplasmic tail portion of GPIb $\alpha$  interacts with actin-associated proteins, supporting the maintenance of the

platelet shape, size, and adhesive function. The interaction of GPIb $\alpha$  with VWF mediates platelet adherence to the vascular subendothelium, which can be investigated in ristocetin-induced platelet agglutination (RIPA).<sup>33,34</sup> Different mutations in the GP1BA gene, encoding the GPIb $\alpha$  subunit or in GP1BB (GPIb $\beta$  subunit) or in GP9 (GPIX), prevent or reduce the surface expression of the whole GPIb/IX/V complex and are typically inherited in an autosomal-recessive mode (biallelic BSS). Few cases with single dominant mutations mainly impair the function (monoallelic BSS).<sup>7</sup> GPV deficiency does not cause BSS, but modulates thrombin-mediated platelet activation and fibrin formation.<sup>35</sup>

The first step in laboratory testing includes the measurement of the blood cell count ranging between 20 and  $100 \times 10^9/\text{L}$  in BSS patients.<sup>28</sup> The mean platelet volume (MPV) varies from 9.3 to 27 fL (mean 14.8 fL).<sup>8</sup> Of note, automated impedance-based measurement of the platelet count does not recognize very large platelets and should be verified by microscope technique. Examination of the stained blood smear includes the assessment of platelet size, and platelet morphology typically showing very large or even giant platelets.<sup>7,28,29</sup> Classical preparation of platelet-rich plasma (PRP) by centrifugation leads to the loss of enlarged platelets. As for other platelet disorders combined with dysfunction and reduced platelet count, the WHO bleeding score does not always correlate with platelet count in BSS.<sup>8</sup>

In the second step, platelet function assessment with the RIPA assay and/or flow cytometry for the examination of GPIb/X/V platelet surface expression are useful.<sup>36,37</sup> Typically, RIPA is absent or reduced in BSS, while the responses to adenosine-diphosphate (ADP), collagen, and arachidonic acid in the LTA are normal if the platelet mass is sufficient for LTA. Depending on the underlying mutation, the expression of this receptor is reduced.<sup>29,36</sup> Importantly, RIPA cannot be corrected by addition of normal plasma to exclude differential diagnosis of von Willebrand disease.<sup>7</sup> In some cases, RIPA assay in patients with Bolzano mutation may be not sufficiently sensitive for diagnosis.<sup>28</sup>

Defects in prothrombin consumption in serum from patients with BSS have been described. GPIb $\alpha$  carries several binding sites for different coagulation factors, and GPIb $\alpha$  is essential for thrombin generation on platelets (thrombin burst).<sup>38</sup>

Immunofluorescence microscopy of blood smears, using antibodies against specific proteins affected in different hereditary platelet disorders including BSS, can be an alternative, requiring, like flow cytometry, only a small volume of blood.<sup>24</sup>

The final step in laboratory testing includes molecular diagnostics. Usually, BSS is characterized by autosomal-recessive inheritance with mutations in GP1BA, GP1BB, or GP9 genes, including missense (54%), frameshifts (38%), and nonsense (8%) mutations; however, none has been described for GPV.<sup>7,8,33</sup> In a large cohort of 211 families with biallelic BSS, 112 mutations in the GP1BA (28%), GP1BB (28%), or GP9 (44%) have been described, while 85% were homozygous and 13% were compound heterozygous.<sup>8</sup> However, mutations can

also be monoallelic (i.e., only one allele is affected with autosomal dominant inheritance). The best-known mutation is the Bolzano variant (p.Ala172Val of GPIb $\alpha$ ) with usually only mild thrombocytopenia.<sup>39</sup>

