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International collaboration as a tool for diagnosis of patients with inherited thrombocytopenia in the setting of a developing country

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International collaboration for diagnosis of inherited thrombocytopenia

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Summary

Background

Inherited thrombocytopenias (IT) are heterogeneous genetic disorders which frequently represent a diagnostic challenge. Requirement of highly specialized tests for diagnosis represents a particular problem in resource-limited settings. To overcome this difficulty, we applied a diagnostic algorithm and developed a collaboration program with a specialized international center in order to increase the diagnostic yield in a cohort of patients in Argentina.

Methods

Based on the algorithm, initial evaluation included collection of clinical data, platelet size, blood smear examination and platelet aggregation tests. Confirmatory tests were performed according to diagnostic suspicion, and included platelet glycoprotein expression, immunofluorescence for myosin-9 in granulocytes and platelet thrombospondin-1 and molecular screening of candidate genes.

Results

Thirty-one patients from 14 pedigrees were included, age was 32 (4-72) years, platelet count was 72 (4-147) $x10^9$/L. Autosomal dominant inheritance was found in 9 (64%) pedigrees, 10 (71%) had large platelets and 9 (29%) patients presented with syndromic forms. A definitive diagnosis was made in 10 of 14 pedigrees and comprised *MYH9*-related disease in 4, while classic and monoallelic Bernard-Soulier syndrome, gray platelet syndrome, X-linked thrombocytopenia, Thrombocytopenia 2 (*ANKRD26* mutation) and familial platelet disorder with predisposition to acute myelogenous leukemia were diagnosed in 1 pedigree each.

Conclusions

Adoption of an established diagnostic algorithm and collaboration with an expert referral center proved useful for diagnosis of IT patients in the setting of a developing country. This initiative may serve as a model to develop international networks with the goal of improving diagnosis and care of patients with these rare diseases.

Inherited thrombocytopenias (IT) are relatively rare and underdiagnosed genetic disorders. They comprise heterogeneous medical conditions which range from severe diseases which present since birth with profuse bleeding to others in which patients are asymptomatic and thrombocytopenia is identified during routine laboratory examination. Recognition of these disorders is essential to avoid misdiagnosis as immune thrombocytopenia, which leads to useless and potentially harmful therapy, such as corticosteroids or splenectomy. Significant advances have been made in recent years regarding the molecular etiology of IT. Most of the identified genetic defects involve key regulators of megakaryopoiesis and thrombopoiesis that lead to defects in platelet production, although in certain disorders shortened platelet life-span represents an additional mechanism underlying thrombocytopenia [1]. Newly identified mutations in *ANKRD26* in patients with Thrombocytopenia 2 (THC2) has contributed to diagnosis of patients with IT harbouring normal-sized platelets [2], while efforts to discover the genetic defect underlying Gray Platelet Syndrome (GPS) have led to the recent finding of mutations in *NBEAL2* [3-5]. Appropriate characterization of IT is important for patient diagnosis, prognosis and genetic counselling and may help guide treatment decisions, especially regarding the use of Thrombopoietin-mimetics, which have been recently shown to improve platelet counts and reduce bleeding in patients with *MYH9*-related disease (*MYH9*-RD) [6].

However, IT frequently represent a diagnostic challenge, even after extensive diagnostic work-up. This is due to the requirement of specialized laboratory and molecular tests and to the fact that the disease-causing mutation remains in many cases still unknown. To guide the diagnostic approach, an algorithm was proposed by the Italian *Gruppo di Studio delle Piastrine.* This is based on assessment of platelet size and the presence or absence of additional clinical features associated with thrombocytopenia, followed by simple laboratory screening tests and, finally, according to diagnostic suspicion, by more complex studies for diagnostic confirmation [7]. Requirement of highly specialized tests represents a particular problem in resource-limited countries, in which stepwise use of available resources is critical for adequate classification and accurate diagnosis of patients. To circumvent this problem, we applied the aforementioned diagnostic algorithm, optimized the use of local resources and developed a collaborative initiative with a specialized center in Italy with the goal of increasing the diagnostic yield in a cohort of patients with IT in Argentina. Using this approach, a definitive diagnosis could be established in 10 (71.4%) of 14 IT pedigrees included in this study, demonstrating the feasibility of this strategy to maximize resources and improve diagnosis and care of IT patients in this setting.

