

Strategies for the diagnosis of inherited platelet disorders

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Inherited platelet disorders (IPDs) represent a heterogeneous group of rare conditions that affect platelet number and/or function, resulting in variable bleeding tendencies and clinical consequences. Two decades ago, IPDs were considered exceptionally rare, with only the most severe and well-characterized forms being widely recognized. However, it has become increasingly evident that milder forms, particularly inherited thrombocytopenias (ITs), are more common than previously thought. These often present with mild to moderate thrombocytopenia and mild bleeding symptoms, and are frequently underdiagnosed, incidentally discovered, or misdiagnosed as immune thrombocytopenia. Notably, several IPDs have been shown to predispose patients to additional clinical manifestations, which in many cases have a greater impact on overall health than the thrombocytopenia itself¹.

To date, more than 60 distinct IPDs have been described, caused by mutations in over 75 genes², although the exact prevalence of each disorder remains unclear. While individually rare, IPDs collectively represent a relatively common cause of hereditary bleeding. A recent genome database analysis estimated the prevalence of IPDs at 329/100,000 in the general population³. At our Center for Congenital Coagulopathies, based in a tertiary care hospital in northern Portugal, IPDs account for 27% of hereditary bleeding disorders, ranking second most common category after von Willebrand disease (VWD). Based on our data, the

estimated prevalence of IPDs is approximately 29 cases/100,000 population. This is markedly higher than figures reported by the U.S. Hemophilia Treatment Centers (2.0/100,000)⁴ and the UK Hemophilia Center Doctors' Organization (5.0/100,000)^{4,5}, though it remains substantially lower than estimates derived from genomic database analysis³.

IPDs present a diagnostic challenge due to their clinical diversity, variable penetrance, and the complexity of functional testing, which is limited to specialized laboratories. As a result, many cases remain undiagnosed. However, advances in genetic testing over the past decade have significantly improved diagnostic accuracy, enabling the identification of previously underdiagnosed or misclassified disorders.

Clinical assessment: the clinical evaluation of suspected IPDs is based on a thorough personal and family history and physical examination. Key goals include assessing bleeding history, identifying features suggestive of syndromic conditions, and excluding acquired platelet disorders. Bleeding history should include both spontaneous and provoked episodes across the patient's lifetime. The number and the intensity of bleeding are substantially higher in IPDs with defective platelet function than in IPDs with thrombocytopenia only⁶. Validated tools, such as the International Society of Thrombosis and Hemostasis' bleeding assessment tool (ISTH-BAT)⁷ help quantify bleeding severity and guide further investigation, although they have limitations, especially in

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children and premenarchal girls⁸. Non-hematological features suggestive of syndromic conditions should be actively sought. These features may precede the bleeding phenotype, or, more commonly, the platelet disorder is identified first, prompting further screening for associated abnormalities. Ruling out acquired platelet dysfunction (e.g., drug-induced or secondary to other conditions) is essential to optimize the diagnostic yield of genetic testing. Finally, family screening can clarify inheritance patterns, although a negative history does not exclude IPDs due to the possibility of *de novo* mutations.

Platelet count, volume, and morphology: routine platelet counting is typically performed using automated analyzers, most commonly through electrical impedance. While accurate in individuals with normal platelet size and morphology, this method underestimates platelet count when platelet volume or shape is altered, as seen in macrothrombocytopenia and, to a lesser extent, microthrombocytopenia. Optical counting based on light scatter offers improved performance but may still fail to detect large or abnormally shaped platelets. More recently, fluorescence-based methods have improved accuracy⁹, although flow cytometry using antibodies against platelet glycoproteins remains the gold standard¹⁰, albeit rarely used in routine practice. At our center, we employ flow cytometry for platelet counting in IT, especially before invasive procedures requiring precise platelet thresholds. Assessing platelet size presents similar challenges. Mean platelet volume (MPV) varies with the analyzer used and is often underestimated in the presence of very large platelets. While mean platelet diameter (MPD) has been proposed for ITs classification¹¹, its clinical use is limited by its time-consuming nature. Instead, we use the platelet Forward Scatter (FSC) index by flow cytometry, a validated and reliable marker of platelet size that can discriminate ITs with high sensitivity and specificity¹². Immature platelet fraction (IPF) quantifies the population of newly released platelets with higher ribonucleic acid content, reflecting thrombopoietic activity. Available on modern analyzers using flow cytometry, IPF correlates with platelet size. IPF values can support the diagnosis of syndromes, such as MYH9-related disease (RD) and Bernard Soulier syndrome (BSS), which present disproportionately elevated values¹³. Peripheral blood smear review remains a valuable diagnostic tool. When combined with immunofluorescence microscopy using antibodies targeting key platelet proteins, it can detect structural abnormalities¹⁴. Electron microscopy provides

the most detailed morphological evaluation, though it is generally unavailable in routine clinical settings.