## Platelet-Type von Willebrand Disease

The platelet-type von Willebrand disease (PT-VWD) is a rare, autosomal-dominant bleeding disorder with dysfunctional platelets.<sup>26</sup> Bleeding becomes more severe in situations like childbirth and surgery. Laboratory testing shows low VWF:ristocetin cofactor and low or normal VWF:antigen and characteristically an enhanced RIPA. This disease can be identified by LTA, when a subthreshold concentration of ristocetin (0.5 mg/mL) does induce platelet agglutination. Of note, in neonates VWF binding induced by 0.5 mg/mL of ristocetin measured by LTA or by flow cytometry is normal.<sup>40</sup> Differentiating between von Willebrand type IIB and platelet-type VWD by plasma exchange experiments requires experience and diagnostic options. Here, fresh platelets and plasma from healthy volunteers are required to perform cross-comparisons by incubation in the plasma of a healthy donor with platelets from the patient and vice versa. In the case of PT-VWD, a RIPA is normal when using platelets from healthy volunteers and patient plasma, whereas when using patient platelets, a pathologically increased agglutination is observed when using a low concentration of ristocetin (e.g., 0.5 mg/mL). This makes it possible to differentiate whether the platelets or the plasma is the cause of the phenotype.<sup>41,42</sup> However, thrombocytopenia due to increased platelet binding to VWF does not fully explain the bleeding tendency. It has recently been described that in this disease, a lack of signaling due to the malfunction of the GPIb receptor can also increase the bleeding tendency in these patients.<sup>26,43</sup>

## Platelet Granule Defects

Platelets contain three types of granules that can be readily distinguished by their content, structure, release, and function: (1)  $\alpha$ -granules, (2)  $\delta$ -granules (dense bodies), and (3) lysosomal granules.

During megakaryopoiesis, a common immature precursor of  $\alpha$ - and  $\delta$ -granules is formed and the immature granules in megakaryocytes are referred to “multivesicular bodies” (MVB I and II). These structures are initially formed by clathrin-dependent endosomes, but also by endocytosis from the plasma membrane. Early MVB I contains only internal vesicles; MVB II additionally has an electron-dense matrix. Sorting into  $\alpha$ - and  $\delta$ -granules occurs before finally mature granules are formed.

### $\alpha$ -Granule Defects

$\alpha$ -Granules are the most abundant ones with ~50 to 80 per platelet.<sup>44</sup> They contain numerous proteins, including platelet-derived growth factor (PDGF), platelet factor 4 (PF4), or VWF, most of which are synthesized in megakaryocytes, while some, like fibrinogen or factor V, are taken up from plasma.  $\alpha$ -Granules are still modified in circulating platelets

due to a continuous shuttling between membrane receptors and the MVB and to further differentiate into distinct  $\alpha$ - and  $\delta$ -granules.

Several transmembrane proteins are incorporated into the  $\alpha$ -granule membrane, including P-selectin (CD62P), which is virtually absent from the plasma membrane of resting platelets. CD62P is a well-established marker for platelet activation in response to stimulation. It is also expressed on Weibel-Palade bodies in endothelial cells, where it can be exocytosed to the plasma membrane upon endothelial cell activation. CD62P can be cleaved from the cell surface by sheddases, resulting in a cleaved transmembrane protein and a soluble ectodomain (sCD62P), which can be detected in plasma and whose expression level has been assayed in distinct disease conditions, such as sickle cell anemia.<sup>45</sup> Triggering receptor expressed on myeloid cells (TREM)-like transcript-1 (TLT-1) has been considered to be a more sensitive marker for  $\alpha$ -granule release with a comparable specificity,<sup>46</sup> but not yet been broadly established.

The most common inherited disorder affecting  $\alpha$ -granule number and content is the gray platelet syndrome (GPS; OMIM #139090)<sup>47</sup> which is associated with a mild to moderate bleeding tendency, macrothrombocytopenia, and elevated vitamin B12 levels. Bone marrow fibrosis and splenomegaly may occur during disease progression.<sup>47,48</sup> In May-Gruenwald-Giemsa-stained blood smears, platelets appear larger and greyish rather than purple as dyes are not incorporated into granules. GPS is caused by homozygous or compound heterozygous mutations in NBEAL2 which encodes neurobeachin-like 2, a protein that is essential for the formation of  $\alpha$ -granules and deficiency thus resulting in loss of  $\alpha$ -granules.<sup>49–51</sup>

In addition, NBEAL-2 is required for neutrophil- and natural killer-cell function and pathogen defense<sup>52,53</sup> and recently Delage et al described a low CTA4 expression in activated T cells from NBEAL-2-deficient patients.