Patients and Methods

Patients

Patients with platelet counts below 150 x 10^9/L and a history of thrombocytopenia in first- or second-degree relatives and/or features suggestive of IT, such as sensorineural hearing loss, nephropathy, Döhle-like bodies, congenital malformations, family history positive for myeloid neoplasms and immunodeficiency, who were followed at the Instituto de Investigaciones Médicas Alfredo Lanari from 2001 to 2011, were recruited in a consecutive manner. In addition, after exclusion of non-genetic causes of thrombocytopenia, patients with giant platelets, microthrombocytes or those with thrombocytopenia refractory to ITP treatment in whom platelet kinetic studies showed normal platelet life-span, were also considered eligible for this study. Our institution is a referral center for patients with platelet disorders, primarily involved in the care of adult patients, while children are referred for diagnostic evaluation from affiliated pediatric institutions. The study was approved by the local Ethics Committee and patients or their legal guardians signed an informed consent. Diagnostic work-up was carried out following an algorithm proposed by the Italian *Gruppo di Studio delle Piastrine* [7], which is based on two diagnostic phases with increasing levels of complexity. Initial evaluation includes platelet size and the presence or absence of clinical features other than thrombocytopenia, according to which patients are classified as presenting with syndromic or non-syndromic forms, and categorized based on the presence of large, normal-sized or small platelets. Platelet function tests and blood smear examination are performed at this stage and may further guide subsequent approach. According to initial suspicion, specialized tests are performed, including flow cytometry, analysis of myosin-9 distribution and molecular studies. A government-supported collaboration program for diagnosis of IT was developed between centers in Argentina and Italy. Studies unavailable at the local institution were performed at the referral center in the setting of this collaboration, the aims of which were, in addition, to train health professionals and to help build local capacities.

Platelet count and size and blood smear examination

Platelet count was determined by microscopy, while automated platelet count and mean platelet volume (MPV) were assessed in an impedance-based electronic cell counter (Coulter, FL). Mean platelet diameter (MPD) was determined by measuring the largest diameter of 100 platelets in May-Grünwald-Giemsa-stained blood smears by using the VideoTesT-Master image analysis software (St. Petersburg, USSR). Smears

were examined for the presence of Döhle-like bodies and cell morphology was recorded.

Platelet aggregation tests

Platelet-rich plasma (PRP) was prepared by centrifugation or, in patients with large platelets, by allowing the sample to sediment. Aggregation in response to adenosine diphosphate (ADP), 2μM; epinephrine, 1μM; collagen, 4μg/ml, arachidonic acid, 1mM (Biopool, Bray, Ireland) was evaluated in a lumiaggregometer (Chrono-Log Corp, PA, USA) and compared to a control sample adjusted to the same platelet count. In addition, spontaneous platelet aggregation (SPA) and ristocetin-induced platelet aggregation (RIPA) with 1.5 mg/mL and 0.5 mg/mL (low-dose) ristocetin (Biopool) was assessed.

Flow cytometry analysis for platelet glycoproteins

Platelet surface glycoprotein (GP) expression was analyzed in a flow cytometer after incubation of PRP with fluorescein isothiocyanate (FITC)- or phycoerythrin-conjugated monoclonal antibodies to GPIIIa (CD61), GPIbα (CD42b) and GPIX (CD42a) (BD Biosciences, San José, CA, USA). Both the percentage of positive platelets and the ratio between mean fluorescence intensity (MFI) for the patient and the control were calculated.

Myosin-9 distribution pattern by immunofluorescence

Analysis of myosin-9 distribution on granulocytes was performed on blood smears after staining with mouse NMG2 monoclonal antibody followed by detection with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody and analyzed on a fluorescence microscope, as described [8].

Immunofluorescence analysis for thrombospondin-1

Platelet thrombospondin-1 (TSP1) content was assessed on blood smears by immunostaining with a mouse anti-Thrombospondin-1 antibody (Sigma Aldrich, St. Louis, MO, USA) followed by a FITC or Alexa Fluor 594-conjugated goat anti-mouse secondary antibody. Platelets were identified by F-actin labeling with tetramethyl rhodamine isothiocyanate (TRITC)- or Alexa Fluor 488-conjugated Phalloidin and examined under a fluorescence microscope. For analysis, platelets were classified according to the number of TSP1-positive granules into platelets displaying >5 granules and those with <5 granules, as reported [9].

