Review

Cecilia Carubbia, Elena Masselli, Antonio Nouvenne, Domenico Russo, Daniela Galli, Prisco Mirandola, Giuliana Gobbi* and Marco Vitale*

Laboratory diagnostics of inherited platelet disorders

Abstract: Inherited platelet disorders (IPDs) are the general and common denomination of a broad number of different rare and congenital pathologies affecting platelets. Even if these disorders are characterized by widely heterogeneous clinical presentations, all of them are commonly present as defects in hemostasis. Platelet number and/or function are affected by a wide spectrum of severity. IPDs might be associated with defects in bone marrow megakaryocytopenia and, rarely, with somatic defects. Although in the last few years new insights in the genetic bases and pathophysiology of IPDs have greatly improved our knowledge of these disorders, much effort still needs to be made in the field of laboratory diagnosis. This review discusses the laboratory approach for the differential diagnosis of the most common IPDs, suggesting a common multistep flowchart model which starts from the simpler test (platelet count) ending with the more selective and sophisticated analyses.

Keywords: bleeding; laboratory tests; platelets; rare diseases.

Introduction

Inherited platelet disorders (IPDs) are rare, congenital diseases of platelet number and/or function characterized by a widely heterogeneous clinical presentation and a challenging diagnostic work-up. They usually present as primary hemostatic defects that may range from a prolonged bleeding after trauma or surgery in adulthood to life-threatening, spontaneous hemorrhages (gastrointestinal, genitourinary or even intracranial) occurring early in life [1, 2].

Defects in platelet function are frequently accompanied by abnormalities in bone marrow megakaryocytopenia, resulting in reduced platelet counts and altered platelet morphology.

In some cases, platelet disorders are associated with somatic defects, such as reduced or delayed pigmentation Hermansky-Pudlak syndrome (HPS), hearing loss, renal impairment and/or cataracts (MYH9-related diseases), eczema and susceptibility to infections Wiskott-Aldrich syndrome (WAS), limb abnormalities (thrombocytopenias associated with skeletal defects).

While the process of IPDs characterization significantly contributed to our understanding of the genetic bases of megakaryocytopenia and platelet function, unfortunately little progress has been made in the field of laboratory diagnostics of these diseases. In fact, diagnosis of the majority of IPDs still primarily relies on personal and family history, co-morbidities (i.e., skeletal defects), peripheral blood smear analysis and genetic testing, when available [3, 4].

This review will discuss the diagnostic algorithm – focusing on laboratory tests – for the most common IPDs; diseases are presented on the basis of their involvement of platelet count, function, or both.

Disorders of platelet number

This group encompasses MYH9-related disorders, congenital amegakaryocytic thrombocytopenia (CAMT),
thrombocytopenias associated with skeletal defects, X-linked thrombocytopenia (XLT) with dyserythropoiesis, familial platelet disorders with predisposition to acute leukemia (FDP/AML), benign Mediterranean macrothrombocytopenia (BBM), familial thrombocytopenia 2 (THC2).

Genetic analysis is the sole specific laboratory test for these IPDs and is currently clinically available for CAMT, thrombocytopenia with absent radii (TAR), thrombocytopenia with radio-ulnar synostosis (ATRUS), XLT with dyserythropoiesis and FDP/AML [5] (Table 1).

Beyond genetic testing, diagnosis is essentially based on family history (usually relevant in autosomal dominant disorders such as MYH9-related disorders, ATRUS, Di George/Velocardiofacial syndrome (VCFS), thrombocytopenia with predisposition to leukemia, BBM, THC 2) and presence of patognomonic-associated defects affecting other organs (see Figures 1 and 2). During the laboratory diagnostic work-up, a key role is detained by peripheral blood smear analysis for platelet morphology (i.e., presence of macrothrombocytes – as in the case of MYH9-related disorders and BBM – or detection of spindle-shaped Döhle leukocyte inclusions, as in the case of MYH9-related diseases) [6].

**MYH9-related disorders**

This term indicates a group of autosomal dominant thrombocytopenias (May-Hegglin anomaly, Sebastian Syndrome, Fechtner Syndrome and Epstein syndrome) caused by mutations within the MYH9 gene, encoding for the non-muscle myosin IIA heavy chain which is part of the contractile cytoskeleton of megakaryocytes, platelets and neutrophils [7, 8].

As a result, bone marrow megakaryocytic thrombocytopenia is ineffective and platelets display abnormally larger dimensions (macrothrombocytes). Platelet aggregation tests are normal. Thrombocytopenia ranges from 20×10⁹/L to 130×10⁹/L, but bleeding phenotype is usually mild. Diagnosis is suggested by peculiar co-morbidities, such as sensorineural hearing loss, cataracts, glomerulonephritis, but definitive identification of this IPD requires the demonstration of a causative mutation within MYH9 [9].

**Congenital amegakaryocytic thrombocytopenia (CAMT)**

CAMT is an autosomal recessive disorder characterized by symptomatic thrombocytopenia manifesting early in life, occasionally with a severe bleeding phenotype. Platelet count is usually below 50×10⁹/L and their size is normal [10].