Certain mutations in the transcription factors GFI1B and GATA1 also lead to gray platelets,<sup>54</sup> as many genes are not adequately expressed. The genes VPS33B and VIPAS39 are involved in MVB and granule maturation and variants in these genes cause the arthrogryposis–renal dysfunction–cholestasis (ARC) syndrome (OMIM #208085).<sup>55</sup> These disorders share several platelet features with GPS, but are not referred to as GPS.<sup>54</sup>

Blood smears are often a primary hint, but the same staining pattern can be attributed to several gene defects. This holds also true for immunofluorescence of platelet proteins performed on blood smears, but these approaches can clearly help with the diagnosis.<sup>24</sup> Defects in  $\alpha$ -granules are best detected by flow cytometry using antibodies against CD62P or TLT-1, which should be absent on resting platelets, but becomes readily detectable after adding a high dose of a strong agonist like thrombin. Transmission electron microscopy (TEM) is often considered the gold standard to verify a lack of  $\alpha$ -granules, but typically time- and labor-intensive and restricted to specialized laboratories.

There is at the moment no curative approach for patients with  $\alpha$ -granule defects: GPS is associated with an increased,



but overall mild bleeding risk that can be treated with platelet transfusions, tranexamic acid, or desmopressin.<sup>5,47</sup> Patients with ARC and GPS syndrome require detailed clinical observations. Patients harboring variants in transcription factors GATA1 should also be followed up carefully over time; both transcription factors are associated with red blood cell defects, and a transition into a pre-leukemic condition may occur depending on the causative mutation.<sup>56</sup>

An altered composition of  $\alpha$ -granules is found in the Quebec platelet disorder (QPD), a disease with autosomal dominant inheritance, in which a gain-of-function defect in fibrinolysis leads to a more than 100-fold increase in platelet stores of urokinase plasminogen activator. This leads to increased plasmin-mediated degradation of  $\alpha$ -granule proteins.<sup>57</sup>

### $\delta$ -Granule Defects

Dense bodies ( $\delta$ -granules) contain ADP, adenosine triphosphate (ATP), serotonin,  $\text{Ca}^{2+}$  cations, pyrophosphate, and polyphosphate and express lysosomal membrane proteins (LAMP1/2; CD107a/b), which can be detected on the platelet surface upon stimulation. Granulophysin (CD63), another marker for lysosomes, is also expressed in  $\delta$ -granules and can be used to detect the release of both granule types.  $\delta$ -Granules appear often as “eyed” granules in TEM micrographs, but are best visualized by whole-mount TEM where they appear as electron-dense dots on the micrographs.

Upon platelet activation, small molecules are released from the  $\delta$ -granules, which act as important autocrine platelet agonists, such as ADP, ATP, and serotonin. Released polyphosphates and  $\text{Ca}^{2+}$  cations essentially contribute to the platelet-based amplification of thrombin generation during the coagulation process. Disorders of platelet  $\delta$ -granule formation are characterized by quantitative or qualitative defects manifested as isolated or syndromic forms.<sup>4,58</sup> The bleeding severity of  $\delta$ -storage pool disorders ( $\delta$ -SPDs) is variable, ranging from mild to severe when assessed by the ISTH-BAT bleeding score.<sup>6</sup> However, a mild spontaneous bleeding diathesis can be life-threatening after trauma or surgery.

Congenital  $\delta$ -SPDs are typically caused by pathogenic variants in genes either responsible for the biogenesis and trafficking of lysosome-related organelles (melanosomes, lysosomes) including platelet  $\delta$ -granules during late megakaryopoiesis, or encoding distinct transcription factors (e.g., GATA1, RUNX1, FLI1) essentially involved in early megakaryopoiesis. The syndromic  $\delta$ -SPDs, Hermansky-Pudlak syndrome (HPS), Chediak-Higashi syndrome (CHS), and Griscelli's syndrome (GS) with autosomal recessive inheritance are predominantly recognized rather by clinical manifestations. Currently, 11 different HPS subtypes are known based on the mutated gene (ISTH TIER1 genes: HPS1, AP3B1 [HPS2], HPS3-HPS6, AP3D1, BLOC1S3, BLOC1S5, BLOC1S6, DTNBP1). Prominent clinical associations of HPS are oculocutaneous albinism, nystagmus, immunodeficiency (i.e., HPS-2), granulomatous colitis (i.e., HPS-1, HPS-4), hemophagocytic lymphohistiocytosis (i.e., HPS-2), and pulmonary fibrosis. Patients with CHS caused by pathogenic variants

in LYST present with partial albinism, immunodeficiency, hemophagocytic lymphohistiocytosis, neurological defects, and hepatosplenomegaly. For all these syndromes (HPS, CHS, GS), platelet  $\delta$ -granules are absent or the number is strongly reduced, despite normal platelet count. In comparison, GS is caused by pathogenic variants in MYO5A, RAB27A, or MLPH and patients present with normal or reduced platelet count and partial albinism, silver hair, neurological defects, and immunodeficiency. SLFN14 gene mutation causes a combined defect with mild thrombocytopenia, enlarged platelets, and reduced ATP secretion. Patients show a disproportionately high bleeding tendency compared with the rather mild thrombocytopenia.<sup>59</sup>