Mutation analysis

For mutation testing, sequencing of all exons and exon/intron splice junctions of *GP1BA*, *NBEAL2* and *RUNX1* and of the 5'-untranslated region (5'-UTR) of *ANKRD26* was performed. For *MYH9* analysis, exons where mutations are clustered (exons 16, 30, 38 and 40) were studied first, followed by screening of exons 1, 10, 24, 25, 26, 31, 32 and 37 and then of all the other exons. For *WAS* analysis, single strand conformation polymorphism (SSCP) was followed by sequencing of exons with mobility shifts, as described [10]. To screen for the c.515C>T Bolzano mutation in *GP1BA*, a fragment spanning the mutation was amplified and digested with *Hpa*I, as described [11]. Sequence analysis of *GP1BA* was carried out in the family with the p.Ala172Val mutation to genotype two polymorphic intragenic loci, rs6065 and a variable number of tandem repeats (VNTR), for haplotype determination. For nomenclature, NCBI reference sequences NM_002473.3, NM_000173.5, NM_015175.2, NM_000377, and NM_014915.2 and D43968 were used for *MYH9*, *GP1BA, NBEAL2*, *WAS, ANKRD26* and *RUNX1,* respectively.

Statistical analysis

Comparison between continuous variables was performed using the student *t* test or the Mann-Whitney-Wilcoxon test, while correlation between MPV and MPD was assessed using the Spearman rank correlation coefficient.

Results

Patients

Thirty-one patients belonging to 14 pedigrees were included, median age was 31 (2-72), 17 patients were female. Fourteen patients had been misdiagnosed as ITP and treated with corticosteroids, danazol or IVIG, with no or transient partial response, while no patient had been splenectomized. Whereas in 9 (64%) pedigrees the transmission pattern was clearly autosomal dominant, in the other pedigrees we could not determine whether thrombocytopenia was transmitted as X-linked or autosomal recessive trait or as the result of a de novo event. In five pedigrees, there were clinical features suggestive of syndromic thrombocytopenias. In particular, 5 patients from pedigrees I and II had end-stage renal failure and/or hearing loss, patient from pedigree X had birth defects including mitral valve dysplasia, annular pancreas and psychomotor retardation, while 2 patients from pedigree XIII and one from pedigree XIV developed myeloid neoplasms. Bleeding was mild to moderate in most patients, except for 4 patients who had severe bleeding. WHO bleeding score was grade 0, 1, 2, 3 and 4 in

3, 11, 9, 4 and 4 patients, respectively. Five patients died, two because of central nervous system bleeding after transformation to myeloid neoplasms, while subarachnoid hemorrhage during endovascular surgery, stroke and AML led to death in one patient each, respectively. Follow-up was 73 (1-259) months.

Screening-phase studies

Platelet count

Platelet counts evaluated by microscopy were 77 (4-147) $x10^9$/L, while automated platelet counts were 63 (2-148) $x10^9$/L. In patients with macrothrombocytopenia lower automated compared to manual platelet counts were found, 32 (2-120) $x10^9$/L vs 65 (12-145) $x10^9$/L, $p<0.0001$, reflecting underestimation of platelet counts by electronic counters.

Platelet size and blood smear examination

MPV was 9.05 (5.45-19) fL, while MPD was 3.24 (1.46-5.94) μm, reference values 6.2-10.4 fL and 2.27-3.08 μm, respectively. MPV could not be determined in 4 patients with large platelets because of abnormalities in cell distribution histograms. Correlation was found between MPV and MPD, $r=0.61$, $p=0.002$, figure 1. However, discrepancy between the two parameters was found in patients from pedigree IX, who had normal MPV, while a population of large platelets was evident in the smear and MPD was increased. Overall, ten pedigrees (I to X) had large platelets, pedigree XI had small platelets and three pedigrees (XII to XIV) harboured normal-sized platelets. In patients with macrothrombocytopenia, MPV was 11.6 (8.4-19) fL and MPD was 3.91 (3.09-5.94) μm, which differed significantly from controls, $p<0.0001$ for both. Granulocyte inclusions reminiscent of Döhle bodies were present on smears from pedigrees II, III and IV, consistent with *MYH9*-RD. Besides, large platelets with a pale appearance were evident in pedigree IX.

