Diagnostics of Inherited platelet disorders (IPD) in the Nordic Countries

Background
Inherited platelet disorders (IPD) are a highly heterogeneous group of bleeding diseases caused by germline mutations in genes expressed in platelet and megakaryocytes.
Inherited platelet disorders may be associated with thrombocytopenia and can be divided into subgroups depending on the type of functional abnormality: Disorders of a) adhesion, b) aggregation, c) secretion and d) procoagulant activity. Some IPD are not associated with obvious bleeding symptoms and patients with inherited thrombocytopenia may be identified incidentally during routine investigations that include a complete full blood count.

For diagnostics of IPD, we recommend the following strategy:

1. Obtain family history of bleeding and comorbidities

2. Obtain detailed history of bleeding, comorbidities and medication of the patient

3. Consider if the platelet disorder can be part of a syndrome or perhaps is acquired

4. Physical examination: bleeding manifestations of the skin and oral cavity (bruises, hematomas, petechiae) and syndromic manifestations (see below)

5. Standard coagulation laboratory testing

6. Before specific platelet function testing:

   A. **Clear preanalytical patient guidance with regard to infection, time period after potential surgery, smoking, fasting or heavy exertion (should not be less than 24 hours)**
      Recommendations:
      - Samples should be collected from fasting and resting subjects who have refrained from smoking and caffeine ingestion on the day of testing
      - Use a standardized, atraumatic blood collection protocol with minimal stasis
      - Use needles between 19 and 21 gauge to prevent vein trauma or reduced blood flow, leading to activated platelets
      - The first 3-5 ml of blood should not be used for platelet function testing
      - Most platelet function tests require the use of 105–109 mmol/l buffered trisodium citrate tubes
      - Collection tubes must be filled completely to ensure the proper 9:1 ratio of blood to anticoagulant
      - The collection tubes should be mixed gently immediately upon filling, do not subject to excessive agitation or mixing
      - Maintain specimens at room temperature (18-24°C) only and not refrigerated or frozen
      - Specimens should rest on the laboratory bench top for 30 minutes prior to platelet function testing
      - Platelet function testing should be completed within 3-4 hours of collection

B. Recorded questionnaire on the avoidance of drugs affecting platelet function

Clinical History
For a detailed history of bleeding, the ISTH bleeding assessment tool (BAT) is recommended [1, 2]. It is cautioned, that the negative predictive value is low, meaning that especially males and children can have normal scores even in the presence of an identifiable bleeding disorder [3].

Reference values, normal range: Men 0-3, Women 0-5, Children 0-2 [4].

Current medication
A complete record of current medication taken. Table 1 (appendix) lists substances known to interfere with platelet function [5].

***Recommendations**:*
Before performing platelet function tests patients should defer from taking the following substances for 14 days:

- Inhibitors of platelet receptors
- Acetylsalisylic acid
- NSAID
- Dipyramidole
- Selective serotonin reuptake inhibitors (SSRIs)
- Propranolol
- Nitroprusside, nitroglycerin
- Furosemide
- Calcium channel blockers
- Herbal remedies
- Theophyllin, caffeine
- Antibiotics (penicillins, sulfonamides)
- Antihistamines
- Fish oil
- Ibrutinib
- Melatonin

Platelet function tests performed on patients taking any of the drugs listed below should be repeated when not taking the substance for at least 14 days before sampling.

Focus on syndromic features
Several IPD:s are part of syndromes and other organs might be affected. Therefore, presence of

- Albinism
- Pulmonary fibrosis
- Colitis
- Myelofibrosis (*NBEAL2*)
- Cryptorchism (*GATA1*)
- Bone marrow failure
- Sitosterolemia
- Bone abnormalities
- Immunodeficiency (*WAS, FERMT3*, *NBEAL2*)
- Autism (*NBEA*)

- Developmental impairment (*CDC42*)
- Kidney impairment, *(MYH-9)*
- Deafness *(MYH-9)*
- Cataract (*MYH-9*)
- Myopathia (*STIM1, GNE)*
- Eczema (*WAS*)
- Heart defects
- Gastroduodenal ulcers (*PLA2G4A*)
- Arthrogryposis with renal dysfunction and cholestasis (*VIPAS39, VPS33B*)