Bone marrow aspirate reveals marked megakaryocytic hypoplasia that may progress to bone marrow failure. Mutations of the thrombopoietin (TPO) receptor (c-mpl) underlie this disease and can be detected by genetic testing [11].

**Thrombocytopenias associated with skeletal defects**

This group of IPDs encompasses three different thrombocytopenias associated with peculiar skeletal defects: TAR, ATRUS and Di George/VCFS.

TAR is an autosomal recessive disease that manifests early in life with a bleeding diathesis that tends to ameliorate with time due to a progressive increase of the platelet count. Diagnosis is straightforward given the presence of the typical bilateral radial anomalies that affect TAR patients. Bone marrow megakaryocytes are reduced, but platelet size and function are normal. Defects in the TPO/c-mpl signaling may be found with appropriate genetic tests [12].

ATRUS is an autosomal dominant amegakaryocytic thrombocytopenia associated with aplastic anemia, radio-ulnar synostosis, clinodactyly, syndactyly, hip dysplasia and sensorineural hearing loss. Like TAR, also ATRUS presents with severe thrombocytopenia at birth that may improve with time [13]. Detection of mutations of HOXA11, a member of the homeobox genes encoding for proteins that detain a central role in skeletal development and hematopoiesis, allows final diagnosis [14].

Di George/VCFS is an autosomal dominant macrothrombocytopenia typified by cardiac abnormalities, cleft palate, thymic aplasia, mental retardation, disimmunity. The gene involved, usually with a hemizygous loss of allele, is GPIBB, located at 22q11, one of the three genes defective in Bernard-Soulier syndrome (BSS). Thus, clinical manifestations are usually mild and may overlap with heterozygous BSS. Again, clinical diagnosis is suggested by the relevant co-morbidities and requires confirmation by genetic testing [15].

**X-linked thrombocytopenia with dyserythropoiesis**

This X-linked platelet disorder is due to mutations of the GATA1 gene, encoding for the transcription factor GATA1,
| Inherited Platelet Disorders and Laboratory Tests Currently Available |
|---|---|---|---|
| **MYH9-related disorders** | MYH9 (22q12-13) | AD | Yes | PLT count
Peripheral blood smear
Platelet aggregometry |
| **Congenital amegakaryocytic thrombocytopenia** | c-MPL (1p34) | AR | Yes | PLT count
Peripheral blood smear
Bone marrow aspirate |
| **Thrombocytopenias associated with skeletal defects** | Y19 (1q21.1), HOXA11 (7p15-p14.2), GPIBB (22q11) | AD/AR | Yes | PLT count
Bone marrow aspirate |
| **X-linked thrombocytopenia with dyserythropoiesis** | GATA1 (Xp11.23) | X-linked | Yes | PLT count
Peripheral blood smear
Bone marrow aspirate |
| **Familial platelet disorders with predisposition to leukemia** | RUNX1 (21q22) | AD | Yes | PLT count
Peripheral blood smear
Platelet aggregometry |
| **Benign Mediterranean macrothrombocytopenia** | GPIBA | AD | No | PLT count
Peripheral blood smear |
| **Familial thrombocytopenia 2** | ANKRD26, MASTL, ACBD5 | AD | No | PLT count
Bone marrow aspirate |
| **Bernard-Soulier syndrome** | GPIBA (17p13) | AR | Yes | PLT count
Bleeding time
Closure time
Peripheral blood smear
Platelet aggregometry
Flow cytometry |
| **Glanzmann Thrombasthenia** | ITGA2B, ITGB3 (17q21-23) | AR | No | PLT count
Bleeding time
Closure time
Peripheral blood smear
Platelet aggregometry
Flow cytometry |
| **Platelet-type von Willebrand disease** | GPIBA (17p13) | AD | Yes | PLT count
Bleeding time
Closure time
Peripheral blood smear
Platelet aggregometry
Protein electrophoresis |
| **Wiskott-Aldrich syndrome** | WAS | X-linked | Yes | PLT count
Bleeding time
Closure time
Peripheral blood smear |
| **Storage pool diseases** | LYST (1q42.1-42.2), HPS1, AP3B1, HPS3, HPS4, HPS5, HPS6, DTNB1, HPS8, FLI1 (11q23) | AD/AR | No | PLT count
Bleeding time
Closure time
Peripheral blood smear
Platelet aggregometry
Flow cytometry
Electron microscopy |
| **Scott syndrome** | ABCA1 | AR | No | PLT count
Bleeding time
Closure time |
Table 1

<table>
<thead>
<tr>
<th>Gene (location)</th>
<th>Inheritance</th>
<th>Genetic testing clinically available</th>
<th>Lab test(s)</th>
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<td>Agonist PLT receptor defects</td>
<td>TBXA2R, P2Y12</td>
<td>AD/AR</td>
<td>No</td>
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whose role in both erythroid and megakaryocytic differentiation of hematopoietic progenitors is well established. As a result, patients present with marked thrombocytopenia sustained by a defective megakaryocitopoiesis. Platelets are large with a decreased granular content. Dyserythropoiesis usually accompanies megakaryocytic defects and may account for transfusion dependence. Due to the complexity and heterogeneity of the disease, diagnosis may be challenging and requires demonstration of GATA1 mutation(s) [16].