Platelet function tests: light-transmission aggregometry remains the gold standard for assessing platelet function, despite being labor-intensive, time-consuming, and dependent on expert interpretation, with limited standardization and significant inter-laboratory variability. At our center, we use a Lumi-aggregometer, which simultaneously measures platelet aggregation and adenosine triphosphate (ATP) release through luminescence. This dual testing increases sensitivity for detecting dense granule deficiencies and secretion defects. ATP release testing is recommended when platelet dysfunction is strongly suspected, even if standard aggregation appears normal. Impedance aggregometry, performed in whole blood, provides an alternative. Studies show that mild abnormalities may be detected by one method but not the other¹⁵. Flow cytometry is commonly used to quantify platelet glycoproteins, especially in diagnosing receptor deficiencies in BSS, Glanzmann thrombasthenia (GT), and occasionally collagen receptor defects. Additional assays assess α - and δ -granule defects, agonist-induced fibrinogen binding, and procoagulant activity, offering further insight into functional abnormalities⁶.

Genetic testing: sanger sequencing has traditionally been used to confirm a limited number of IPDs with well-established genetic causes and remains a valuable first-line tool in selected cases. However, the introduction of high-throughput sequencing (HTS), including targeted gene sequencing (TGS), whole-exome sequencing (WES), and whole-genome sequencing (WGS), has revolutionized IPDs diagnostic by allowing faster, broader, and more cost-effective genetic analysis⁶. Several TGS platforms have been developed, and the ISTH Genomics Subcommittee maintains a curated list of genes with confirmed roles in hemostatic disorders¹⁶. Diagnostic yields are currently around 50% for ITs and 25% for inherited platelet function disorders¹⁷. A major limitation of HTS lies in the interpretation of variants of uncertain significance (VUS). In such cases, functional assays, family segregation analysis, and clinical phenotype correlation are essential to support or refute pathogenicity⁶. The development of shared VUS databases has also been proposed to enhance variant classification and improve diagnostic accuracy¹⁸. Finally, structural variants may go undetected even with advanced HTS methods, such as WGS, highlighting the need for complementary diagnostic strategies.

How we diagnose IPDs: at our center, the diagnostic approach to IPDs builds on extensive experience with

