Inherited thrombocytopenias: a proposed diagnostic algorithm from the Italian Gruppo di Studio delle Piastrine

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Background. Although no epidemiological study has so far been performed, inherited thrombocytopenias are considered to be very rare based on the number of case reports in the literature. However, diagnosis of these disorders is often difficult and requires competences that are limited to specialized centers. We, therefore, suspect that inherited thrombocytopenias are underreported because their diagnosis is often missed.

Objectives. The aim of this study was to develop a diagnostic algorithm that utilizes the simplest possible diagnostic tests and so can also be used in centers that are not highly specialized.

Methods. The basic features of the diagnostic algorithm (definition and classification of hereditary thrombocytopenias, laboratory tests, sequence of investigations) were discussed by the members of the Italian Gruppo di Studio delle Piastrine during two meetings held in October 2000 and 2001. The sources of information were literature and personal experience. The final proposed diagnostic algorithm was produced by the authors of this paper and approved during a third meeting in May 2002.

Perspectives. A definite diagnosis for patients with hereditary thrombocytopenias serves many purposes. It is essential to define the prognosis of the patients and to identify the best therapeutic approach. It also defines the risk of transmitting the disorder to progeny and, in many cases, allows prenatal diagnosis. Finally, several pieces of evidence indicate that not all the genetic thrombocytopenias have yet been identified. Exclusion of known disorders by accurate investigation is the starting point for the discovery of these new illnesses.

Key words: inherited thrombocytopenias, diagnostic algorithm, platelets.

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Although inherited thrombocytopenias are rare, their frequency is probably underestimated because of diagnostic difficulties. Moreover, not all the existing forms have yet been characterized, and many patients remain without a definite diagnosis despite accurate investigation. For instance, we recently studied 22 families referred to us as having an undefined macrothrombocytopenia. After an exhaustive clinical and laboratory evaluation, 10 families were classified as having heterozygous Bernard-Soulier syndrome, 4 were found to be affected by MYH9-related disease, and one by X-linked thrombocytopenia and thalassemia. The remaining 7 families did not meet the criteria for any known inherited macrothrombocytopenia (unpublished data).

Here, we propose an algorithm to assist clinicians in the diagnosis of inherited thrombocytopenias. A correct diagnostic approach is essential not only to classify patients with known disorders, but also to identify families with uncharacterized forms. The co-ordinated efforts of physicians, biologists and geneticists in studying these new entities will improve our diagnostic skill and provide insights into the molecular basis of platelet production and function.

Classification of inherited thrombocytopenias

In this study, we will consider only those inherited diseases that manifest thrombocytopenia as an outstanding and constant feature. The disorders with generalized bone marrow failure (for instance Fanconi’s anemia) and the complex clinical syndromes that only seldom present thrombocytopenia as an accompanying feature (for example some inherited metabolic disorders) will not be considered.

The initial phase in creating a diagnostic algorithm for any category of illnesses is to identify a system that appropriately classifies the disorders. The most rationale classification, which is based on pathogenetic mechanisms, was not practical for our purpose because the pathogenic mechanisms of most inherited thrombocytopenias are still unknown. We, therefore, considered other criteria, including severity of thrombocytopenia, and the inheritance pattern. However, the large majority of genetic thrombocytopenias are transmitted in an autosomal dominant fashion. As a consequence, this method, too, has little practical value. Moreover, sporadic cases due to de novo mutations sometimes occur.
The severity of thrombocytopenia also proved to be of limited value, because platelet count may range from severely reduced to nearly normal in almost all inherited forms. For instance, thrombocytopenia is usually mild in subjects with MYH9-related disease, but sometimes can be very severe. Likewise, the platelet count in homozygous Bernard Soulier syndrome can vary from low, as in the majority of cases, to borderline values in some patients. We, therefore, chose to classify inherited thrombocytopenias based on both platelet size and the presence (syndromic) or the absence (non-syndromic) of clinical features other than those deriving from the platelet defects (Table 1). The latter criterion has a strong practical impact, since medical history and examination may discover abnormalities that are pathognomonic of single illnesses. We therefore utilized the categorization into syndromic or non-syndromic forms for the initial approach to the patients with inherited thrombocytopenias. For non-syndromic thrombocytopenias, further distinctions rely on platelet size. The most important advantage of this parameter is that abnormalities of platelet size are constant features of many diseases and can easily be ascertained even in non-specialized laboratories.

Table 1 reports the classification of inherited thrombocytopenias according to these criteria and describes the most important features of the disorders. For a detailed discussion of the clinical and biological features of genetic thrombocytopenias we refer the reader to a review that was published recently in this journal.  