For the laboratory diagnosis of quantitative  $\delta$ -SPD with reduced or absent  $\delta$ -granules, the whole mount TEM represents the “gold standard” method.<sup>60</sup> The sensitive and specific quantification of ADP, ATP (HPLC, luminescence), and serotonin (HPLC, ELISA) in platelet lysates allows the evaluation of the major  $\delta$ -granule cargo.<sup>61</sup> However, these technologies and assays are not available in routine laboratories. Alternatively, a combination of different platelet function assays allows the characterization of intrinsic or secretory platelet  $\delta$ -granule defects. LTA may indicate a  $\delta$ -SPD when the second aggregation wave is missing, reduced, and/or characterized by a clear disaggregation in response to the weak agonists ADP and epinephrine or to the strong agonist collagen in a dose-dependent manner. However, normal aggregation responses especially with high agonist concentrations do not exclude a mild or moderate  $\delta$ -SPD.<sup>62</sup> Therefore, a combination of LTA with lumiaggregometry, flow cytometry ( $\delta$ -granule and lysosome membrane marker CD63, mepacrine assay), and immunofluorescence (CD63, LAMP1, and LAMP-2) is recommended to prevent missing and misdiagnosis of this platelet function disorder.<sup>63,64</sup>

### Combined $\alpha$ -Granule and $\delta$ -Granule Defects

Pathogenic variants in the transcription factor genes GATA1, RUNX1, and FLI1 can also cause combined  $\alpha$ -granule and  $\delta$ -granule defects combined with thrombocytopenia with or without enlarged platelets.<sup>65–67</sup>

### Defects of Platelet Signaling Pathways

Inherited disorders of platelet receptors, namely, fibrinogen receptor  $\alpha\text{IIb}\beta 3$  integrin deficiency/dysfunction (GT), von Willebrand factor receptor GPIb–V–IX deficiency/dysfunction (BSS, PT-VWD), as well as deficiencies of the collagen receptor GPVI, ADP receptor P2Y<sub>12</sub>, and thromboxane A<sub>2</sub> (TxA<sub>2</sub>) receptor TP $\alpha$ , are well known to cause dysfunctional signal transduction in platelets. In comparison, intrinsic defects of platelet signaling affect specific or common receptor-mediated pathways, including TxA<sub>2</sub>-, guanosine-triphosphate (GTP)-binding protein and  $\alpha\text{IIb}\beta 3$  integrin inside-out signaling.<sup>68,69</sup> Defects of the GPVI receptor are gaining increasing attention. The receptor plays a significant role in thrombus formation and growth. Especially the procoagulant activity of platelets is triggered via GPVI.<sup>70</sup> Alterations in GPVI signaling can be manifold. For instance, the *protooncogenic tyrosine-protein*

kinase SRC (SRC) plays a crucial role in the signaling by GPVI. SRC is also involved in the signaling processes of the integrin and GPIb-V-IX receptors on platelets. A mutation in the *PTPRJ* gene is associated with absent CD148 and reduced SRC activation in platelets. The *PTPRJ* gene encodes a receptor-like protein tyrosine phosphatase, known as PTPRJ or CD148, which is abundantly expressed in platelets and megakaryocytes. Clinically, the platelets of these patients have a reduced response to convulxin via the GPVI receptor, are morphologically reduced in number and size, and patients have a clear clinical tendency to bleed.<sup>71</sup> A mutation in the *EPHB2* gene leads to reduced crosstalk between GPVI and G protein-coupled receptor signaling. This results in reduced responsiveness of the GPVI receptor, the consequences of which are not yet fully understood.<sup>72</sup>

The ADP receptor P2Y<sub>12</sub> is predominantly expressed on platelets and enables the amplification of platelet activation leading to irreversible platelet aggregation. *Disorders associated with P2Y<sub>12</sub> receptor include conditions like bleeding disorders and certain types of thromboembolic diseases.* The bleeding tendency is variable and often exacerbated by comedication. In particular, bleeding during surgical interventions and trauma has been described.<sup>73</sup> TxA<sub>2</sub> receptor defects are rare and not easy to distinguish from cyclooxygenase inhibitor/aspirin intake or cyclooxygenase defects. Here, the receptor itself is affected. Patients with this defect show a complete lack of response to thromboxane analogs such as U46619.