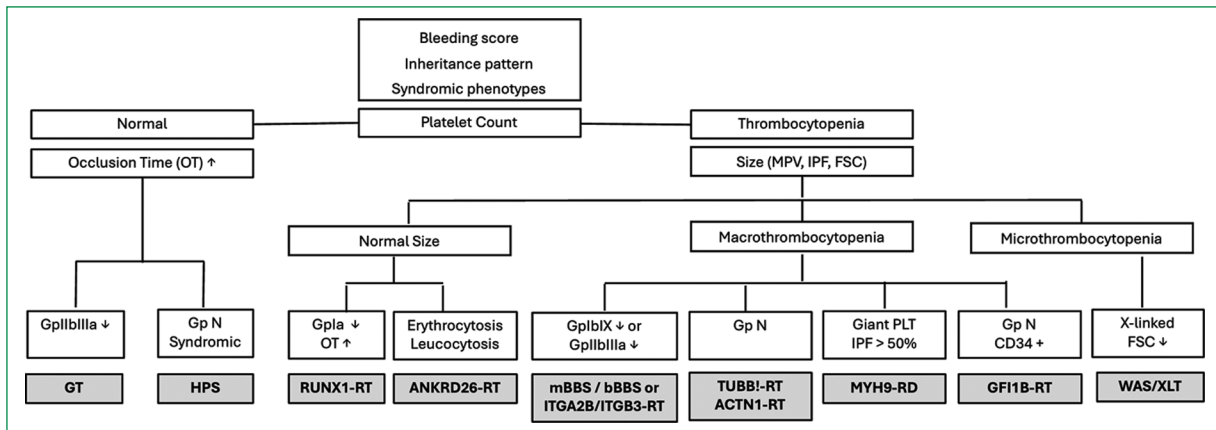


Figure 1. Diagnostic algorithm for the twelve most common inherited platelet disorders in our cohort. GP: glycoprotein; MPV: mean platelet volume; IPF: immature platelet fraction; FSC: forward scatter; RT: related thrombocytopenia; RD: related disease; mBSS: monoallelic Bernard-Soulier syndrome; bBSS: biallelic Bernard-Soulier syndrome; GT: glanzmann thrombasthenia; HPS: Hermansky-Pudlack syndrome; WAS/XLT: wiskott-Aldrich syndrome/X-linked thrombocytopenia.

platelet aggregometry and flow cytometry and has recently been strengthened through the integration of genetic testing. This comprehensive, stepwise strategy involves four key stages: thorough clinical evaluation, assessment of platelet count, size, and morphology, functional testing, and molecular analysis. All patients undergo detailed clinical evaluation, with emphasis on bleeding assessment using the ISTH-BAT, identification of non-hematological features suggestive of syndromic conditions, and family history to evaluate inheritance patterns or predisposition to hematologic malignancies. Platelet analysis includes quantification by optical methods and, in selected cases, flow cytometry; size assessment using MPV, IPF, and FSC index, while morphology is evaluated through peripheral blood smear review. Platelet glycoprotein expression is quantified in all cases. In patients with suspected platelet dysfunction, platelet function is assessed using light transmission aggregometry. Additional studies, such as platelet activation assays¹⁹ and electron microscopy, are performed in selected cases to further characterize the underlying defect. Genetic analysis is performed through targeted Sanger sequencing of suspected genes, or a custom-designed TGS panel for hemostatic disorders, and, in specific cases, Multiplex ligation-dependent probe amplification (MLPA). At present, our standard practice involves WES-based analysis of 81 genes known to be associated with platelet defects.

This initial evaluation enables the identification of distinct phenotypic patterns, at least for the most common IPDs in our cohort, which can guide subsequent genetic testing and support diagnostic confirmation, particularly

in cases involving VUS. For example: mild autosomal dominant thrombocytopenia with normal platelet size (normal MPV and IPF), bleeding tendency and reduced GpIa expression suggests *RUNX1*-related thrombocytopenia (RT); A similar thrombocytopenia pattern without bleeding, but with leucocytosis and/or erythrocytosis, supports *ANKRD26*-RT; severe macrothrombocytopenia with giant platelet, IPF > 50%, and moderate bleeding tendency, is suggestive of *MYH9*-RD; severe macrothrombocytopenia with giant platelet, marked reduction of *GpIb/IX* expression, and severe bleeding tendency, indicates biallelic BSS; in contrast, mild autosomal dominant macrothrombocytopenia, mildly enlarged platelet, partial *GpIb/IX* reduction and no bleeding symptoms, suggests monoallelic BSS; autosomal dominant macrothrombocytopenia with similar platelet features but fewer platelet than monoallelic BSS, partial *GpIb/IIIa* reduction and moderate bleeding, points toward *ITGA2B/ITGB3*-RT; moderate macrothrombocytopenia with increased CD34 expression on platelets is indicative of *GFI1B*-RT (Fig. 1). Identifying these phenotypes is particularly important when using panel-based genetic tests, which may miss non-coding pathogenic variants, such as 5' UTR mutations in *ANKRD26*, or structural variants, as seen in some *RUNX1*-RT cases; in the past scenario, complementary methods, such as MLPA are crucial. When a specific phenotype is highly suggestive, especially in platelet receptor defects, Sanger sequencing remains a cost-effective and reliable diagnostic option.