Laboratory evaluation of inherited thrombocytopenias

In most cases, the differential diagnosis of inherited thrombocytopenias requires numerous competences and skilled laboratory investigations that are not always all available in a single clinical center. In this section we briefly describe the techniques subdivided in two groups: simple laboratory investigations for screening for the inherited thrombocytopenias and more specialized tests for diagnostic confirmation.

Screening tests

Platelet count. Automated cell counters enumerate particles within a specified volume window (e.g., 2–20 fl for platelets). As a consequence, platelet concentration is underestimated in patients with platelets outside the set values. For instance, in subjects with platelet macromegaly, thrombocytopenia might be overestimated by this automatic counting. In a case series of 15 patients with May–Hegglin anomaly, the mean platelet counts were considerably different, being 17 and 87×10^9/L using an electronic method and optical microscopy, respectively. The largest discrepancy was observed in a patient with a high proportion of giant platelets: an automated counter measured 4×10^9 platelets/L whereas manual counting estimated 178×10^9/L. Similar results were obtained in patients with MYH9–related disease and Bernard–Soulier syndrome. On this basis, platelet counts obtained by electronic counters should be regarded with caution in inherited thrombocytopenias and should be confirmed by a careful examination of peripheral blood smears demonstrating the absence of unusually large or small platelets. In the presence of altered size, a correct estimation of platelet count must be made by optical microscopy analysis of a whole blood sample, diluted in a hemolysing solution, in a Neubauer or Burker counting chamber. Alternatively, platelets can be counted on blood smears using white blood cells as a reference: the absolute value is subsequently derived based on the leukocyte count obtained by an automated instrument.

Platelet size. The pitfalls of electronic instruments should also be considered in the evaluation of platelet size. Since cell counters do not recognize large or small platelets, they underestimate platelet volume in macrothrombocytopenias and microthrombocytopenias, respectively. In a case series of patients with MYH9–related disease, the mean platelet volume reported by an automatic counter was at the upper limit of the normal range (12.5 fl) whereas microscopic examination of peripheral blood films revealed that on average 40% of platelets had a diameter larger than 4 mm and 12% larger than 8 mm (the diameter of normal platelets ranges from 1.5 to 3 μm). Other parameters related to platelet size, such as the platelet volume distribution width and the percentage of platelets larger than normal, are likewise not correctly determined by electronic instruments. Thus, careful observation of blood films is essential in order to determine platelet size. It is important to highlight that EDTA-anticoagulated platelets are susceptible to time-dependent swelling in vitro. Blood films should, therefore, be prepared from freshly drawn anticoagulated samples or directly from a drop of blood obtained from a finger-stick or from the venipuncture needle. The values obtained by this method in healthy subjects and in macrothrombocytopenic patients are reported in Table 2 (Balduini CL, personal communication).

Morphologic examination of peripheral blood films. Optical microscopy of peripheral blood smears stained with Wright’s or May–Grünwald–Giemsa allows the identification of peculiar elements that might be determinant for a correct diagnosis. Platelet clumps are suggestive of Montreal platelet syndrome, while pale platelets without granules suggest gray platelet syndrome. Red
Table 1. Main features of inherited thrombocytopenias classified according to platelet size. The clinical and laboratory features with the strongest diagnostic value are in bold.