Although rare, defects in the common pathway of TxA<sub>2</sub> generation are most frequent within the group of congenital platelet signal transduction disorders. They are caused by pathogenic variants in *PLA2G4A*, encoding the phospholipase A<sub>2</sub> (group IV); in *PTGS1*, encoding the cyclooxygenase-1 (COX-1)/prostaglandin-endoperoxide synthase 1; or in *TBXAS1*, encoding the TxA synthase 1 (Ghosal syndrome), with autosomal recessive or dominant inheritance. Usually, the platelet count is normal, and patients present with mild to moderate bleeding diathesis, which could be life-threatening under hemostatic challenges. Similar to aspirin treatment, resulting in irreversible inhibition of COX-1, platelets from patients with deficient COX-1 or TxA<sub>2</sub> synthase showed absent or reduced arachidonic acid-mediated aggregation in the LTA ("aspirin-like defect").<sup>62,74</sup> However, platelet cytosolic phospholipase A<sub>2</sub> (PLA<sub>2</sub>) deficiency cannot be identified by arachidonic acid-induced platelet aggregation tests because PLA<sub>2</sub> itself liberates arachidonic acid through hydrolysis of membrane phospholipids.<sup>75</sup> Inherited and acquired platelet disorders of TxA<sub>2</sub> synthesis can be accompanied by impaired aggregation induced by weak agonists, such as ADP and epinephrine, and by low concentrations of strong agonists, such as collagen and the thrombin receptor PAR-1 agonist activating peptide TRAP-6, but the inter-individual responses are variable. Platelet aggregation with the TxA<sub>2</sub> analog U46619 is normal due to the direct activation of the TP receptor. The quantification of serum TxB<sub>2</sub>, a stable metabolite of TxA<sub>2</sub>, by liquid chromatography-mass spectrometry or ELISA-based assays is recommended as a reference test, since it specifically detects all enzymatic dysfunctions involved in platelet TxA<sub>2</sub> formation.<sup>76</sup>

Soluble platelet agonists such as ADP, TxA<sub>2</sub>, epinephrine, serotonin (5-HT), and thrombin act as important platelet

feedback agonists via guanine nucleotide-binding protein-coupled receptors. Upon receptor ligation, the  $\alpha$ -subunit of the heterotrimeric G proteins converts from the GDP- to the active GTP-bound form, dissociates from the receptor-complexed  $\beta$ - and  $\gamma$ -subunits, and transmits downstream signaling.<sup>77</sup> Congenital platelet function disorders with pathogenic variants in genes of distinct subunits from different G protein subfamilies have been described. Patients with defects of platelet-activating G proteins frequently present with mild to moderate bleeding symptoms, predominantly upon hemostatic challenges. Platelet deficiency in G $\alpha_q$ , encoded by *GNAQ*, impairs platelet activation via the thrombin receptors PAR-1 and PAR-4, ADP receptor P2Y<sub>1</sub>, TxA<sub>2</sub> receptor TP $\alpha$ , and the serotonin receptor 5HT2A and can be functionally analyzed by LTA and flow cytometry in response to intermediate concentrations of PAR-1 and PAR-4-activating peptides and the TxA<sub>2</sub> analog U46619. In contrast, normal platelet function is expected in response to a high concentration of a GPVI agonist, Ca<sup>2+</sup> ionophore, and phorbol ester, as these pathways are insensitive to ADP- and TxA<sub>2</sub>-mediated signaling.<sup>62</sup> However, a similar dysfunctional platelet phenotype is expected when the expression of phospholipase- $\beta$ 2 (PLC $\beta$ 2), encoded by *PLCB2*, is absent or reduced as PLC $\beta$  is a central downstream target of Gq protein-coupled receptors.<sup>78</sup> Here, only the application of Western analysis, GTPase activity assay, and phospho-proteomics, in combination with genetic screening, can specify the Gq protein-coupled receptor signaling defect.