This approach enabled the identification of IPDs in 268 patients from 115 families (Fig. 2). The most frequent

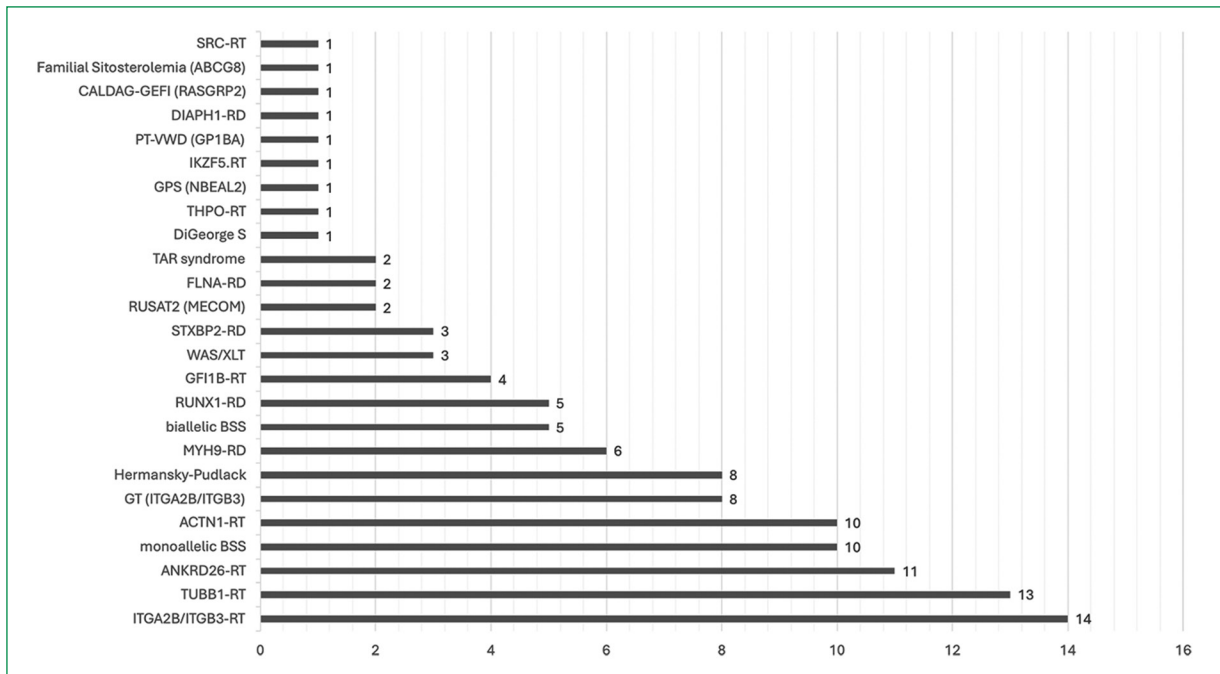


Figure 2. Genetic distribution of IPDs identified in a cohort of 115 families. Detected variants are classified as pathogenic, likely pathogenic, or VUS according to the guidelines of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology²⁰. VUS are included when additional evidence, such as functional studies, family-based segregation, or clinical phenotype, supports their contribution to IPDs. Heterozygous carriers of pathogenic/likely pathogenic variants in recessive disorders are not included. IPDs: inherited platelet disorders; VUS: variants of uncertain significance, RT: related thrombocytopenia; RD: related disease; BSS: Bernard-Soulier syndrome; GT: Glanzmann Thrombasthenia; WAS/XLT: Wiskott-Aldrich syndrome/X-linked thrombocytopenia, GPS: gray platelet syndrome; PT: platelet type; TAR: thrombocytopenia-absent radius, VWD: von Willebrand disease.

disorder in our cohort was ITGA2B/ITGB3-RT, present in 16% of patients (14 families), a notably higher frequency than reported in other series. A similar frequency was observed for *ANKRD26*-RT, affecting 16% of patients (11 families). Cytoskeleton-related disorders were also common, with *TUBB1*-RT and *ACTN1*-RT found in 11.6% (13 families) and 10.1% (10 families), respectively. Along with cases of GT (8 families), Hermansky-Pudlack syndrome (8 families), monoallelic BSS (10 families), biallelic BSS (5 families), *MYH9*-RD (6 families), *RUNX1*-RT (5 families) and *GFI1B*-RT (4 families), these diagnoses accounted for 86% of patients with a confirmed molecular diagnosis. The remaining 14% represented a heterogeneous group of rare IPDs, including Wiskott-Aldrich syndrome, CalDAG-GEFI deficiency, thrombocytopenia-absent radius syndrome, Gray platelet syndrome, platelet type-VWD, *DIAPH1*-RT, *MECOM*-RT, *FLNA*-RD, *THPO*-RT, *IKZF5*-RT, *SRC*-RT, and others (Fig. 2). With the recent introduction of WES, we anticipate achieving diagnoses in some previously undiagnosed patients, further expanding the identification of IPDs within our cohort.

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