Platelet aggregation

Platelet aggregation was evaluable in 18 patients, who had platelet counts above 100 $x10^9$/L in PRP. Decreased aggregation responses to all agonists, including ADP, epinephrine, arachidonic acid and collagen, were found in 7 patients (pedigrees IV, IX, XII, XIII). Agglutination induced by 1.5mg/mL ristocetin was absent in the patient from pedigree V, who had absent response to von Willebrand factor (VWF)-containing bovine fibrinogen, pointing to Bernard-Soulier syndrome (BSS). In addition, SPA and enhanced RIPA were found in the patient from pedigree VII on repeated testing, suggesting platelet-type (PT-VWD) or type 2B von Willebrand disease (2BVWD).

Diagnostic suspicion raised during the screening phase

First-step evaluation studies, which are summarized in table 1, raised the diagnostic suspicion of specific disorders in 9 of 14 pedigrees. In particular, diagnosis of *MYH9*-RD, BSS, P-T/2B VWD, GPS, X-linked thrombocytopenia (XLT) and familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML) were considered in pedigrees I to IV, V, VII, IX, XI and XIII, respectively.

Confirmatory tests

Surface glycoprotein expression by flow cytometry

Platelet glycoprotein expression was evaluated in 15 patients with macrothrombocytopenia, revealing high MFI for CD61, 181 (125-377) %, CD42a, 150 (129-263) %, and CD42b, 137 (126-222) %, in 11, 7 and 8 patients, respectively, reflecting larger surface area. However, in 6 cases (pedigrees II, IV, VI, and VII), decreased CD42a and CD42b relative to CD61 values were found, 53.8 (41.1-69.2) % and 52.4 (21.6-69.7) % of control, respectively. In addition, markedly reduced GPIbα expression was shown for patient from pedigree V, who showed less than 1% CD42b-positive platelets, MFI was 2% of control, while GPIX expression was moderately decreased, 64% of platelets stained positive for CD42a and MFI was 43% of control, consistent with BSS.

Myosin-9 distribution pattern in granulocytes

Myosin-9 aggregates were evident in granulocytes of patients from pedigrees I to IV. Immunofluorescence pattern in pedigrees I and II was characterized by numerous small (<0.5μm) aggregates, while one to four main large inclusions (2-5μm), round or oval, often together with further small aggregates were evident in pedigrees III and IV. *MYH9*-RD was excluded by the absence of myosin-9 aggregates in probands with large platelets and no definite diagnostic suspicion.

Analysis of platelet thrombospondin-1 content

A marked reduction in α-granule content was observed in pedigree IX, as 93% and 96% of platelets from both patients displayed <5 TSP1-positive granules, respectively, whereas platelets from control individuals showed a packed granular pattern, with only 4.4 (2-6.2)% of platelets harbouring <5 granules. A substantial, albeit more moderate, decrease in α-granule content was also found in the patient from pedigree X, in whom 62.7% of platelets had <5 granules. Electron microscopy was available for this patient, confirming the reduction in α-granules (data not shown). Besides, a mild reduction in α-

granule content, ranging from 12.5 to 14.5% of platelets <5 granules, was evident in 3 patients from pedigree XII.