<table>
<thead>
<tr>
<th>Disease (abbreviation, OMIM*)</th>
<th>Inheritancea</th>
<th>Gene (localization)</th>
<th>Clinical and laboratory features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Small platelets</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Wiskott-Aldrich syndrome (WAS, 301000)</td>
<td>X-L</td>
<td>WAS (Xp11)</td>
<td>Thrombocytopenia usually severe. <strong>Severe immunodeficiency. Defective WAS protein.</strong></td>
</tr>
<tr>
<td>°X-linked thrombocytopenia (XLT, 313900)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Normal-sized platelets</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Familial platelet disorder and predisposition to acute myelogenous leukemia (FPD/AML, 601399)</td>
<td>A.D.</td>
<td>CBFA2 (21q22)</td>
<td>Propensity to develop myelodysplastic syndrome or acute myelogenous leukemia.</td>
</tr>
<tr>
<td>°Congenital amegakaryocytic thrombocytopenia (GAMT, 604948)</td>
<td>A.R.</td>
<td>c-mpl (1p34)</td>
<td>Thrombocytopenia usually severe. Defective WAS protein.</td>
</tr>
<tr>
<td>*Amegakaryocytic thrombocytopenia with radio-ulnar synostosis (CITRUS, 605432)</td>
<td>A.D.</td>
<td>HOXA11 (7p15-14)</td>
<td>Thrombocytopenia usually severe. <strong>Reduced absent megakaryocytes.</strong> Possible aplastic anemia. <strong>Radio-ulnar synostosis ± other malformations.</strong> Possible sensorineural hearing loss.</td>
</tr>
<tr>
<td>°Thrombocytopenia with absent radii (TAR, 274000)</td>
<td>A.R.</td>
<td>n.d.</td>
<td>Thrombocytopenia usually severe in the first years of life. <strong>Reduced megakaryocytes. Bilateral radial aplasia ± other malformations.</strong></td>
</tr>
<tr>
<td>*Autosomal dominant thrombocytopenia (THC2, 188000)</td>
<td>A.D.</td>
<td>n.d.</td>
<td>None</td>
</tr>
<tr>
<td><strong>Large platelets</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>°VEGOCARDIFACIAL syndrome (VCS, 192430)</td>
<td>A.D.</td>
<td>GS5-GP Ibβ</td>
<td>Clift palate, cardiac anomalies, typical facies, learning disabilities, defective GP Ib/IX/V.</td>
</tr>
<tr>
<td>*Platelet-type or pseudo von Willebrand syndrome (PvWD, 177820)</td>
<td>A.D.</td>
<td>GP Ibα (17p13)</td>
<td>Spontaneous platelet aggregation in vitro and/or increased platelet agglutination to low-dose ristocetin. Reduction of high molecular weight vWF multimers in plasma.</td>
</tr>
<tr>
<td>*Benign Mediterranean macrothrombocytopenia (n.d., 153670)</td>
<td>A.D.</td>
<td>n.d.</td>
<td>None</td>
</tr>
<tr>
<td>*Dyserythropoietic anemia with thrombocytopenia (n.d., 303937)</td>
<td>X-L</td>
<td>GATA1 (Xq11)</td>
<td>Thrombocytopenia usually severe. <strong>Anemia from mild to severe, red cell anisopoikilocytosis, reduced expression of GP Ib in a subpopulation of large platelets, dysmegakaryopoiesis.</strong></td>
</tr>
<tr>
<td>**X-linked thrombocytopenia with thalassemia (XTT, 314040)</td>
<td>X-L</td>
<td>MYH9 (22q12-13)</td>
<td>Giant platelets, neutrophil inclusions ± hearing loss ± cataract ± renal defect.</td>
</tr>
<tr>
<td>*Paris-Trousseau type thrombocytopenia (TCPT, 188025/600568)</td>
<td>A.D.</td>
<td>CTS1, ETS1</td>
<td>Cardiac and facial anomalies. Mental retardation. Giant platelet granules.</td>
</tr>
<tr>
<td>°Montreal platelet syndrome (MPS, n.d.)</td>
<td>A.D.</td>
<td>n.d.</td>
<td>Thrombocytopenia usually severe. <strong>Spontaneous platelet aggregation in vitro.</strong></td>
</tr>
<tr>
<td>°Macrothrombocytopenia with platelet expression of glycoporphin A (n.d., n.d.)</td>
<td>A.D.</td>
<td>n.d.</td>
<td>Large platelets express glycoporphin A. <strong>Defective platelet aggregation induced by arachidonic acid.</strong></td>
</tr>
</tbody>
</table>

*a Syndromic form ° Non-syndromic form ** Both syndromic and non-syndromic forms exist. ~Online mendelian inheritance in man; A.D., autosomal dominant; A.R., autosomal recessive; X-L, X-linked; Contiguous gene syndrome; MHA, SBS, FTNS and EPS have so far been considered distinct entities, but it is now clear that they are different clinical expressions of a single disease due to MYH9 mutations.
cells and leukocytes may provide further insights: anisopoiikilocytosis of erythrocytes has been observed in X-linked thrombocytopenias caused by mutations of GATA-1,\(^1\),\(^2\) and light-blue inclusions in the cytoplasm of polymorphonuclear leukocytes (Döhle-like bodies) are often detected in MYH9-related disease.\(^3\)

**Platelet function studies.** Abnormalities of in vitro platelet function have been described in almost all inherited thrombocytopenias. Although these defects are usually non-specific, there are a few exceptions. In vitro platelet agglutination induced by ristocetin is absent or severely reduced in homozygous Bernard-Soulier syndrome,\(^12\) whereas low concentrations of ristocetin, which have no effect on normal platelets, are sufficient to induce platelet clumping in patients with platelet-type von Willebrand disease.\(^13\) In addition, stirred platelets undergo spontaneous aggregation in Montreal platelet syndrome\(^8\) and in some patients with platelet-type von Willebrand’s disease.\(^13\)

The study of platelet aggregation in platelet-rich plasma by the densitometric method, proposed 40 years ago by Born,\(^14\) still represents the most popular method to evaluate platelet function. It is cheap and technically easy to perform, although many variables affect the results, including processing time, methods for separating platelet-rich plasma and platelet-poor plasma, platelet concent-

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### Table 2. Platelet size determined by microscopic observation of peripheral blood films. Three hundred platelets were studied in each subject.

