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Perspective

Diagnosis of inherited platelet function disorders

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Abstract

Inherited platelet function disorders (IPFDs) are rare, congenital diseases characterized by a widely heterogeneous clinical presentation. Diagnosis of IPFDs is challenging as there is no consensus or standardized approach and many laboratory techniques used for assessment are insufficiently standardized, technically challenging and poorly reproducible. Aim of this article is to describe the correct approach for laboratory diagnosis of IPFDs.

Background

Inherited platelet function disorders (IPFDs) are congenital haemorrhagic disorders characterized by mild to severe mucocutaneous bleeding and by a wide phenotypic and genotypic heterogeneity. Though IPFD make up a significant proportion of congenital bleeding diatheses, they remain poorly understood and often difficult to diagnose. The exact prevalence of IPFDs in the world is unknown, but it may range from as low as 2/1,000,000 for some autosomal recessive forms to up to 1/100 for some mild platelet secretion defects[1]. The prevalence is probably underestimated due to under-diagnosis. The diagnostic evaluation of IPFDs is complex, poorly standardized and time consuming. The diagnosis of IPFDs is sometimes a significant challenge to even laboratories with good facilities[2]. Approach for the laboratory diagnosis of IPFDs should be stepwise, starting from the simpler tests, followed by more selective, sophisticated analyses and laboratory findings should always be correlated with clinical presentation.

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Identification of patients with a suspected bleeding disorder

The initial diagnosis of IPFD relies on careful evaluation of the clinical findings. A detailed medical history, including personal, drug and family history including consanguinity is important. Reviewing the medical history can establish whether the disorder is hereditary or acquired. e.g. taking non-steroidal anti-inflammatory drugs, renal failure and myeloid neoplasms can be associated with platelet dysfunction with clinically significant bleeding[3]. A family history of mucocutaneous bleeding helps to orient toward an IPFD, however, the absence of a familial bleeding history is not exclusive. Presence of consanguinity increases the likelihood of autosomal recessive disease. A de novo mutation must also be considered[1,3].

Bleeding manifestations typical of platelet function defects include unexplained or extensive bruising, particularly associated with soft tissue haematoma, epistaxis, particularly lasting more than 30 minutes or causing anemia or admission to hospital, menorrhagia, particularly if present since menarche, gingival bleeding, bleeding during childbirth, bleeding following invasive procedures e.g. dental extraction, tonsillectomy, adenoidectomy[1,4]. The timing of onset of haemorrhagic features is important. Severe disorders are likely to present at a younger age, sometimes with severe neonatal bleeding. A later presentation with haemorrhagic symptoms is more typical of a less severe disorder[3].

An accurate assessment of the presence and severity of bleeding symptoms using a standardized bleeding assessment tool (BAT) may be of great help in diagnosing IPFDs especially for the mild forms. The ISTH-BAT is a largely used tool, but it has not been sufficiently validated for patients with IPFDs yet[1,3].

Platelet disorders are sometimes 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))[3].

Indications for testing

Laboratory investigations for IPFDs are indicated for patients with a history of mucocutaneous bleeding (familial or not) in whom an acquired or drug-induced cause of platelet dysfunction, immune thrombocytopenia (ITP), von Willebrand disease, a blood clotting defect or afibrinogenemia have been ruled out. For that, preliminary laboratory investigations, including full blood count (FBC), prothrombin time (PT), activated partial thromboplastin time (APTT), and von Willebrand factor (VWF) screening tests (VWF antigen, ristocetin cofactor activity, and factor VIII coagulant activity) should show normal results[1,7]. Given that several IPFDs are associated with thrombocytopenia, a mildly reduced platelet count should not halt further testing[7].

Laboratory tests for platelet disorders

Laboratory tests for platelet disorders comprise:

1 Measurement of platelet number and size
2 Global screening tests of platelet haemostatic function
3 Specific assays of platelet haemostatic function[3]

Platelet number, size and morphology

FBC should be performed on all patients suspected of IPFD to assess platelet number and size (mean platelet volume, (MPV)). In samples with abnormalities in platelet count or size distribution (as indicated by an automated analyser), a blood film should be examined. Platelets may be large in Bernard-Soulier syndrome and May-Hegglin anomaly. In Wiskott-Aldrich syndrome, platelets are small in size and decreased in number. Platelets may appear gray or washed out in gray platelet syndrome. Large inclusion granules in white cells are visible on smears in Chediak Higashi syndrome[1]. However, in the majority of platelet function defects such as release and storage pool defects and Glanzmann's thrombasthenia, platelet number and morphology are normal.