Mutational screening of candidate genes

MYH9 mutations were found in pedigrees I to IV, two of which were located in frequently affected hotspots, including c.2105G>A (p.Arg702His) (pedigree I) and c.4270G>A (p.Asp1424Asn) (pedigree III), while identification of the c.284C>A (p.Ala95Asp) mutation, in pedigree II has been recently reported [12]. In addition, a novel c.2539_2559dup (p.Met847_Glu853) in exon 20 was identified in pedigree IV (De Rocco et al, submitted). Of the four mutations, p.Asp1424Asn is a de novo event as it was not detected in the proband's parents. In the patient from pedigree V, who had markedly reduced GPIbα, a novel homozygous c.882C>G mutation resulting in p.Tyr294* was detected in *GP1BA*, confirming BSS. Her parents and sister were heterozygous carriers for this mutation and two of them had platelet macrocytosis and moderately decreased GPIbα expression, 33% and 50% of control, respectively, while platelet count was at the lower limit of normal in one case. Besides, the Bolzano variant c.515C>T (p.Ala172Val) in *GP1BA* was found in patients from pedigree VI, who had reduced GPIb/IX relative to GPIIIa expression, and monoallelic BSS was diagnosed. The three patients belonging to this pedigree were all carriers of at least one T allele of SNP rs6065 and B variant of the VNTR, suggesting that they share the same haplotype as that identified in other patients with the same mutation [11]. On the other hand, screening of *GP1BA* and of the region of VWF exon 28 encoding residues 1266-1461, where mutations are usually identified in type 2B VWD, disclosed no abnormality in the patient from pedigree VII, who had SPA and enhanced RIPA. In patients from pedigree IX, who were characterized by markedly reduced α-granules, a novel homozygous c.2187C>A mutation yielding p.Tyr729* in *NBEAL2* was identified, confirming diagnosis of GPS (Bottega et al, submitted). Clinical and molecular features of this pedigree are shown in Figure 2. On the other hand, no *NBEAL2* mutation was identified in patient from pedigree X, even if she had substantial although not complete α-granule reduction. X-linked thrombocytopenia was diagnosed in the only pedigree with non-syndromic thrombocytopenia harbouring small platelets (pedigree XI), based on the finding of c.156G>C (p.Gln52His) mutation in exon 2 of *WAS*, where the majority of mutations causing XLT are located. Regarding patients with normal-sized platelets, identification of c.653delC (p.Thr219Argfs*8) C-terminal *RUNX1* mutation in pedigree XIII has been previously described [13], while no mutation in *RUNX1* was found in pedigree XIV. Patients with thrombocytopenia and normal-sized platelets of unknown molecular etiology (pedigrees XII and XIV) were screened for *ANKRD26* mutations and

the c.-127A>G substitution in 5´-UTR of *ANKDR26* was detected in pedigree XII, where mutations causing THC2 have been reported to be clustered. Family tree for this pedigree is shown in Figure 3. Results of confirmatory tests are summarized in Table 2.

Discussion

Recent progress in the field of inherited platelet disorders has contributed to better understanding of these genetic conditions, leading to improved approach to patient diagnosis and care. Accurate diagnosis of IT may be challenging due to the relative rarity of these heterogeneous conditions, as well as atypical presentation of known disorders and the need to identify new genetic defects in patients who do not fit into established categories. Adoption of established algorithms may be useful to optimize diagnostic strategies. In this regard, application of the algorithm proposed by the italian group led to diagnosis in 22 of 46 unrelated patients studied in Italy [15]. However, even when diagnostic guidelines have been established, the required diagnostic technologies are not always readily available and referral to specialized centers may be necessary for definitive diagnosis. To maximize utilization of local, low-cost resources, we applied the aforementioned algorithm to patients with IT in a single center in Argentina, which proved useful as an initial approach to diagnosis and helped guide further, more complex studies, some of which were performed at a referral center with known expertise in this field. This approach led to a definitive diagnosis in 10 (71.4%) of 14 pedigrees included in this study. This figure compares favorably with those reported in the literature, in which only around half of the patients with IT were given a definitive diagnosis [15]. Feasibility of this strategy was based on the development of a collaboration program between centers in Argentina and Italy and its success was facilitated by sustained commitment of both partner sites. Extension of this project to other regional centers would be useful to assess whether this high diagnostic yield is maintained. This initiative may serve as a model for developing international networks in which centers from developing or middle-income countries team up with referral centers from resourceful nations.

As described in other cohorts, the majority of patients included in this series harbored large platelets and *MYH9*-RD comprised the most frequent single disorder. Consistent with findings raised by a genotype-phenotype study [16], patients with mutations at position 702 (pedigree I) had severe disease with early-onset extrahematological involvement, while patients carrying tail domain mutations (pedigrees III and IV) presented with a milder phenotype characterized by isolated moderate thrombocytopenia. Besides, patients harboring the p.Ala95Asn mutation (pedigree II)

had severe thrombocytopenia and early-onset hearing loss but no renal disease, highlighting that the risk of renal impairment in patients with motor domain mutations located at positions other than 702 is not clear [12]. The pattern of myosin-9 distribution differed between patients with motor domain respect to those with tail domain mutations, as the former displayed a speckled pattern while the latter showed large aggregates. This observation is in agreement with a recent study, in which immunofluorescence pattern was proposed as a useful clue to guide the choice of candidate exons for optimized mutational screening [8]. Besides, the finding of low GPIb-IX relative to GPIIIa expression in two of four *MYH9*-RD pedigrees in this cohort has been previously observed in other *MYH9*-RD patients [17].