<table>
<thead>
<tr>
<th></th>
<th>n of patients</th>
<th>% of platelets with a diameter</th>
<th>platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;4 µm</td>
<td>4-8 µm</td>
</tr>
<tr>
<td>Heterozygous Bernard-Soulier syndrome</td>
<td>9</td>
<td>77.0±11.0</td>
<td>20.1±10.4</td>
</tr>
<tr>
<td>Homozygous Bernard-Soulier syndrome</td>
<td>4</td>
<td>64.3±19.0</td>
<td>29.7±13.8</td>
</tr>
<tr>
<td>MYH9-related disease</td>
<td>34</td>
<td>52.0±19.8</td>
<td>31.2±9.5</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>44</td>
<td>96.7±4.3</td>
<td>3.1±3.2</td>
</tr>
</tbody>
</table>

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Figure 1. Diagnostic algorithm for hereditary thrombocytopenias. XLT: X-linked thrombocytopenia; CAMT: congenital amegakaryocytic thrombocytopenia; THC2: autosomal dominant thrombocytopenia; VCFS: velocardiofacial syndrome; TCPT: Paris-Trousseau type thrombocytopenia; JBS: Jacobsen’s syndrome; CTRUS: amegakaryocytic thrombocytopenia with radio-ulnar synostosis; TAR: thrombocytopenia with absent radii; WAS: Wiskott-Aldrich syndrome; FPD/AML: familial platelet disorder and predisposition to acute myelogenous leukemia.
Concentration, rate of platelet-rich plasma stirring during the test, processing temperature, and agonist source. As a consequence, each laboratory must strictly standardize its methodology and identify the normal range for each aggregating agent. Since a low concentration of platelets in blood may prevent preparation of platelet-rich plasma with a standard platelet concentration, in patients with severe thrombocytopenia it is often necessary to determine the normal range of platelet aggregation in samples with a platelet concentration similar to that of the patient's platelet-rich plasma.

Alternative methods have been proposed to overcome the limitations of optical aggregometry. The impedance technique allows the determination of platelet aggregation in whole blood, thus avoiding the risk of losing platelets of unusually large size during sample preparation due to centrifugation. However, the instrument required is available only in specialized centers and the results obtained with this method in the study of inherited or acquired platelet defects did not differ from those obtained with the conventional optical method. The platelet function analyzer, PFA-100, has been designed to provide an in vitro measure of primary hemostasis for routine screening of patients with a potential risk of hemorrhage. This very simple and quick test identifies patients with defective platelet function without, however, providing insights into differential diagnosis. Moreover, the results of the PFA-100 are unreliable in patients with very low platelet counts.

Confirmatory tests

Electron microscopy. Electron microscopy is useful in ascertaining the lack of α-granules that characterizes platelets of patients with gray platelet syndrome, and for studying the fine ultrastructural features of Döhle-like bodies in MYH9-related diseases (microfilaments and ribosomes in an amorphous area of neutrophil cytoplasm devoid of specific granules), differentiating them from Döhle bodies of infectious diseases (several rows of rough endoplasmic reticulum).
Platelet composition. Inherited thrombocytopenias might have well-defined abnormalities of platelet proteins. In Wiskott–Aldrich syndrome and X-linked thrombocytopenia due to mutations of the WAS gene, platelets (and mononuclear cells) have defective WAS protein (WASp). In congenital amegakaryocytic thrombocytopenia the receptor for thrombopoietin (c-mpl) is absent or reduced, whereas a quantitative or qualitative defect of platelet glycoprotein (GP) Ib–IX–V complex is the distinguishing feature of Bernard–Soulier syndrome. Proteins stored in the α-granules (thrombospondin, fibrinogen, fibronectin, von Willebrand factor, etc.) are absent or reduced in gray platelet syndrome. Finally, in a rare form of inherited macrothrombocytopenia platelets express glycophorin A, an erythroid-specific protein, on their surface. Since these abnormalities are often sufficient differential elements for a correct diagnosis, we will briefly report the methodological approaches used for this purpose.