Activating signaling via the Gi- and Gz-coupled receptors P2Y<sub>12</sub> for ADP and  $\alpha$ 2 adrenergic receptor for epinephrine, respectively, interact with the inhibitory Gs-coupled IP receptor pathway by regulating the cytoplasmic level of the inhibitory second messenger cyclic adenosine-monophosphate (cAMP). While signaling via G $\alpha_i/z$ -coupled receptors leads to the inhibition of the adenylate cyclase and its cAMP synthesis, prostacyclin-mediated activation of the G $\alpha_s$ -coupled receptor IP results in adenylate cyclase activation, increase in cytoplasmic cAMP-level, activation to protein kinase A and platelet inhibition.<sup>79</sup> Gi-like platelet defects are functionally detected by absent or reduced platelet aggregation in response to epinephrine and no second wave of aggregation in response to ADP. In comparison, maximal aggregation responses induced by arachidonic acid and high concentration of the PAR-1 agonist TRAP-6 and collagen are within the reference range.<sup>62</sup> Although dysfunction of the G $\alpha_i$  subunit is more frequent than that of the  $\alpha_q$  subunit, cases of congenital Gi deficiency (i.e., pathogenic variants in *GNAI1-3*) are very rare.

Inherited platelet defects affecting  $\alpha$ IIb $\beta$ 3 integrin inside-out signaling are characterized by GT-like platelet dysfunction and bleeding symptoms.<sup>80</sup> Leukocyte adhesion deficiency syndrome type 3 (LAD-III) is an autosomal recessive disease caused by pathogenic variants in *FERMT3*, encoding the focal adhesion protein kindlin-3. Kindlin-3 binds to the cytoplasmic tails of the integrin  $\beta$ -subunit and cooperates with talin in integrin activation. Infections, impaired wound healing, and osteopetrosis are frequently observed in patients with LAD-III. Kindlin-3 deficiency does not affect integrin expression, but agonist-induced activation of integrins in leukocytes and

especially of the platelet  $\alpha\text{IIb}\beta 3$  integrin.<sup>81</sup> Another GT-like platelet disorder is associated with mutations in RASGRP2 encoding diacylglycerol-regulated guanine nucleotide exchange factor I (CalDAG-GEFI), a  $\text{Ca}^{2+}$ -dependent activator of Rap-1, which is essentially involved in  $\alpha\text{IIb}\beta 3$  integrin inside-out and outside-in signaling.<sup>82–84</sup> Therefore, patients with a CalDAG-GEFI defect showed reduced platelet spreading on fibrinogen and impaired platelet aggregation in response to weak agonists and lower concentrations of strong agonists with the exception of protein kinase C (PKC)-stimulating phorbol esters. Specialized assays for the measurement of Rap-1 activation are established only in research laboratories.

## Defects of Platelet Procoagulant Activity

Dysregulation of  $\text{Ca}^{2+}$ -dependent platelet functions primarily leads to alterations of procoagulant activity and the surface presentation of negatively charged phospholipids such as phosphatidylserine (PS). Platelet PS exposure is

important for  $\text{Ca}^{2+}$ -dependent binding of the Gla-domain containing coagulation factors (e.g., FX, FII of the final prothrombinase complex) to enable platelet-dependent amplification of thrombin generation.<sup>85,86</sup> Loss-of-function variants in ANO6/TMEM16F encoding anoctamin-6, a transmembrane ion channel protein, scrambling  $\text{Ca}^{2+}$ -dependent phospholipids from the inner to the outer leaflet of the cell membrane, cause the very rare autosomal recessive Scott syndrome. Scott patients have a normal platelet count, morphology, and aggregation responses, but platelets fail to expose PS and subsequently to generate thrombin, fibrin, and extracellular vesicles. These dysfunctional platelets do not promote the stabilization of the thrombus and therefore, Scott patients present with moderate to severe bleeding diathesis during and after surgery.<sup>87,88</sup> A bleeding tendency can also be associated with spontaneously enhanced platelet PS exposure, caused by inherited defects of  $\text{Ca}^{2+}$ -release-activated  $\text{Ca}^{2+}$  channels as observed for the rare Stormorken's syndrome, which is also linked to immune