**Familial platelet disorders with predisposition to acute leukemia (FDP/AML)**

This is a rare, autosomal dominant condition sustained by mutations in the RUNXI gene, encoding for a key transcription factor in hematopoiesis. Haploinsufficiency of RUNX1 accounts for inadequate transcription of genes involved in megakaryocytic differentiation [17].

Although platelet count is only moderately reduced (80–150×10^9/L), patients may experience severe bleeding diathesis due to aspirin-like platelet dysfunction [18, 19]. Consequently, laboratory diagnosis, beyond genetic testing, requires platelet aggregometry.

Involvement of RUNX1 in the disease pathogenesis may explain the tendency to develop hematologic malignancies, such as myelodisplastic syndrome and acute myeloid leukemia.

**Benign mediterranean macrothrombocytopenia (BMM)**

Described in Greek and Italian population, this IPD is inherited with an autosomal dominant pattern. Thrombocytopenia ranges from mild to moderate and is usually asymptomatic. Peripheral blood smear reveals large size platelets with a conserved functionality in in vitro studies. Bone marrow megakaryocytes are normal. No genetic test is currently available [20, 21].

**Familial thrombocytopenia 2 (THC2)**

THC2 patients present with a mild to moderate thrombocytopenia that becomes usually symptomatic only during hemostatic stress (trauma, major surgery). Peripheral blood smear is not helpful since platelet size is normal although bone marrow megakaryocytes show abnormal morphology, typified by a smaller size and hypolobated nuclei [22]. Several gene mutations have been described and can be screened to rule out other normothrombocytic thrombocytopenias [23–25] (see Table 1).

**Disorders of platelet function (and number)**

Among inherited platelet disorders, platelet function disorders are characterized by normal or reduced platelet number (reference range 140–400×10^9/L) associated with impaired physiological mechanisms of activation and aggregation. Main causes are represented by: dysfunctions of surface platelet receptors, deficiency of platelet granule content and abnormalities of degranulation.

Diagnosis of platelet function disorders is initially based on traditional laboratory tests including bleeding time, platelet aggregometry and platelet function analyzers (PFA) (see Table 2), but definitive differential diagnosis, requires more specific tests such as platelet secretion assay, measure of granule content and flow cytometric analysis of surface receptors [26–28].
Figure 1  Proposed diagnostic algorithm for suspected IPDs in case of low platelet count.

AA, arachidonic acid; AML, acute myeloid leukemia; ATRUS, thrombocytopenia with radio-ulnar synostosis; BMM, benign mediterranean macrothrombocytopenia; BSS, Bernard-Soulier syndrome; C, collage; CAMT, congenital amegakaryocytic thrombocytopenia; E, epinephrine; FC, flow cytometry; FDP/AML, familial platelet disorders with predisposition to acute leukemia; GPS, gray platelet syndrome; MK, megakaryocytes; PB, peripheral blood; PT/J, Paris-Trousseau/Jacobsen syndrome; PT-VWD, platelet-type von Willebrand disease; QPD, Quebec syndrome; R, ristocetin; TAR, thrombocytopenia with absent radii; THC2, familial thrombocytopenia 2; VCFS, Velocardiofacial syndrome; WAS, Wiskott-Aldrich syndrome.
Figure 2 Proposed diagnostic algorithm for suspected IPDs in case of normal platelet count.
AA, arachidonic acid; CHS, Chediak-Higashi syndrome; E, epinephrine; FC, flow cytometry; EM, electron microscopy; GT, Glanzmann thrombastenia; HPS, Hermansky-Pudlack syndrome; PB, peripheral blood; PT-VWD, platelet-type von Willebrand disease; QPD, Quebec syndrome; R, ristocetin; SS, Scott syndrome.

Bleeding time (BT) is the time taken for bleeding to stop after an incision is made into the skin. The test is not expensive and allows studying the entire hemostatic process, including the role played by the vessel wall. However, its low sensitivity, specificity and poor reproducibility, make it a suitable test only for preliminary screening [29].

In the Platelet Function Analyzer (PFA-100) test, citrate blood is aspirated from the sample reservoir through a capillary and a microscopic aperture cut into a membrane, coated with collagen/epinephrine or collagen/ADP. The presence of platelet activators and high shear rates result in platelet adhesion, activation, aggregation and, eventually, formation of a plug within the aperture. The time taken to occlude the aperture is reported as closure time and depends on platelet function [29]. PFA-100 is now currently used in place of BT because of its better standardization. PFA-100 is the most widely used PFA; other commercially available instruments are represented by VerifyNow, Plateletworks and IMPACT-R.

Light transmission aggregometry (LTA) tests agonist-induced changes in the turbidity (or optical density) of a platelet suspension while stirring the sample in a clear cuvette at 37 °C. The test is commonly done using platelet
rich plasma (PRP) and agonists used are ADP, epinephrine, collagen, arachidonic acid, tromboxane analogues and ristocetin. Parameters measured include the rate of aggregation and the maximal percentage of aggregation after a fixed period of time [29, 30].