Flow cytometry analysis detects cell surface proteins by fluorescently labeled specific antibodies. This technique is also applicable for intracellular proteins after permeabilization of the cytoplasmic membrane. Since many antibodies against platelet proteins are commercially available, flow cytometry is one of the preferred methods for the analysis of platelet proteins. This approach is being applied to the study of Wiskott–Aldrich syndrome, X-linked thrombocytopenias due to WAS or GATA-1 mutations, congenital amegakaryocytic thrombocytopenia, hereditary macrothrombocytopenia with platelet expression of glycoporphin A, an erythroid-specific protein, on their surface.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting detect proteins, providing a semiquantitative estimation of their amount. This method is being used for the diagnosis of Bernard–Soulier syndrome, gray platelet syndrome, Wiskott–Aldrich syndrome and X-linked thrombocytopenia. While flow cytometry is the best choice to study surface GPs, immunoblotting is preferable for intracellular molecules. In addition to quantitative defects, it can also reveal other protein abnormalities, such as post-translational alterations or truncated products, as seen in patients with Wiskott–Aldrich syndrome.

Immunocytochemistry or immunofluorescence using antibodies against the heavy chain of non-muscle myosin IIα (NMMHC-IIα, the protein encoded by the MYH9 gene) detects a spotty abnormal distribution of these molecules within neutrophils in all patients with MYH9-related disease, even when Döhle-like bodies are not recognizable on May–Grünwald–Giemsa blood films because of the very small size of the bodies (less than 2μm). Since NMMHC-IIα was found to be normally organized in neutrophils of all investigated inherited thrombocytopenias without MYH9 mutations, we conclude that this assay is highly specific and should always be implemented for a correct diagnosis of patients with suspected MYH9-mutations.

Genetic studies

When a gene causing a disease is identified, molecular diagnostic tests based on mutational screening offer the possibility to make a correct diagnosis, to perform prenatal diagnosis, and to identify asymptomatic carriers. In addition, the discovery of mutations has a fundamental impact on studying the molecular basis of different mutations and on establishing the role of specific functional protein domains. However, as discussed below, the search for mutations is essential for the diagnosis of only a few diseases. For others, alternative assays are sufficiently reliable to define the clinical entity and/or the molecular defects responsible for the disease. The choice of the strategy for mutational screening is usually based on the evaluation of some aspects, including the size of the gene and the number of exons to be analyzed, hot spots of mutations either in patients from all over the world or in populations of particular ethnic origin, the distribution of mutations through the entire gene, as well as the category of mutations (stop codon and frameshift or missense mutations) that are listed in the Human Gene Mutation Database at http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html. Direct sequencing of products obtained by amplification of either genomic DNA or RNA (if available) is the procedure that better guarantees results. Alternatively, for instance when the gene is large and/or mutations are spread through the entire gene, a prescreening procedure using cost-efficient
and rapid methodologies, such as single-strand conformation polymorphism (SSCP) or denaturing high-pressure liquid chromatography (DHPLC), allows the identification of altered patterns that are likely to carry variations in the nucleotide sequence. 

Platelet survival studies

In vivo kinetic studies of autologous and/or homologous (normal) platelets can be of help in differentiating between thrombocytopenias due to decreased production and those due to enhanced destruction. Few patients with well-defined inherited thrombocytopenias have been studied by this technique: a defect of platelet production was identified in all of them, with the only exception of those with Wiskott–Aldrich syndrome and X-linked thrombocytopenia, which are characterized by a shortened platelet life-span. Platelet life-span is always reduced in idiopathic (or autoimmune) thrombocytopenic purpura, which is by far the most frequent cause of isolated thrombocytopenia. This test may, therefore, be used in selected cases when the differential diagnosis between inherited or acquired thrombocytopenia is otherwise impossible (see below). Unfortunately, this study is complex and cumbersome and is available only in a few specialized centers.

Differential diagnosis of thrombocytopenias: hereditary vs acquired

The genetic origin of hereditary thrombocytopenias is not always obvious. Patients with mild platelet deficiency may not have a lifelong bleeding diathesis and thrombocytopenia may be diagnosed only incidentally in adult life. Moreover, the patient may not have thrombocytopenic relatives because recessive inheritance or de novo mutations can occur in sporadic cases. For instance, a recent molecular analysis of MYH9-related disease revealed de novo mutations in 9 out of 19 families. Therefore, a genetic thrombocytopenia should be suspected in all patients with no record of a previous normal platelet count. A careful medical history of the propositus and his or her relatives should be taken in order to reveal even minimal signs of bleeding diathesis at any time since birth. Such signs include easy bruising, menometrorrhagia or prolonged bleeding after a minor surgical procedure or a tooth extraction. Platelet survival studies may be occasionally required to differentiate inherited thrombocytopenias with normal or increased platelet size from idiopathic thrombocytopenic purpura.