Should be noted, for comprehensive list, see (Table 2)

Laboratory algorithm

- Laboratory testing only with clinical suspicion
- First excluding VWD and coagulation deficiencies by screening laboratory tests

Screening blood tests

***Recommendations**:*

- Full blood count and MPV
- Blood smear
- Activated partial thromboplastin time (APTT)
- Prothrombin Time (PT) or INR
- Thrombin Time
- Clauss Fibrinogen
- VWD testing: VWF activity (ristocetin co-factor or GpIb binding assay) and factor VIII and IX level
- FVIII, FIX

If the initial coagulation factor assays are normal, it may be considered to exclude the extremely rare

- Factor XIII, V, or XI deficiency

Platelet function tests

Recommendation:
When history and clinical features of the patient supports the suspicion of an IPD, and no abnormalities in plasma coagulation factors are found that could explain the bleeding tendency, platelet function tests should be ordered in a step wise fashion as described in [6].

First step tests

- *Blood smear* to evaluate platelet size, giant platelets, grey appearance of platelets, red cell abnormalities and neutrophil inclusion bodies

- *Light transmission aggregometry (LTA) in platelet-rich plasma (PRP)*
  - Golden standard but poorly standardized and should be repeated on at least once occasion before it can be considered diagnostic of a platelet dysfunction disorder.
  - For details on agonists and recommended concentrations see [7]
  - Assess performance of new batches of agonists by comparison with a previous batch

- The platelet count of PRP samples should NOT be adjusted to a standardized value with autologous PPP [8]

- Platelet counts below 150×10^9/L in PRP can severely influence responses to some agonists [7]

- *Lumi-aggregometry*
  Assessment of ATP/ADP release from platelet granules

- *Flow cytometry*
  To investigate or confirm
    Glanzmann thrombasthenia (GT), with abnormalities in the fibrinogen receptor Glycoprotein GPIIb/IIIa)
    Bernard-Soulier Syndrome (BSS, with abnormalities in the VWF receptor GPIb/IX/V)

Second-step tests

- LTA with an expanded agonist panel
- Flow-cytometry with additional antibodies, assessment of platelet activation as well as phophatidylserine expression to detect Scott syndrome
- Transmission electron microscopy (TEM) for counting α-and dense granules and to detect structural abnormalities

Genetic screening by high-throughput sequencing

In the past, functional platelet testing had a central role in the diagnostics of IPD, with Sanger sequencing only performed in genes where a defect was suspected. Clinical testing of candidate genes by Sanger sequencing and linkage analysis have now largely been replaced by high-throughput sequencing (HTS) techniques, mostly using targeted gene panel tests based on whole exome sequencing (WES) or whole genome sequencing (WGS).

Recommendation
For diagnostic purposes HTS for IPD should be performed using a validated platform containing a multi-gene panel of curated diagnostic-grade genes (genes in which variants have been proven to cause human disease, for example through biochemical studies or segregation with the phenotype in at least 4 independent pedigrees) as recommend by the ISTH [9].

Genetic screening can be considered in patients:

- suspected of hereditary thrombocytopenia
- with syndromic features
- with a strong family history
- with a significant bleeding score and where clinical and laboratory features strongly suggest a platelet disorder, but where further diagnostic assays are not available

Interpretation of results from genetic screening

Usually, several candidate disease-causing DNA variants are identified when using HTS technology. Correct interpretation and pathogenicity scoring of these variants are crucial for diagnosis and appropriate genetic counselling. A molecular diagnostic service should therefore have an array of available expertise, including a multidisciplinary team comprising of clinicians, geneticists, non-clinical platelet experts and bioinformaticians [10]. Genetic testing for a heterogeneous condition such as IPD and understanding the impact is not straightforward and clinicians should consider referring their patients to expert centers that perform in-house HTS instead of sending patient samples to external laboratories [11].

To avoid misinterpretation of variants of unknown significance (VUS) findings and provide appropriate counselling it is strongly advised that a HTS test for clinical diagnostics and inclusion of VUS reporting should only be requested by clinicians experienced in genetic interpretation.