Global tests of platelet haemostatic function

Global tests do not enable a diagnosis of a specific platelet disorder. They are normally performed as the first part of a strategy that requires further testing with more specialized assays of platelet function. The most widely performed tests for screening platelet function disorders are the template bleeding time (BT) and the platelet function analyser (PFA-100) closure time. Thromboelastography (TEG) and rotational thromboelastometry (ROTEM) provide global tests of haemostasis and platelet function which are mainly used within the surgical setting.

The BT is not recommended as a screening test as it has poor reproducibility, sensitivity and specificity, as well as being invasive. PFA-100 has better standardization; however, it is not sensitive or diagnostic for mild IPFD while abnormal results may be observed in severe IPFD such as GT, BSS[3,8]. The utility of TEG/ROTEM for the screening and diagnosis of platelet function defects has not yet been examined systematically and their use for this application is therefore not currently recommended[3].

Specific assays of platelet function

Light transmission aggregometry (LTA)

LTA is the gold standard for platelet function testing[1,3]. 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, thromboxane analogues and ristocetin. Parameters measured include the rate of aggregation and the maximal percentage of aggregation after a fixed period of time.

LTA must be performed by experienced technicians on samples that are properly obtained and promptly transported to prevent activation of platelets before testing. Temperature, lipemia,

sample collection, interval from venipuncture, and preparation of PRP may all affect platelet aggregation profiles[3,8].

The pattern of impairment of platelet aggregation is characteristic of several specific IPFDs and is sometimes diagnostic (e.g. GT and BSS)[1,3].

Despite its widespread use, LTA is poorly standardized, preparation of PRP and analysis is time-consuming, and interpretation of results and correlation with clinical phenotype still needs to be undertaken by those with experience[1,3]. LTA may be normal or minimally abnormal in some IPFD (e.g. storage pool defect). Hence, normal LTA does not completely rule out IPFD[1].

Flow cytometry

The most commonly used flow cytometry tests relevant to platelet function are the quantification of glycoprotein receptor density in the diagnosis of GT and BSS, and detecting their heterozygous states. It should be performed with resting platelets using antibodies towards the components of GPIIb/IIIa and GPIb/IX/V. Flow cytometry is highly recommended for the diagnosis of BSS and GT variant forms, in which LTA is normal. Flow cytometry can also be used to measure the collagen (GpIa/IIa and GpVI) and PAR-1 receptor densities if LTA testing suggests any abnormalities in these receptors. There are also tests available to measure platelet activation in response to classical agonists, dense granule content, and exposure of anionic phospholipids[3].

As flow cytometry is expensive, time consuming and requires specialized training, only those patients with an appropriate clinical history and/or abnormalities of other platelet function tests should be assessed for receptor defects[3].

Measurement of platelet granule release

The measurement of total and/or released adenine nucleotides provides an important additional diagnostic tool usually in conjunction with aggregometry for determining whether there is any specific deficiency in dense granule numbers or their content (e.g. storage pool disease), or specific defect(s) in degranulation (e.g. release defects).

1. Lumi-Aggregometer:

The simplest assay of released platelet nucleotides can be performed in real time with a Lumi-Aggregometer and platelet-rich plasma or whole blood. It simultaneously measures LTA and ATP secretion. The assay is performed by adding a luciferin-luciferase reagent, along with an agonist (to stimulate the release of platelet dense granule contents), while stirring the sample at low shear to promote platelet activation and aggregation. The ATP released from platelets reacts with the luciferin-luciferase reagent, resulting in light emission that is usually quantified relative to an ATP standard.

While ATP release assays are typically used to assess people suspected of having a platelet disorder, their sensitivity and specificity have never been reported. Furthermore, uncertainties exist about the number and type of agonists that are needed to detect impaired platelet ATP release due to common platelet function disorders[5,12].

Although the manufacturer suggests to use platelet ATP release response with thrombin to differentiate storage from release defects, it is practically difficult using this approach.

2. ATP: ADP ratio:

Many laboratories determine the total platelet content of both ADP and ATP with lysed platelet preparations (at standardized platelet counts) and sometimes after a degranulation step to induce release. There are two nucleotide pools within the platelet: the metabolic pool and the dense granular/storage pool, the latter comprising about 60% of the total content. The ratio of ATP: ADP is therefore of fundamental diagnostic importance as there are pronounced differences between the relative concentrations in the two pools. Any storage defects are associated with a decrease in the amount of stored and released ADP with an increased ratio of ATP: ADP. Normal ADP levels and ATP: ADP ratios but decreased ADP release are indicative of a release-defect[3].

3. Serotonin uptake and release test:

Secretion can also be measured by allowing platelets to take up ^{14}C labelled serotonin; its release is then measured in response to agonists.

This test has a significant technical component, and is performed only in selected laboratories. Enzyme-linked immunosorbent assays (ELISA) for platelet serotonin content are also available[3].