Differential diagnosis of hereditary thrombocytopenias

We propose a two-step diagnostic algorithm: a first phase of clinical and/or simple laboratory investigations (screening tests) to provide a diagnostic hypothesis, followed by a second phase of specialized investigations (confirmatory tests) to confirm the diagnostic suspicion. All institutions should be able to conduct the first phase and formulate a potential diagnosis. Thereafter the patient should be referred to a specialized center for diagnostic confirmation.

Syndromic vs. non-syndromic hereditary thrombocytopenias

The medical history and examination are the starting points of the diagnostic algorithm and allow differentiation of syndromic thrombocytopenias from non-syndromic forms. In syndromic forms, history and examination are often sufficient to raise a diagnostic suspicion. In non-syndromic forms, subsequent investigation depends on platelet size (Table 1 and Figure 1).

Syndromic thrombocytopenias: differential diagnosis

Signs of severe immunodeficiency, such as recurrent infections, allergy, autoimmune diseases or lymphoreticular malignancies, are typical of Wiskott–Aldrich syndrome. Its less severe form, known as X-linked thrombocytopenia, is characterized by thrombocytopenia but no, or minimal, immunologic disturbances, although in some patients immune dysregulation may develop over time (see below: non-syndromic thrombocytopenias with small platelets).

Since patients with this syndrome are the only people known to have an inherited reduction of platelet size, examination of peripheral blood smears can support the diagnostic suspicion. An accurate diagnosis must reveal the absence or severe reduction of Wiskott–Aldrich syndrome protein (WASP) in hematopoietic stem-cell-derived lineages, such as peripheral blood mononuclear cells. Quantitative defects of WASP can be detected using anti-WASP antibodies, either by flow cytometry of permeabilized cells or by SDS–PAGE with immunoblotting, but the latter has the additional advantage of recognizing qualitative defects (truncated forms) resulting in altered electrophoretic mobility. Further molecular confirmation relies on screening for mutations in the Wiskott–Aldrich syndrome gene. Hearing loss, abnormal renal function, and cataract may be the accompanying features of MYH9-related disease, an autosomal dominant macrothrombocytopenia induced by mutations of the MYH9 gene for NMMHC-IIA.

The clinical expression of this disorder varies wide-
ly, and a few patients present with only macrothrombocytopenia (see below: non-syndromic thrombocytopenias with large platelets). In addition to macrothrombocytopenia, an abnormal distribution of neutrophil NMMHC-IIA is a constant feature of this disorder,10 and therefore immunofluorescence or immunocytochemistry with specific, commercially available, antibodies is recommended in all patients with suspected MYH9-related disease. Electron microscopy can also be used to detect leukocyte inclusions, although this requires specialized competence. The search for MYH9 mutations is an alternative procedure to confirm the diagnosis.

The occurrence of acute myelogenous leukemia in a family of patients with thrombocytopenia might suggest a diagnosis of familial platelet disorder with predisposition to myeloid malignancy.24 Screening for mutations is the only diagnostic tool available to confirm a suspicion of this disease.

Macrothrombocytopenia and anemia, sometimes requiring transfusions, in males are findings compatible with dyserythropoietic anemia with thrombocytopenia.10,11,35 Although red cell anisopoikilocytosis in peripheral blood and dyserythropoiesis with abnormal megakaryocytes in the bone marrow support the diagnostic suspicion, identification of the GATA-1 mutation is the only way to make a certain diagnosis of this disease. Together with the patient's history, a careful examination can provide valuable information on the phenotypic anomalies of syndromic thrombocytopenias. Typical abnormalities characterize congenital amegakaryocytic thrombocytopenia with radio-ulnar synostosis,36 thrombocytopenia with absent radii,27 velocardiofacial syndrome,38,39 Paris-Trousseau type of thrombocytopenia40 and Jacobsen's syndrome41 (Table 1 and Figure 1). Whenever not contraindicated, a bone marrow examination should be performed in patients with suspected congenital amegakaryocytic thrombocytopenia with radio-ulnar synostosis or thrombocytopenia with absent radii to identify the typical features of megakaryocytes. Flow cytometry or SDS-PAGE of platelets showing defective GPIb/IX/V complex supports the diagnosis of velocardiofacial syndrome, which is confirmed by the karyotype. Since there are no alternative tests specific enough to diagnose Paris-Trousseau type of thrombocytopenia and Jacobsen's syndrome, identification of deletions on chromosomes 22q11 or 11q23.3, respectively, is essential to confirm the diagnostic suspicion.