**Table 1** Overview of some medications altering platelet function, modified after Scharf<sup>91</sup>

Substance class	Drugs	Suspected mechanism
NSAIDs	Diclofenac, ibuprofen, indomethacin, naproxen	Reversible COX-1-inhibition
Beta-lactam antibiotics	Ampicillin, penicillin G, carbenicillin, piperacillin	Platelet receptor a/o VWF receptor interaction
Cephalosporins	Cefotaxime, cefoperazone, cephalothin	platelet membrane constituents interaction
Other antibiotics	nitrofurantoin, miconazole	Inhibition of platelet cox-1
PDE inhibitors	Sildenafil, caffeine, theophylline, aminophylline	Inhibition of PDE5; activation defect of integrin $\alpha\text{IIb}\beta 3$
Nitrates	Nitroprusside, nitroglycerin, isosorbide dinitrate	Nitric oxide increases with the increase in platelet cAMP/cGMP
Beta-adrenergic receptor blockers	Propranolol, nebivolol, pindolol	Independent of $\beta$ -adrenoreceptor blockade
Calcium channel blockers	Verapamil, nifedipine, diltiazem	Inhibition of epinephrine to $\alpha 2$ -adrenergic receptors and of platelet response to TXA2 and serotonin
Angiotensin-converting enzyme inhibitors	Ramipril, captopril, enalapril, lisinopril	Downregulation of $\alpha\text{IIb}\beta 3$ ? NO-mediated drug effects
Angiotensin receptor blockers	Valsartan, losartan, olmesartan	Downregulation of $\alpha\text{IIb}\beta 3$ Interference with TXA2 receptor
Antiarrhythmic drugs	Quinidine	Blockade of $\alpha 2$ -adrenergic receptors
HMG-CoA inhibitors (statins)	Atorvastatin, cerivastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin	Changes in lipid composition of the platelet plasma membrane; inhibition of protein prenylation with subsequent inhibition of GTP-binding proteins
Plasma expanders	Dextrans, hydroxyethyl starch	Interaction with platelet membrane constituents
Tricyclic antidepressants	Imipramine, amitriptyline, nortriptyline	Unclear
Phenothiazines	Chlorpromazine, promethazine, trifluoperazine	Inhibition of calmodulin-dependent pathways
Selective serotonin reuptake inhibitors	Fluoxetine, paroxetine, sertraline	Multifactorial mechanisms suggested

Abbreviations: cAMP, cyclic adenosine-monophosphate; cGMP, cyclic guanosine-monophosphate; COX-1, cyclooxygenase-1; GTP, guanosine-triphosphate; NSAIDs, nonsteroidal anti-inflammatory drugs; PDE5, phosphodiesterase type 5; TXA2, thromboxane A2.

deficiencies.<sup>89</sup> Autosomal dominant gain-of-function variants in sensor stromal interaction molecule 1 (STIM1), encoding the  $\text{Ca}^{2+}$  STIM1 within the dense tubular system, or in ORAI1, encoding Orai1 within the plasma membrane, cause impaired store-operated  $\text{Ca}^{2+}$  entry accompanied by elevated cytosolic  $\text{Ca}^{2+}$  levels and preactivated platelets in vivo. Thus, patients with Stormorken's syndrome showed moderate thrombocytopenia associated with increased basal platelet surface expression of PS and P-selectin but reduced agonist-induced  $\alpha\text{IIb}\beta 3$  integrin activation capacity, assessed by flow cytometry, and impaired thrombus formation under flow in a multiparameter-based flow chamber assay.<sup>90</sup> Flow cytometric detection of fluorochrome-

conjugated annexin-V binding to platelets stimulated by strong agonists such as thrombin, thrombin plus a GPVI agonist (COAT, coated platelets), and  $\text{Ca}^{2+}$  ionophore in the presence of  $\text{Ca}^{2+}$  is recommended to analyze platelet PS translocation.<sup>85</sup> Semiautomated thrombin generation assays in PRP provide functional readouts for altered platelet coagulation capacity.

## Acquired Platelet Defects

Patients can present with acquired platelet defects due to external factors or conditions that develop during a person's lifetime.