Flow cytometry (FCM) is a key technique to confirm suspected platelet disorders [28]. By using specific monoclonal antibodies, it allows to detect specific glycoprotein receptor deficiencies [31, 32].

In addition to these techniques, specific tests are available for the assessment of platelet granule number, contents and release [33, 34]. Specifically, the α-granules transmembrane protein P-selectin, expressed on platelets surface once activated, is commonly detected by FCM [35].

Transmission electron microscopy allows detecting platelet granule deficiency or abnormal morphology based on their characteristic ultrastructure [36]. Even though electron microscopy is rarely available, it is usually required for diagnostic confirmation of specific platelet disorders.

Analysis of granule content and secretion relies on the detection of proteins selectively contained in platelet granules or expressed on the granule membrane. Among these tests, detection of ATP release using LTA combined with firefly luciferase is the first-choice method to assess δ-granules secretion [37]. Radioimmunoassays (i.e., 14C-serotonin secretion from prelabeled platelets) are rarely utilized to investigate δ-granule disorders [38], while are more common to assess α-granule content release [i.e., detection of plasma platelet factor 4 (PF4) and β-thromboglobulin (βTG)] [39, 40].

### Bernard-Soulier syndrome (BSS)

BSS is an autosomal recessive disorder with a quantitative or qualitative platelet defect due to a lack or dysfunctional von Willebrand factor (VWF) receptor, also known as GpIb-IX-V complex, resulting in a defective adhesion of platelet to subendothelium [41].

Clinically, bleeding tendency is usually evident during early childhood with easy bruising, petechiae, epistaxes and gastrointestinal bleeds. The severity of these symptoms may worsen during puberty and adult life (i.e., menorrhagia). Severe bleeding usually accompanies hematologic stress such as surgery, trauma or tooth extraction. Joint bleeds and intracranial hemorrhages are rare but may occur [42]. Heterozygous patients usually present a milder bleeding tendency.

Laboratory diagnosis includes: detection of a variable degree of thrombocytopenia (30−150×10^9/L), but platelet count can also be normal [43]; peripheral blood smear shows increased platelet size (even in patients with normal platelet count), that in one third of platelets may
be as large as half a red blood cell, and a few may even be as large as a lymphocyte [44]. BT and PFA-100 are prolonged but sensitivity of these tests is variable, depending on the severity of the defect [45]. In vitro platelet aggregation studies are normal with physiological agonists such as epinephrine, ADP, collagen and arachidonic acid, except for low dose of thrombin and ristocetin that are not capable to induce platelet agglutination [41, 44].

Platelets also fail to agglutinate when exposed to ristocetin and the addition of normal plasma is not effective in rescuing this defect [44]. Confirmation of the diagnosis is primarily based on FCM assessment of GpIb-IX-V expression on platelet surface. The use of antibodies that specifically recognize CD42b reveals a severe reduction or complete lack of GpIb, while other platelet antigens [i.e., CD41 (GpIIb) and CD61 (GpIIa)] are normal [46].

Differential diagnosis includes other inherited giant platelet disorders, such as May-Hegglin anomaly or Gray platelet syndrome (GPS), and von Willebrand disease (VWD). May-Hegglin anomaly is a relatively common giant platelet disorder with clinical manifestations matching with BSS; however, aggregation to thrombin and ristocetin is normal, while impaired response to ephinephrine has been described [47]. In GPS, instead, collagen-induced aggregation is reduced and response to ristocetin is normal or slightly impaired but never absent. Additionally, large platelets are typically agranular at the blood smear [6]. VWD symptoms (see below for details) may overlap with BSS and, similarly, platelet aggregation studies reveal failure to ristocetin-induced platelet aggregation. However, the ristocetin cofactor activity measured in VWD patients’ plasma and freshly washed platelets is uniquely reduced. Moreover, unlike BSS, VWD is not typically associated with thrombocytopenia.

Glanzmann thrombastenia (GT)

GT is a rare autosomal recessive disease due to a deficiency or dysfunction of the platelet membrane GpIIbβ3 integrin (also known as GpIIb-IIIa) which, upon platelet activation, binds adhesive glycoproteins such as fibrinogen, VWF and fibronectin, mediating platelet aggregation [48]. In addition to impaired aggregation, GPIIb-IIIa deficiency accounts also for: 1) abnormal spreading of platelets on subendothelial matrix; 2) decreased fibrinogen content in platelet α-granule; 3) reduced clot retraction; and 4) impaired phosphorylation of multiple platelet proteins [49].

GT presents as a bleeding syndrome: purpura, epistaxis, gingival hemorrhage and menorrhagia are almost constant features while gastrointestinal bleeding and hematuria are less common and deep visceral hematomas are rare [50]. Bleeding symptoms occur only in homozygous patients and manifest rapidly after birth, while heterozygous condition is mostly asymptomatic since 50% of the normal amount of GPIIb-IIIa is at least sufficient for platelet aggregation [51]. Consequently, clinical variability of this disease is high, ranging from minimal bruising to severe and potentially fatal hemorrhages; anyway, generally, bleeding tendency decreases with age.