Macrothrombocytopenias involving abnormalities in GPIbα were identified in two additional pedigrees. Markedly decreased GPIbα levels were found in a patient with classical BSS, in whom a novel *GP1BA* homozygous mutation was found. Besides, the finding of decreased GPIb-IX relative to GPIIIa expression in another pedigree led us to consider the diagnosis of monoallelic form of BSS, which was confirmed by the identification of a heterozygous Bolzano mutation. Monoallelic dominant BSS due to the Bolzano variant represents a frequent cause of macrothrombocytopenia in Italy and a founder effect for this mutation has been described in Southern Italy [11]. However, it is unknown whether this mutation occurs outside this country. The fact that patients from the Argentinian pedigree are likely to share the same haplotype as that described in Italian patients suggests a common origin for this mutation, and may reflect immigration of the Italian population to Argentina. Finally, SPA and enhanced RIPA, suggesting increased affinity between VWF and GP1bα, was a prominent feature in another patient in this cohort, although inconclusive results of mixing assays did not allow us to determine the origin of this abnormality (data not shown). No mutation was identified in *GP1BA* and in the mutational hot spot region of *VWF* exon 28. Failure to identify mutations in both *VWF* exon 28 and *GP1BA* in patients with phenotypic features of P-T/2B VWD has been recently highlighted, suggesting involvement of other genes or alternative diagnosis [18].

The finding of markedly decreased platelet TSP1-positive granules in another pedigree, strengthened by the detection of myelofibrosis, splenomegaly and high B_{12} levels, led to diagnostic suspicion of GPS, which was confirmed by the identification of a novel homozygous *NBEAL2* mutation, illustrating the usefulness of assessing TSP1 content by immunofluorescence as a marker for α-granules, as shown previously [9]. In addition, a mild reduction in α-granules was found in patients with THC2, as reported for other pedigrees [19], indicating that, although not present in all patients, this finding may suggest the presence of *ANKRD26* mutations in patients with normal-sized

platelets. Staining for TSP1 could thus be included among the second-phase evaluation studies proposed in the algorithm, especially if electron microscopy is unavailable. On the other hand, no *NBEAL2* mutation was found in the patient with large platelets and birth defects, who had substantial α-granule reduction. Among macrothrombocytopenias associated with congenital malformations, Jacobsen syndrome was excluded because of absence of typical giant α-granules, normal karyotype and normal MPLA for subtelomeric regions, which ruled out 11q terminal deletion, while 22q11.2 microdeletion syndrome was deemed unlikely because of normal GPIb expression, although more specific molecular studies could not be performed to definitively exclude this possibility. More sophisticated genetic technologies, such as next generation sequencing, SNP genotyping arrays and array-comparative genomic hybridization (CGH), may help identify new disease loci in patients with unrecognized forms of thrombocytopenia associated with congenital malformations.

Regarding disorders with normal-sized platelets, the recent identification of *ANKRD26* mutations in THC2 has contributed to diagnosis in this subset of patients, and, to date, 21 such pedigrees have been reported [2,19]. Although the pathogenic effects of these mutations are currently under investigation, they may lead to increased *ANKRD26* expression which may interfere with megakaryocyte maturation and platelet production. In our cohort, c.-127A>G substitution in *ANKRD26* was found in a pedigree with clinical features resembling those of previously described patients [19]. Affected individuals have non-syndromic autosomal dominant mild to moderate thrombocytopenia with few bleeding manifestations and normal or mildly decreased platelet aggregation. Of interest considering the increased frequency of leukemia found in some THC2 pedigrees [19], a relative of affected individuals died of acute leukemia, highlightening the need for further studies to define the risk of leukemia in this disorder. With regards to other patients characterized by normal-sized platelets, the presence of severe platelet dysfunction, development of myeloid neoplasms and the finding of a *RUNX1* mutation led to diagnosis of FPD/AML in another pedigree [13], while no mutations in *ANKRD26* or *RUNX1* were found in the remaining one and molecular diagnosis remained undetermined in this family, in which one affected member had developed a myeloid neoplasm. Of note, platelet dysfunction, which seems to be a constant feature of FPD/AML, was absent in this pedigree. Microdeletions involving RUNX1, which may have been missed by sequencing, have been reported to cause thrombocytopenia and leukemia predisposition although, unlike these patients, most affected individuals display dysmorphic features or mental retardation. Array-CGH may be useful in this setting [20].