4. Mepacrine uptake and release:

The release by the dense granules can be measured by flow cytometry[3].

5. α-granular content release:

The release of α-granules is commonly evaluated by flow cytometry by measuring surface P-selectin expressed on platelets surface after platelet activation. The release of specific α-granule proteins (β-TG, TSP-1, VWF, fibrinogen, P-selectin) can also be assessed in platelet supernatants by HPLC or ELISA[3].

Clot retraction

Clot retraction is assessed by incubating non-anticoagulated whole blood for 60 min at 37°C in glass tubes. A long clot retraction time is abnormal and suggests an abnormality somewhere in the coagulation cascade after integrin IIb/IIIa interacts with fibrin. Therefore, may suggest GT, fibrinogen deficiency or Stormorken Syndrome.

Serum thromboxane B2

Serum thromboxane B2 can be assessed by ELISA or RIA. It detects defects of arachidonic acid release or metabolism.

Transmission Electron Microscopy (TEM)

TEM reveals structural platelet abnormalities, including a decreased number or abnormal morphology of platelet alpha and dense granules. EM studies are helpful in diagnosing platelet granule defects.

Molecular studies

Table 1. Mutations identified for some IPFDs

Mutant gene	IPFD
ITGA2B, ITGB3,	GT
GP1BA, GP1BB, GP9	BSS
GP1BA	PT-VWD
WAS	WAS50
NBEAL2, GFI1B	GPS

Targeted familial variant analysis (usually by Sanger sequencing) is the simplest genetic test that is used in the evaluation of patients with IPFDs. It is highly cost-effective when an affected family member has a confirmed diagnosis and the specific familial genetic variant is known. Single gene analysis is useful when the phenotype for a monogenic disorder is highly penetrant and clinical or laboratory features are diagnostic, for example, analysis of MYH9 in a patient with macrothrombocytopenia and Dohle bodies, or analysis of ITGA2B and ITGB3 in a patient with a laboratory diagnosis of GT. Genetic panels have become available and currently are the most widely used testing strategy. Panels are built by each laboratory based on the genes that are considered to be of clinical interest. Next-generation sequencing (NGS) most effectively detects sequence variants and small insertions or deletions[3,8].

The first screening should include an evaluation of a blood smear, light transmission aggregometry (LTA) using a limited number of agonists (epinephrine, ADP, collagen, arachidonic acid and ristocetin), the measurement of platelet granule release and the evaluation of the main surface glycoproteins (GPs) by flow cytometry.

The measurement of platelet granule release should include an assay for ATP/ADP and at least one marker of alpha-granules.

Flow cytometry screening should be carried out on resting platelets using antibodies against GPIIb/IIIa (CD41), GPIIIa (CD61), GPIb (CD42b) and GPIb/IX (CD42a), and on activated platelets using an antibody against a GPIIb/IIIa activation epitope (PAC-1). Reduced glycoprotein expression of GPIIb/IIIa and GPIIIa suggests GT and GPIb/IX suggests BSS.

The first-step tests have the potential to diagnose up to ~40% of all IPFD[7].

A second set of laboratory tests, to be performed in patients for whom the first step has not yielded a conclusive diagnosis.
It should include LTA with an expanded range of

How to investigate for an IPFD:

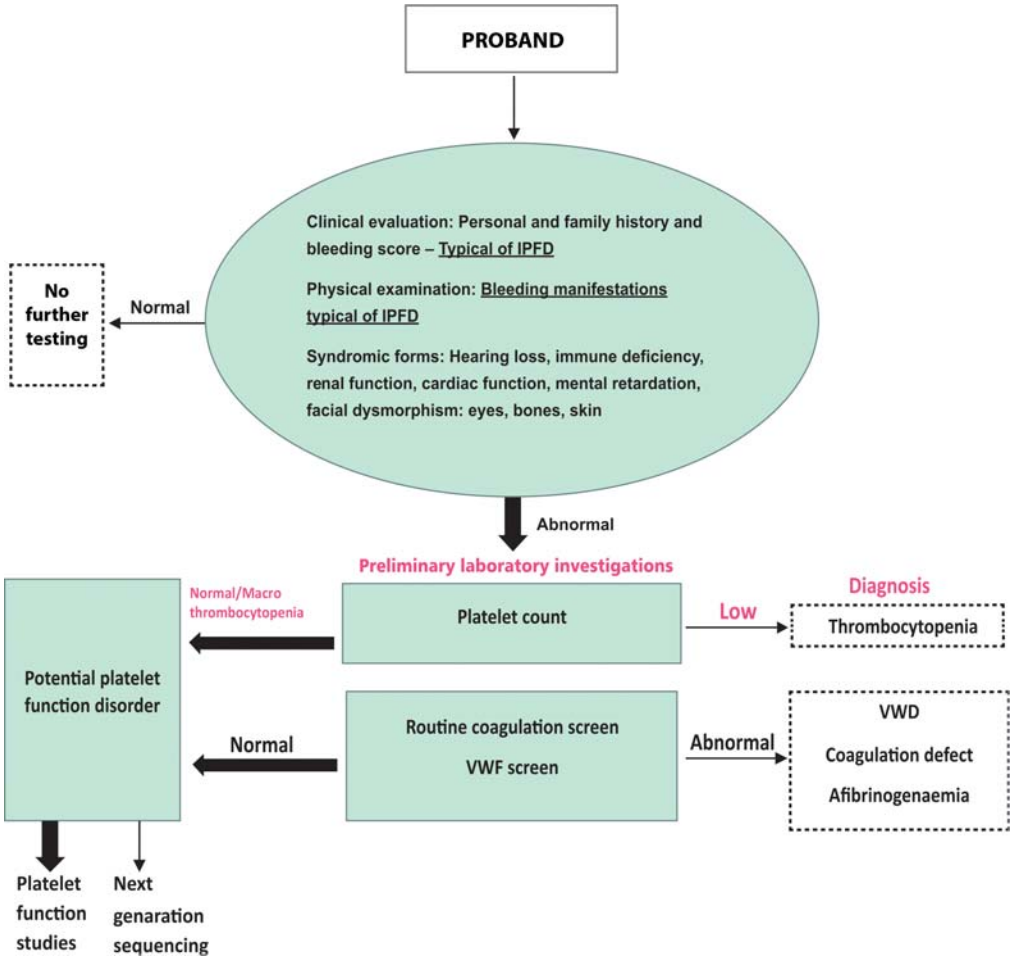

Figure 1. Diagnostic algorithm flowchart.

agonists, flow cytometry with antibodies directed towards additional surface glycoproteins, granule content, clot retraction, serum TxB2, and transmission electron microscopy (TEM).

Repeated LTA should evaluate responses to alpha-thrombin, TRAP-6 (Thrombin receptor agonist peptide), U46619 (thromboxane A2 receptor agonist), CRP, convulxin, PAR-4 activating peptide, PMA and A23287, to confirm platelet dysfunction and expand diagnostic options.

Expanded flow-cytometry should evaluate the glycoproteins GPIa/IIa (CD31 and CD49b), GPIV (CD36) and GPVI to identify rarer platelet glycoprotein abnormalities. Assessment of platelet procoagulant activity by flow-cytometry, for example annexin V binding, can help to detect disorders with enhanced (Stormorken Syndrome) or impaired (Scott Syndrome) platelet procoagulant activity.

Transmission electron microscopy is recommended at this stage for counting platelet alpha- or dense-granules and/ or for the identification of structural alterations. Alpha and dense-granule content may be measured in platelet lysates using ELISA, immunofluorescence, luminometry or HPLC.

Finally, if enhanced ristocetin-induced platelet aggregation is detected, mixing tests should be performed to distinguish the plasmatic (VWD type 2B) from the platelet (PT-VWD) origin of platelet hyper-reactivity.

The second-step tests have the potential to

diagnose a further 7% of all IPFD. They can raise diagnostic suspicion for a further 40%, although confirmation needs additional testing[7].

Third-step tests, which are warranted for cases that are undiagnosed but still are strongly suspected to have an IPFD, include biochemical studies, receptor binding assays and molecular genetics studies. These tests may identify some rare disorders, such as soluble agonist receptor defects, and possibly, previously unrecognized IPFD[7].

Conclusion and recommendation

Although developed countries have extended the diagnostic facilities of IPFD to molecular level, many countries with resource constraints including Sri Lanka still rely on basic tests for the diagnosis of IPFD. We still do bleeding time as a screening test for suspected platelet function defect. LTA is done using only a basic panel of agonists. However, even with the available facilities we are able to diagnose IPFD as testing is done by well experienced personnel in a few specialized laboratories in the country. We are probably missing rarer forms which do not show abnormalities in LTA. Due to non-availability of further testing, these patients are left without a diagnosis, even if they have a clinical phenotype highly suggestive of IPFD. Correct diagnosis is important for the individual patient management and also for finding the correct prevalence of these rare diseases in a country. Therefore, establishing further testing to diagnose IPFD in Sri Lanka is necessary. To complete the panel of first step investigations, evaluation of the main surface glycoproteins by flow cytometry should be added. Although platelet dense granular studies by lumiaggregometry is available, alpha granular studies are yet to be started. It is also necessary to engage in research on IPFD, for better understanding of these diseases.

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