### Medications and Nutritional Deficiencies

Besides platelet function inhibitors, there are a considerable number of drugs that can affect platelet function, as outlined in ▶Table 1.<sup>91</sup> Among the most frequently encountered types are nonsteroidal anti-inflammatory drugs, antihypertensive drugs, antibiotics, and psychotropic medications. Furthermore, certain diets, herbs, alcohol, and nutritional supplements or fasting itself have been shown or are suspected to impair platelet function and laboratory tests (▶Table 2).<sup>92</sup> Certain nutritional deficiencies like vitamin B12 deficiency have also been shown to affect platelet count and function.<sup>93,94</sup>

### Medical Conditions

In uremic patients, platelets have been shown to have similar defects as a  $\delta$ -SPD (reduced ADP and serotonin content in platelet granule; reduced  $\delta$ -granule; impaired ATP release).<sup>95</sup>

Hematologic diseases such as myelodysplastic syndrome, myeloproliferative neoplasms (e.g., essential thrombocythemia), or acute leukemia are associated with acquired  $\delta$ -SPD and can inherently lead to a decreased platelet function.<sup>96</sup>

Moreover, autoantibodies against platelet receptors result in acquired forms of Glanzmann's disease or delta storage pool disease which should be especially considered in elderly patients with new bleeding symptoms. Apart from bleeding control, the therapy consists of immunosuppression.<sup>97</sup>

Platelet aggregation has been shown to be impaired in patients with severe sepsis and is associated with increased mortality.<sup>98</sup>  $\delta$ -SPD can secondarily occur due to partial or complete granule exocytosis of activated platelets in throm-

**Table 2** Overview of some foods and diets altering platelet function, modified after McEwen<sup>92</sup>

- Berries (especially raspberry extract)
- Coffee and caffeine
- Cocoa and dark chocolate
- Energy drink
- Garlic
- Ginger
- Kiwi fruit
- Omega-3 polyunsaturated fatty acids
- Onion
- Purple grape juice
- Red wine
- Tomato
- Turmeric/Curcumin
- Vegetarian diet
- White wine
- Bromelain
- Ginkgo biloba extract

boinflammatory states such as acute ischemic stroke<sup>99</sup> and severe COVID-19.<sup>100</sup>

### Platelet Dysfunction Ex Vivo Due to Platelet Pathological Platelet Activation In Vivo

Certain conditions can lead to a prolonged activation of platelets in vivo and consecutively to a release of platelet granules (also called "exhausted platelet syndrome"). Reasons include prolonged bleeding situations in which platelets are constantly activated and consumed.<sup>101</sup> Limited adhesion and activation of the platelets as well as limited maximum aggregation are described.<sup>102</sup> This is also true for activation through interaction with nonphysiological surfaces such as extracorporeal devices, especially systems with high blood flow rates such as ventricular assist devices, extracorporeal membrane oxygenation, and cardiopulmonary bypass leading to impaired platelet granule release ex vivo.<sup>103–106</sup>

## Conclusion

iPFDs are a heterogeneous group of rare bleeding diseases in terms of frequency, bleeding severity, platelet dysfunction, and platelet count/volume. They are caused by different and multiple gene defects, which result in altered megakaryopoiesis, platelet production, and platelet function. If a reduced platelet count does not explain the bleeding phenotype, a combined iPFD should be considered. Although iPFDs are rare, their prevalence is underestimated due to a limited diagnostic potential and challenging platelet function tests. The complex laboratory diagnosis is usually possible only in specialized laboratories. In case of a positive family history, platelet dysfunction should be considered after the exclusion of a plasmatic coagulation disorder.



However, in some cases, iPFs are also associated with VWD or other coagulation factor disorders. Especially the diagnosis of inherited platelet function defects with mild to moderate bleeding diathesis, determined by a standardized bleeding assessment tool, requires a combination of platelet phenotyping, different platelet function assays, and genotyping. Therefore, a specific diagnosis of iPFs is not only essential for preventive bleeding treatment and advice of affected family members but also for the therapy of bleeding. Despite the upcoming automation of classical platelet function tests to improve standardization, the establishment of additional specialized platelet function assays in combination with whole genome sequencing will help identify and phenotype new iPFs, so far classified as a bleeding disorder of unknown cause.

#### Data Availability

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

#### Conflict of Interest

KA: Speaker's honoraria: Meet the experts, Sobi, CLS Behring; Travel support: Sobi, Biomarin, NovoNordisc; Leadership or fiduciary role in other board, society, committee or advocacy group, paid or unpaid: ISTH Platelet immunology (Co-Chair): no payment.

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