In conclusion, given the rarity of IT, increased awareness of these conditions, adoption of established diagnostic algorithms and collaborative international efforts should facilitate advances in patient diagnosis and care, as well as help identify new genetic defects in patients who do not fall into known categories. In addition, despite identification of the causative genetic defect, the intrinsic mechanisms underlying thrombocytopenia in some of these disorders are still incompletely understood and collaborative research may enable the homogeneous study of larger patient cohorts, providing unique opportunities to gain further insight into the molecular regulation of platelet production and function.

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Table 1. Patient features and results of screening studies

Pedigree	Patient features			First diagnostic phase			
[No.of patients]	Age, years (range)	Platelet count, ($x10^9$/L) (range)	WHO bleeding score [No. of patients]	Syndromic	Platelet size	Blood smear	Platelet aggregation
I [2]	25 (10-40)	34.5 (34-35)	3 [1], 4 [1]	HL, CRF	large	---	normal
II [3]	31 (27-70)	20 (12-22)	1 [2], 2 [1]	HL	large	Döhle-like inclusions	NE
III [1]	19	42	1	no	large	Döhle-like inclusions	reduced response to ADP
IV [2]	17.5 (2-33)	86 (76-96)	0 [1], 2 [1]	no	large	Döhle-like inclusions	mildly decreased
V [1]	4	110	4	no	large	---	absent RIPA
VI [3]	34 (24-53)	102 (86-130)	1 [3]	no	large	---	normal
VII [1]	40	145	2	no	large	---	SPA, enhanced RIPA
VIII [1]	25	42	0	no	large	---	normal
IX [2]	56 (53-59)	47.5 (30-65)	2 [2]	no	large	pale platelets	mildly decreased
X [1]	11	65	3	CM	large	---	NE
XI [2]	9.5 (5-14)	25 (15-35)	2 [2]	no	small	---	NE
XII [4]	50 (14-72)	85 (68-147)	0 [1], 1 [3]	no	normal	---	normal/mildly decreased
XIII [5]	32 (9-53)	82 (10-145)	2 [2], 3 [2], 4 [1]	CMML, AML	normal	---	decreased
XIV [3]	32 (26-55)	86 (4-105)	1 [2], 4	MDS/MPN	normal	---	normal

WHO, World Health Organization; HL, hearing loss; CRF, chronic renal failure; CM, congenital malformations; CMML, chronic myelomonocytic leukemia; AML, acute myeloid leukemia; MDS/MPN, mixed myelodysplastic myeloproliferative syndrome; ---, indicates no relevant abnormalities besides abnormal platelet size; NE, non-evaluable; SPA, spontaneous platelet aggregation; RIPA, ristocetin-induced platelet aggregation.

Bleeding was assessed according to WHO bleeding scale: grade 0 indicates no bleeding; 1. petechiae; 2, 3 and 4, mild, gross and debilitating blood loss, respectively.