Non-syndromic thrombocytopenias: differential diagnosis

As previously mentioned, the first criterion used to identify the various forms of non-syndromic thrombocytopenias is based on platelet size.

Non-syndromic thrombocytopenias with small platelets

Reduced platelet size has been reported only in Wiskott-Aldrich syndrome and X-linked thrombocytopenia.22 As discussed above (see: syndromic thrombocytopenias), the former condition is easily recognizable because thrombocytopenia is associated with immunodeficiency, while the latter is characterized by thrombocytopenia only. X-linked thrombocytopenia must, therefore, be suspected in patients with isolated thrombocytopenia and small platelets in the peripheral blood (mean platelet volume < 5FL). Diagnostic confirmation requires the same investigations as those described for Wiskott-Aldrich syndrome.

Non-syndromic thrombocytopenias with normal-sized platelets

When a syndromic form has been excluded in a patient with normal platelet size, cytologic or histologic investigation of bone marrow is required to diagnose congenital amegakaryocytic thrombocytopenia, an amegakaryocytic thrombocytopenia caused by mutations of the gene for thrombopoietin (TPO) receptor (c-mpl).42,43 The defective expression of c-mpl on the platelet surface can be detected by flow cytometry (the antibody is not yet commercially available). That the signal transduction pathway of c-mpl is defective was proven by demonstrating that platelets and hematopoietic progenitor cells did not react to TPO, as measured by testing TPO-synergism with adenosine diphosphate in platelet activation or by megakaryocyte colony assay.23 However, these abnormalities are not specific to congenital amegakaryocytic thrombocytopenia because they have also been observed in thrombocytopenia with absent radii. Moreover, these two tests are poorly standardized and the identification of mutations in the encoding gene is the preferred tool to confirm a diagnostic suspicion of congenital amegakaryocytic thrombocytopenia.

Some patients with inherited thrombocytopenia and normal-sized platelets do not fulfill the criteria for any of the disorders described above and are crudely diagnosed as being affected by inherited thrombocytopenia. There is an exception, a distinct disorder called THC2, characterized by autosomal dominant transmission and defective megakaryocyte maturation. Since these features do not represent a differential criterion, THC2 can be correctly defined only in large families by linkage analysis on chromosome 10p12.1, where the THC2 gene, not yet cloned, has been localized.51,52 There are, however, forms that are undistinguishable from THC2 but that do not map to chromosome 10, suggesting the presence of genetic heterogeneity (Savoia, personal communication). Therefore, it is likely that an undefined number of thrombocytopenic patients still cannot be correctly diagnosed despite accurate clinical and laboratory investigations.
Non-syndromic thrombocytopenias with large platelets

Although epidemiological studies are not available, personal experience suggests that large platelets are a feature of the majority of patients with an inherited thrombocytopenia. Several genetic macrothrombocytopenias are known and their number is expected to increase as soon as differential features are identified in patients with uncharacterized forms.

In non-syndromic patients, morphologic examination of blood films and in vitro platelet aggregation studies often lead to formulation of a diagnosis that requires confirmation by specialized investigations.

In May–Grünwald–Giemsa stained blood films, the presence of pale platelets due to the absence of α-granules strongly suggests gray platelet syndrome. Several defects of platelet aggregation have been reported in this disorder. Since none of them is specific,46 aggregometry has a marginal role in the diagnostic process. Gray platelet syndrome is ascertained if α-granules are absent or severely reduced. Ultrastructural analysis allows the presence of platelet granules to be checked while immunoblotting evaluates the presence of the proteins stored within the α-granules (β-thromboglobulin, thrombospondin, fibrinogen, platelet factor 4, platelet derived growth factor).46

As already discussed (see above: syndromic thrombocytopenias), MYH9-related disease may be either syndromic or non-syndromic. In the latter case, the diagnostic suspicion arises from the observation of Döhle-like bodies in neutrophils and/or giant platelets on May–Grünwald–Giemsa stained peripheral blood films. Immunocytochemistry or immunofluorescence that reveals leukocyte inclusions containing NMMHC-IIA confirms the diagnosis40.