Laboratory findings include markedly prolonged BT and PFA-100 test, while platelet count and morphology are normal. LTA shows lack or severe reduction of platelet aggregation induced by all agonists. Definitive diagnosis relies on flow cytometric analysis of αIIbβ3 integrin expression on platelet membrane by monoclonal antibodies recognizing GpIIb (CD41) and GpIIa (CD61) [52].

Based on the above mentioned laboratory findings, GT has been subclassified in three types according to the amount of GPIIb-IIIa present per platelet, the presence or absence of α-granule fibrinogen and clot retraction. In type I, platelet display <5% of the normal amount of surface GPIIb-IIIa and α-granule fibrinogen and clot retraction are absent. In type II, platelet GPIIIa-IIIb ranges from 10% to 20%, clot retraction is only decreased and α-granule fibrinogen is present. The third type, known as variant thrombasthenia, is characterized by an amount of GPIIb-IIIa equal to 50% of normal, indicating that GPIIb-IIIa defect is qualitative rather than quantitative.

At least 126 unique mutations accounting for GT have been identified in the two genes encoding for GPIIb and GPIIIa: 68 in ITGAB and 58 in ITGB3, including, in order of frequency, missense mutations (52%), nucleotide deletions and insertions (28%), nonsense mutations (14%), and alternative splicing (6%), http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu.

Recently, new diagnostic strategies have been proposed, including ELISA detection of GpIIbIIIa [53], polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) or allele-specific restriction analysis for prenatal diagnosis [50].

Platelet-type von Willebrand disease (PT-VWD)

PT-VWD is a rare autosomal dominant disorder with a quantitative and qualitative platelet defect due to a gain of function of GpIbα, a subunit of VWF receptor, on platelet membrane. In PT-VWD, GpIbα displays an increased avidity for VWF; as a consequence, platelets bind spontaneously high molecular weight VWF multimers, with
consequent platelet clumping and enhanced platelet clearance, causing thrombocytopenia and loss of VWF multimers [54].

Although the enhanced platelet clumping is a risk factor for thrombosis, hemorrhagic phenotype prevails due to thrombocytopenia and augmented clearance of high molecular weight VWF multimers. Bleeding diathesis is usually mild, but may become more severe after injuries, surgery and use of antiplatelet drugs. Laboratory tests assessing platelet function show mild thrombocytopenia, platelet macrocytosis and prolonged BT [55]. Platelet aggregation in response to low dose of ristocetin is enhanced, and multimeric analysis – performed by gel electrophoresis of test plasma – shows a decreased amount of high molecular weight VWF multimers [56]. Plasma VWF testing shows a discordance between functional and non-functional test parameters, such as low ristocetin cofactor activity and/or collagen binding capacity, compared with antigen [48].

Most of the clinical and laboratory features of PT-VWD are similar to VWD, due to a qualitative or quantitative defect VWF molecule. Differential diagnosis is based on ristocetin-induced aggregation. Specifically, platelet aggregation results normal when normal platelet rich plasma (PRP) is mixed with PT-VWD plasma, while it is enhanced when PRP is added to VWD patient. Conversely, when normal VWF is add to PT-VWD patient platelets, aggregation results strongly enhanced, while it is normal in presence of VWD platelets [57]. However, since aggregometry results are often ambiguous, demonstration of mutations in the GPIBA gene is generally necessary to provide final diagnosis.

Recently Giannini et al. proposed also a flow cytometric assay to discriminate PT-VWD from VWD, quantifying VWF-binding induced by ristocetin to fresh autologous or to formalin-fixed donor platelets [58].

**Wiskott-Aldrich syndrome (WAS)**

WAS is a X-linked recessive disease sustained by mutation in the *WAS* gene coding for a protein implicated in actin polymerization, and consequently required for numerous cellular functions such as cell structural changes, division, endomitotic process, migration and chemotaxis [59]. Mutations of WAS are also described in XLT, a variant with a milder clinical phenotype and more favorable prognosis [60]. At present, more than 150 mutations in WAS gene have been identified.

Mucocutaneous bleeding tendency is often the first sign of WAS and it is the only clinically relevant symptom in XLT patients. Diagnosis of WAS requires the presence of the classical triad: microthrombocytopenia, eczema and immunodeficiency. Thrombocytopenia is present at birth, while eczema tends to develop during the first year of life. Infection starts occurring early, usually in the first 6 months [4]. Platelet counts vary from patient to patient – but also within individual patients – between 5×10^9/L and 50×10^9/L. Mean platelet volume is between 3.5 and 5 fl, equal to half that of healthy subjects. Although many reports indicate that platelet functionality in WAS and XLT patients is abnormal, mechanisms underlying this altered activation have not been fully elucidated yet. Additionally, despite the potential pivotal role of WAS protein in the regulation of actin-dependent conformational changes during platelet activation, in vitro data are often contradictory. For example, increase in F-actin content and shape changes in stimulated WAS platelets appear to be normal [61, 62]. For these reasons, platelet function tests are not recommended for the diagnostic work-up of WAS.