Table 2. Results of specialized studies and final diagnosis in IT patients

Pedigree	Second-phase studies			Diagnosis	Ref
	Flow cytometry	Immunofluorescence[†]	Molecular studies		
I	normal	myosin-9 aggregates	p.Arg702His *MYH9* mutation	MYH9-related disease	14
II	low GPIb/GPIIIa ratio	myosin-9 aggregates	p.Ala95Asn *MYH9* mutation	MYH9-related disease	12
III	normal	myosin-9 aggregates	p.Asp1424Asn *MYH9* mutation	MYH9-related disease	-
IV	low GPIb/GPIIIa ratio	myosin-9 aggregates	**p.Met847_Glu853 *MYH9* mutation**	MYH9-related disease	¶
V	markedly reduced GPIbα	-	**p.Tyr294* *GP1BA* mutation[‡]**	Bernard-Soulier syndorme	-
VI	low GPIb/GPIIIa ratio	-	p.Ala172Val *GP1BA* mutation	Monoallelic Bernard-Soulier	-
VII	low GPIb/GPIIIa ratio	-	no mutation in *GP1BA* and *VWF* exon 28	UD	-
VIII	normal	-	ND	UD	-
IX	normal	markedly reduced TSP1	**p.Tyr729* *NBEAL2* mutation**	Gray platelet syndrome	**
X	normal	moderately reduced TSP1	no mutation found in *NBEAL2*	UD	-
XI	normal	-	p.Gln52His *WAS* mutation	X-linked thrombocytopenia	10
XII	normal	mildly reduced TSP1	c.-127A>G *ANKRD26* mutation	Thrombocytopenia 2	-
XIII	normal	-	p.Thr219Argfs*8 *RUNX1* mutation[§]	FPD/AML	13
XIV	normal	-	normal *RUNX1* & *ANKRD26*	UD	-

[†]only positive findings are indicated; [‡]amino acid position of precursor glycoprotein; [§]the p.Thr219Argfs*8 mutation, described according to current nomenclature recommendations (www.hgvs.org/mutnomen), was previously reported as Pro218fsTer225[13], ¶De Rocco et al et al, submitted, **Bottega et al, submitted.

ND, indicates not done; UD, undefined; FPD/AML, familial platelet disorder with predisposition to acute myelogenous leukemia; Ref, reference.

Novel mutations are shown in bold.

Legends to figures

Figure 1. Correlation between mean platelet volume (MPV) and mean platelet diameter (MPD) in patients with inherited thrombocytopenia. MPV was assessed by using an automated cell counter, while MPD was determined on blood smears by measuring the largest platelet diameter with an image analysis software. Dotted lines represent reference values for MPV and MPD. Filled symbols represent patients in whom MPV and MPD values agree while empty symbols show patients from pedigree IX, in whom discrepant results were found between the two parameters, as MPV was normal while MPD was increased.

Figure 2. Features of a pedigree with gray platelet syndrome. (A). Family pedigree. Thrombocytopenia was detected in two brothers (II.1 and II.2) at age 53 and 42 born to consanguineous parents. Squares indicate males; circles, females; black symbols, thrombocytopenia; open symbols, normal platelet counts and question mark, unknown platelet status. A c.2187C>A homozygous mutation in *NBEAL2* was identified in patients II.1 and II.2, while III.1, III.2 and III.3, as expected, were heterozygous carriers for this mutation. (B). Sequencing data. Chromatogram showing the presence of an homozygous c.2187C>A mutation yielding p.Tyr729* in *NBEAL2.* (C). Immunofluorescence analysis for thrombospondin-1. Staining for TSP1 was performed by incubation with an anti-TSP1 antibody followed by a FITC-conjugated secondary antibody (green) and platelets were identified by F-actin staining with TRITC-conjugated Phalloidin (red) and examined under a fluorescence microscope (BX51, Olympus) (x100). Compared to control (top panel), platelets from patient II.2 (lower panel) show decreased numbers of TSP1-positive granules. (D). Gomori´s silver staining for reticulin fibers in the bone marrow. Diffuse and dense fiber network with intersections (grade 2 according to the European Consensus grading system) is shown for patient II.2 by light microscopy (x40).

Figure 3. Features of a pedigree with Thrombocytopenia 2 (THC2). (A). Family pedigree XII. The founder pedigree and partial pedigrees of 3 members of generation II are shown. Squares represent males; circles, females; black symbols indicate thrombocytopenia associated with the presence of heterozygous *ANKRD26* mutation; dark gray symbols, documented thrombocytopenia; pale gray, reported thrombocytopenia; open symbols, normal platelet counts; question mark, unknown platelet status and the striped symbol (II.1) represents an individual who developed acute leukemia. (B). Mutational screening revealed the presence of a heterozygous c.-

127A>G (*) in 5'-UTR of *ANKRD26* in II.3, III.3, IV.6 and IV.7, while it was absent in III.2. Mutational screening was not carried out in the other thrombocytopenic patients.