Red cell anisocytosis on blood smears is compatible with X-linked thrombocytopenia with thalassemia. The affected males sometimes have mild anaemia but always present imbalanced α/non-α globin chain synthesis, increased reticulocyte count, signs of peripheral hemolysis, and severe dysmegakaryocytepoiesis on examination of the bone marrow. Obligate female carriers may have reticulocytosis and imbalanced globin chains.47,48 Although none of these signs is specific, together they strongly indicate X-linked thrombocytopenia with thalassemia. Finding mutations of the transcription factor GATA-1 gene definitively confirms the diagnosis. The mutations are likely to affect one of the two DNA-binding domains of the protein, where the only mutation so far detected (R216Q) has been identified.49

Ristocetin-induced platelet agglutination is absent or strongly reduced in von Willebrand's disease and homozygous Bernard–Soulier syndrome. The former is distinguishable from the latter because the addition of normal plasma corrects the defect. Flow cytometry is the simplest method to confirm the defect of the platelet GPIb/IX/V complex.13 The identification of mutations in the GPIba, GPIbβ or GPIX genes is a useful diagnostic confirmation although it is not strictly required, as severe deficiency of GPIb/IX/V complex and defective platelet response to ristocetin are specific signs of homozygous Bernard–Soulier syndrome.

Ristocetin-induced platelet agglutination tests are also useful in the diagnosis of platelet-type von Willebrand disease or pseudo von Willebrand's disease. In this condition platelet GPIb exhibits an exaggerated affinity for von Willebrand factor so that platelet agglutination is induced in vitro by concentrations of ristocetin that do not elicit any response in normal platelets.50 Moreover, in some cases spontaneous in vitro aggregation occurs in stirred platelets and clumped platelets are observed on blood smears. These functional abnormalities also characterize von Willebrand's disease type 2B. The two conditions can be distinguished by suitable mixing experiments: addition of plasma from von Willebrand disease 2B patients to normal platelet-rich plasma favors spontaneous aggregation and ristocetin-induced platelet agglutination, while plasma from patients with platelet-type von Willebrand disease has no effect on normal platelets. However, since aggregometry results are often ambiguous, the search for mutations in the GPIbα gene is required to provide diagnostic certainty.

Spontaneous platelet aggregation of resting platelets and of platelets in stirred platelet-rich plasma can be observed in patients with Montreal platelet syndrome, a rare and poorly characterized condition.5 Montreal platelet syndrome can be differentiated from platelet-type von Willebrand's disease in that von Willebrand factor is required for spontaneous platelet aggregation in the latter but not in the former. Spontaneous in vitro clumping of platelets also occurs in EDTA-induced pseudo-thrombocytopenia,51 but this phenomenon, at variance with Montreal platelet syndrome, occurs very slowly, strictly depends on the presence of calcium ion-chelating agents and is not increased by stirring. Finally, a consistent group of patients affected by non-syndromic hereditary macrothrombocytopenia do not show any of the above reported abnormalities of platelet aggregation or the peripheral blood film. In these cases, four diagnoses should be considered: heterozygous forms of Bernard–Soulier syndrome, MYH9-related disease, macrothrombocytopenia with platelet expression of glycoprotein A and Mediterranean macrothrombocytopenia.

We look for heterozygous Bernard–Soulier syndrome in the initial screening because this condition is the most frequent cause of macrothrombocytopenia in Italy.22 Flow cytometry is the simplest technique to detect a partial defect of the GPIb/IX/V
complex, because RIPA is not sensitive to a reduction of the number of GP Ib/IX/V complexes to 50% of normal. So far, one mutation, which is responsible for the Bolzano variant, has been found in all the mutated alleles. Since the mutation can be recognized by a digestion with the restriction enzyme HpAl, DNA amplification products can be easily tested in patients with giant platelets and without any obvious clinical or laboratory manifestation of specific forms of inherited thrombocytopenia.

If heterogeneous Bernard–Soulier syndrome is ruled out, the distribution of NMMHC-IIB within leukocytes should be evaluated to diagnose a possible MYH9-related disease since, as previously discussed, Döhle-like bodies are not always recognizable on a May–Grünwald–Giemsa stained blood film because of their small size.

The diagnosis of macrothrombocytopenia with platelet expression of glycophrin A is supported by a defective in vitro platelet aggregation induced by arachidonic acid and is confirmed by flow cytometry, which demonstrates that platelets express glycophrin A.

If both the above conditions are excluded, patients are usually referred to as having an isolated autosomal dominant macrothrombocytopenia, which has been reported in the literature as Mediterranean macrothrombocytopenia, or genetic thrombocytopenia with autosomal dominant transmission, or chronic isolated hereditary macrothrombocytopenia. This is likely to represent a heterogeneous category containing different forms of non-syndromic macrothrombocytopenias which cannot at present be diagnosed because of the lack of specific signs.

Concluding remarks

An accurate diagnosis of subjects with hereditary thrombocytopenias is required for genetic counselling and definition of prognosis. Hematopoietic stem cell transplantation, splenectomy or medical treatment can cure the disease or improve a patient’s quality of life in selected cases. Therefore, a vague diagnosis of inherited thrombocytopenia is not satisfactory and clinicians should make every effort to define the diagnosis in all patients.

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