**Storage pool diseases**

Platelets granules belong to two main categories, namely α- and δ-granules, containing fibrinogen, fibronectin, VWF, thrombospondin, vitronectin, platelet-derived growth factor (PDGF), platelet factor-IV (PF-IV), transforming growth factor (TGF), coagulation factors and ADP (α-granules); ATP, serotonin, calcium, pyrophosphate (δ-granules). These factors are essential for platelet activation and aggregation, and defects in granule content and/or degranulation result in disorders that may accompany both a normal or reduced platelet count. These diseases are known as storage pool diseases and, accordingly to the type of granule content deficiency, are distinguished in δ-storage pool diseases (including CHS and HPS), α-storage pool diseases (including GPS, PT/JS and Quebec syndrome (QPD)) and α/δ-storage pool disease, affecting both granules [63].

CHS is an autosomal recessive disorder with bleeding tendency. Laboratory tests show a normal platelet count with a prolonged BT and abnormal aggregation. Additionally, measurement of platelet granule components reveals a reduced number of dense-granules that frequently display typically larger dimensions (giant granules). Diagnosis is usually suggested by additional phenotypic trait, such as oculocutaneous albinism, symptomatic neutropenia with recurrent infections, presence of large, peroxidase-positive, cytoplasmic granules in a variety of both hematopoietic and non-hematopoietic cells [64].
HPS is a rare autosomal recessive disease of subcellular organelles present in many tissues, such as melanosomes, lysosomes and platelet-dense granules. Deficiency of δ-granules allows including this disorder among δ-storage pool diseases. Indeed patients present with a bleeding diathesis that is similar to other δ-storage pool diseases, causing easy bruising, epistaxis, menorrhagia, postsurgical bleeding; severity varies substantially among patients. BT is prolonged, and variable degrees of abnormalities can be observed by platelet function tests. When analyzed by electron microscopy, δ-granules result markedly reduced in number and characterized by peculiar structural abnormalities that typify HPS [65].

Since this disease involves not only platelet-dense granules, but also melanosomes and lysosomes, diagnosis is based also on other characteristic features: tyrosinase-positive oculocutaneous albinism and ceroid-lipofuscin lysosomal storages [66]. Ceroid-lipofuscin is a lipid-protein complex accumulating in lysosomal organelles, which is believed to be responsible for the development of progressive pulmonary fibrosis and granulomatous colitis [67–69]. To date, HPS has been associated with mutations in eight human genes [65].

**Gray platelet syndrome (GPS)**

In GPS the storage pool deficiency selectively hits α-granules. Its recessive and dominant inheritance pattern raises the possibility that this syndrome may be caused by defects in more than one gene [70]. Gray platelets not only lack the soluble molecules contained in α-granules (PF 4, β-thromboglobulin, VWF, thrombospondin, fibrinogen, fibronectin), but also the proteins that are endocytosed from plasma such as albumin and immunoglobulins [71].

The fact that α-granules membrane proteins are normal [72, 73], suggests that GPS defect specifically targets the process of packaging of endogenously synthesized proteins in the α-granules. This packaging defect seems to be specific for the megakaryocyte lineage, as GPS patients have normal Weibel-Palade bodies in endothelial cells [74, 75].

Patients have a lifelong history of mild to severe mucocutaneous bleeding, including intracranial hemorrhages and life-threatening postsurgical bleedings. Of note, some patients present a mild degree of bone marrow fibrosis [76].

Preliminary laboratory screening shows a prolonged BT and thrombocytopenia, which can be as low as $50 \times 10^9/L$ and severity may increase with time. Peripheral blood smear demonstrates abnormally large, gray platelets, as a consequence of the reduced or absent α-granules content [77].

Platelet aggregation studies are not helpful due to their variability in GPS patients. Platelet aggregation induced by ADP is usually normal, but impaired aggregation responses induced by low concentration of thrombin or collagen have been described in some patients [78].

PT/JS is an autosomal dominant disorder characterized by mild hemorrhagic diathesis. Laboratory tests show thrombocytopenia with normal platelet life span, increased number of bone marrow megakaryocytes with signs of abnormal maturation and intramedullary lysis. Circulating platelets display giant α-granules unable to release their content upon platelet stimulation with thrombin. These laboratory features are usually associated with other abnormalities including mental retardation, heart defect, and facial dysmorphism [79].

In QPD, a moderate to severe bleeding pattern is associated with a gain of function in fibrinolysis. QPD is characterized by low levels of factor V within platelet α-granules, due to increased expression and storage of urokinase plasminogen activator in platelets [80].

Clinically, QPD patients present a prevalent mucocutaneous bleeding diathesis that is difficult to distinguish from those of most platelet disorders, whereas other symptoms are more suggestive of a coagulation defect, like delayed bleeding and joint bleeds. Platelet count is normal or moderately reduced. BT and closure time can be normal. Platelet aggregation is absent in response to low-dose epinephrine, while normal to reduced with ADP and collagen. However, these aggregation findings are not QPD specific [81, 82].

QPD inheritance pattern is autosomal dominant and the genetic lesion involved is a tandem duplication of *PLAU*, encoding for Urokinase plasminogen activator. Genetic testing for PLAU duplication, using PCR and Southern blot, has become the method of choice for definitive diagnosis of QPD. The PCR assay for QPD mutation can be performed on cord blood samples to rapidly determine *PLAU* mutational status in a newborn from affected parents [83].