A diagnostic rate of approximately 50 % in patients with suspected inherited thrombocytopenia and 25% in patients with a known platelet function disorder confirmed by laboratory tests can be obtained by HTS [12, 13].

Patient information

It is important that the patient understands the testing procedure, the benefits and limitations of the test, and the possible consequences of the test results in relation to clinical management options [14]. It may also be relevant to have a talk with patients, both prior to and following testing, regarding the importance of discussing their results with relatives. To improve knowledge about IPD, it should be made clear to a patient that anonymized sharing of data provides a greater understanding of the relevance of particular genetic variants enabling to improve molecular diagnosis in patients with similar conditions. In the majority of the Tier-1 genes associated with IPD, very few variants are published and when performing genetic screening a large number of previously not described variants will be uncovered.

Recommendation

To determine whether such a previously not described variant is disease causing or not, the five-tier scheme as recommended by The American College of Medical Genetics and Genomics (ACMGG)[15], should be used

Genetic screening will typically uncover a number of VUS. Greater certainty regarding a variant's pathogenicity may be achieved by:

- Determination of conservation of the residue across species. Variants of poorly conserved residues are less likely to be pathogenic
- In silico prediction algorithms
- Allele frequency in international databases (gnomAD, ExAc)
- Specific functional studies i.e. transmission electron microscopy or immunofluorescent microscopy
- Co-segregation studies in the patient's family

A variant classified as a VUS does not exclude the possibility of a causal or pathogenic state, but pathogenicity cannot be confirmed or excluded due to lack of evidence. VUS can be upgraded to pathogenic or downgraded to benign as more data become available and so a variant's classification should be periodically reviewed. This will have to be conveyed to the family members.

To date, current practices regarding the inclusion, or exclusion, of VUS in a diagnostic genetic report vary [16]. For laboratories, it is important to report a VUS in a known gene that is relevant to the clinical question of interest. A clinician receiving a report containing a VUS could use this information to order

further platelet tests and family co-segregation studies that could inform on the pathogenicity of the variant. To avoid misinterpretation of VUS findings and provide appropriate counselling it is strongly advised that a HTS test for clinical diagnostics and inclusion of VUS reporting should only be requested by clinicians experienced in genetic interpretation. Alternatively, multidisciplinary meetings can be organized between expert(s) and prescriber, to discuss a patient's suitability for testing, results interpretation and patient and family care. Variants of uncertain significance should not be used to guide clinical practice [17].

Incidental findings and patient consent

When referring a patient for genetic screening there is always a risk of incidental findings. These could be any of:

- A variant which is not involved in the direct cause of the phenotype under investigation (bleeding or thrombocytopenia), but might have other clinical consequences for that person or their relatives. Ex: Finding that a woman with increased bleeding is carrier of haemophilia A, but having normal FVIII levels
- A finding which contradicts stated biological relationships between family members. Ex the finding of a trait that is inherited X-linked is present in the father, but not in his daughter

- The HTS panel recommended by the ISTH-SCC includes 3 genes related to inherited thrombocytopenia (RUNX1, ANKRD26 and ETV6) where pathogenic variants has been shown to convey an increased risk of myelodysplastic syndrome and acute leukemia. We recommend that patients should be well informed about this possibility prior to performing the test.

Recommendation

- Patients should be asked before testing whether they want to receive information on incidental findings (IF) and should be able to opt-out. The informed consent process should explain the likelihood of IF and the reporting approach taken [18].
- Consent should be taken prior to genetic testing for IPD and should cover: breadth of testing, implications of results for patient and extended family, VUS, and incidental findings, data sharing and non-haemostatic effects of some variants.
- Genetic screening of IPD involves the potential detection of pathogenic variants in genes associated with leukemic risk (*RUNX1, ETV6* and *ANKRD26*). Such variants typically present in families with autosomal dominant thrombocytopenia and a history of blood cancer but may also be an accidental finding in a healthy blood donor. We recommend that patients should be well informed about this possibility prior to performing the test.

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