**Scott syndrome (SS)**

SS is a rare autosomal recessive disorder affecting calcium-induced phospholipid scrambling and generation of thrombin on platelets. Activated Scott platelets fail to transport phosphatidylserine (PS) from the inner to the outer phospholipid leaflet of the membrane bilayer. As
a result, factors Va and Xa are unable to bind the activated platelet surface and, consequently, to transform prothrombin into thrombin [84]. This phenotype is not restricted to platelets but can be demonstrated in other blood cells.

Scott patients have a clinical history of provoked bleeding episodes (menorrhagia, trauma-related hematoma, bleeding after tooth extraction, and severe postpartum bleeding).

Routine laboratory tests that investigate disorders of platelet function fail to reveal any deficiencies or functional aberration: BT is within the normal range, platelet count and morphology are normal, and no aberration of platelet aggregation, secretion, granule content have been detected [85]. Scott platelets do, however, fail to promote fibrin formation in the presence of coagulation factors. In fact, prothrombin is not efficiently cleaved during clotting of whole blood but, unlikely coagulation factor deficiencies, this condition is not restored by addition of healthy donor plasma [86]. Since Scott platelets do not have altered phospholipid composition in their membrane after activation [87], flow cytometric analysis can rapidly and effectively detect this defect through the Annexin V binding test. The assay is based on Annexin V-binding to PS exposed after activation, revealing a lack of PS exposure after activation of Scott platelets [88].

A few years ago, Albrecht et al. described a specific genetic lesion associated with SS: a missense mutation in the gene coding for lipid transporter ABCA1 [89].

**Conclusions**

Although in the last few years new insights in the genetic bases and pathophysiology of IPDs have extremely improved our knowledge of these disorders, the causes of many of them, including the most common, are still largely unknown. Next generation sequencing strategies will hopefully help to fill this gap.

Many efforts are required also in order to improve and standardize the laboratory diagnosis of IPDs. In fact, since these diseases are rare, diagnostic tests are available only at specialized laboratory centers. Adequate management of these patients requires initially an accurate bleeding history and evaluation of family history (this aspect is particularly relevant in children since they may have had few hemostatic challenges).

The first and most common laboratory tests are represented by platelet count and peripheral blood smear. These routinely available and relatively simple tests allow to discriminate between bleeding disorders with normal platelet count from those with low platelet count, and, in the latter group, between macro- and micro-thrombocytopenias [103].

In the presence of a bleeding diathesis with a normal platelet count, platelet aggregation tests are mandatory. An impaired aggregation to all agonists associated with pathognomonic clinical features (oculocutaneous albinism, granulomatosis colitis, and pulmonary fibrosis) suggests δ-granule defects such as CHS and HPS; electron microscopy, although not routinely utilized in clinical practice, leads to final diagnosis. FCM is a feasible and rapid technique to discriminate between GT and SS when aggregation and clot formation are lacking [26, 27]. Aggregation studies are extremely informative and, generally conclusive when identifying selective aggregation defects for a specific agonist, such as epinephrine in case of QPD.

**Agonist platelet receptor defects**

Agonist platelet receptor defects are extremely rare diseases typified by impaired platelet aggregation in response to certain agonists, due to lack or decreased functionality of specific receptors. Better characterized diseases are ADP receptor and Thromboxane A₂ receptor defects. ADP receptor defects display a heterozygous inheritance pattern and are due to mutations in P2Y12, accounting for a congenital deficiency or dysfunction of the receptor, leading to impaired downstream signaling [90]. So far, only 13 cases have been reported in the literature [91–97].

Mutations involving TXA₂R lead to reduced receptor coupling with its effector G protein and with phospholipase C. An autosomal inheritance has been described for these diseases [98–100].

Clinical presentation of agonist platelet receptor defects is similar, with mild to moderate mucocutaneous bleeding diathesis accompanied to normal platelet count and morphology. Diagnosis relies on aggregation studies; in case of ADP receptor deficiency a small and rapidly reversible aggregation induced by ADP – even at high concentration (≥10 μM) – is seen. Additionally, other agonists induce a defective aggregation and secretion [93]. Tests assessing the degree of inhibition of adenyl cyclase by ADP by measuring either the platelet levels of cAMP or the phosphorylation of VASP induced by prostaglandin E₁, are recommended to confirm diagnosis [101].

Thromboxane A₂ receptor defects show absent aggregation upon stimulation with arachidonic acid and thromboxane analogues. Response to other agonists is variable, with the exception for ristocetin, which is usually preserved [98, 102].
or ADP and arachidonic acid in case of agonist receptor defects (Figure 2).

In the case of thrombocytopenia, bone marrow aspirate/biopsy is generally required to investigate abnormalities of the megakaryocytic lineage [2]. Co-morbidities and associated somatic defects, together with genetic testing, lead to the final diagnosis of the great majority of inherited thrombocytopenias such as WAS, thrombocytopenias associated with skeletal defects, MHY9-related disorders, XLT and PT/J (Figure 1). Among inherited macrothrombocytopenias, platelet function tests detain a key role in the differential diagnosis between BSS and PT/VWD, which show an opposite response to ristocetin-induced aggregation (absent and enhanced, respectively). Of note, BSS diagnosis relies also on FCM analysis of GpIb expression. A defective aggregation upon stimulation with collagen and arachidonic acid indicates GPS (Figure 1).

Genetic testing is the final step of the diagnostic work-up of many IPDs, but, unfortunately, it is available only for MHY9-related disorders, CAMT, X-linked thrombocytopenia, FDP/AML, BSS, PT-VWD and WAS [5] (Table 1 and Figures 1 and 2).

Acknowledgments: This work was partially supported by Programma di ricerca Area 1 Regione Emilia-Romagna-Università (ER-Università) 2010–2012 and Finanziamento Italiano per la Ricerca di Base (FIRB) RBAP10KCNS_002. We are grateful to Luciana Cerasuolo, Vincenzo Palermo, Domenico Manfredi and Davide Dallatana for technical support.

Conflict of interest statement

Authors’ conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

Research funding: None declared.

Employment or leadership: None declared.

Honorarium: None declared.

Received February 7, 2014; accepted March 11, 2014; previously published online April 3, 2014

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Cecilia Carubbi completed her studies in Medical Biotechnologies, obtaining her MSc in 2007 at the University of Parma, Italy. She is currently attending a PhD program in Biomedical Sciences (University of Ferrara, Italy). Her scientific interests are represented by mechanisms of thrombopoiesis and platelet function, signaling in normal and malignant hematopoiesis.

Elena Masselli obtained her Medical Degree in 2007 and her Hematology Board in 2012 at the University of Parma, Italy. From March 2010 to August 2011 she was appointed as visiting assistant professor at the Department of Hematology/Oncology, Mount Sinai School of Medicine, New York, USA. She is attending a PhD program in Molecular Biology and Pathology at the Department of Biomedical, Biotechnological and Translational Sciences (S.Bi.Bi.T.), University of Parma, Italy. Her research involves the molecular basis of thrombocytosis and thrombocytopenias, polycythemias and myeloproliferative neoplasms.
Antonio Nouvenne graduated with full marks at the University of Parma in 2003, he took his post-graduate degree in Gastroenterology in 2007 and his PhD in osteo-methabolic diseases and acid-base balance disorders at the same university in 2010. He currently works as hospital doctor at the Internal Medicine and Subacute Critical Care Clinic of the University Hospital of Parma.

Domenico Russo, MD is Full Professor and Chair of Hematology, Department of Medical and Surgical Sciences, University of Brescia, Italy. He obtained his Medical Degree in 1982 from the University of Bologna, Italy, completed his residency in Hematology in 1986. He had a 2-year research appointment at the Laboratory of Cellular Kinetics, Hotel Dieu Hospital, University of Paris (VI), Paris, France. His scientific interests are focused on hematological diseases and stem cell transplantation.

Daniela Galli obtained her PhD in 2004 at the University of Rome “La Sapienza”, Italy. From 2005 to 2007 she worked as post-doc at Pasteur Institute, Paris, France. From June 2010 she has been working at University of Parma, Department of Biomedical, Biotechnological and Translational Sciences (S.Bi.Bi.T.). Her scientific interests cover the studies of signal transduction in vascular and hematopoietic models.

Prisco Mirandola obtained his Degree in Biology (1992) and PhD in Molecular and Cell Biology (2001). Since 2006 he has been Associate Professor of Human Anatomy at the Department of Biomedical, Biotechnological and Translational Sciences, University of Parma, Italy. Scientific interests areas: differentiation, signaling, proliferation and apoptosis of blood cells.

Giuliana Gobbi, PhD, is Assistant Professor of Human Anatomy at the Department of Biomedical, Biotechnological and Translational Sciences, University of Parma, Italy. She obtained her Degree in Biology in 1996 and 4 years later completed her residency in Clinical Pathology at the University of Bologna, Italy. In 2004 she obtained her Master’s Degree in Clinical and Experimental Flow Cytometry and 3 years later a PhD in Human Morphological and Molecular Sciences. She was appointed as visiting scientist at the National Human Genome Research Institute (NHGRI) Bethesda, Maryland, USA in 2000 and at the Brigham and Women Hospital (Harvard University), Boston, MA, in 2011. Her major active research interest is focused on human platelets function and megakaryocytopoiesis.

Marco Vitale, MD is Full Professor of Human Anatomy and Chair of the Department of Biomedical, Biotechnological and Translational Sciences, University of Parma, Italy. He obtained his Medical Degree in 1985 from the University of Bologna, Italy, completed his residency in Hematology in 1988 and in Biomedical Technologies 4 years later. He had a 1-year research appointment at the Jefferson Cancer Institute, Thomas Jefferson University School of Medicine (Philadelphia, USA). Since 2012 he has been Adjunct Professor of Medicine and Member at the Institute of Human Virology at the University of Maryland School of Medicine, Baltimore, MD, USA. His scientific interests are focused on molecular biology and signaling of hematopoietic cells.