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Inherited thrombocytopenias: from genes to therapy

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Background and Objectives. Inherited thrombocytopenias are a heterogeneous group of rare diseases characterized by a reduced number of blood platelets. Some of these diseases are exclusive to megakaryocytes and platelets, while in others the pathology extends to other cell types. Although the defective genes, coding for membrane glyoproteins, cytoskeleton components and intracellular signaling pathways, as well as transcription factors, have been identified in most cases, the pathophysiology of these disorders is often unknown. This review describes recent contributions to clinical and diagnostic aspects, biology and treatments of familial thrombocytopenias.

Evidence and Information Sources. The information presented here derives from literature and the experience of the authors. The most relevant studies are critically analyzed and discussed.

State of Art. The clinical and laboratory features of most of the inherited thrombocytopenias have been reviewed. The different forms have been classified into 3 groups depending on platelet volume. Although this criterion is not completely satisfactory, it is one of the most useful in diagnostic algorithms. We report on recent advances in Wiskott-Aldrich and Bernard-Soulier syndromes, as well as in MYH9-related diseases, a new nosological entity that groups old distinct forms known as May-Hegglin anomaly, Sebastian, Fetchner, and Epstein syndromes. Other, less frequent forms are also discussed, including non-syndromic forms of mild thrombocytopenia that are genetically heterogeneous.

Perspectives. In the past, inherited thrombocytopenias were considered exceedingly rare and the number of well-defined forms was very small. In the last few years, the widespread diffusion of electronic cell counters has allowed these conditions to be detected more frequently and several new entities have been identified through the co-ordinated efforts of physicians, biologists and geneticists. The pathogenesis of many *new* and *old* forms is being unraveled, thus providing insights on the molecular basis of platelet production and function. This knowledge will be a valuable resource for clinicians in the diagnostic approaches to such disorders.

Key words: platelets; inherited thrombocytopenias; bleeding.

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The classification of any category of illnesses should group diseases according to pathogenesis and facilitate and improve the diagnosis. There are, however, several difficulties for inherited thrombocytopenias, including the paucity of information on the pathogenetic mechanisms and the extreme heterogeneity of these diseases. Different parameters can been considered, such as the inheritance pattern or the presence of symptoms other than thrombocytopenia. However, the inheritance pattern is not always helpful because transmission of some disorders may be both dominant and recessive, and sporadic cases due to *de novo* mutations sometimes occur. Likewise, a classification based on clinical symptoms is not always reliable, as syndromic and non-syndromic forms may result from mutations of the same gene. Among other different possibilities, one of the most satisfactory classification relies on platelet size (Table 1), which has several advantages because platelet size is easy to determine by microscopic observation of peripheral blood smears and represents the most constant feature of each illness. For instance, subjects with heterozygous Bernard-Soulier syndrome may have a normal platelet count, but the size of their platelets is always increased (*see below Bernard-Soulier syndrome*). This criterion for classification does, however, have some disadvantages. A borderline platelet size is sometimes observed and, since electronic counters underestimate volume in patients with macrothrombocytopenia, time-consuming observation of blood smears is usually

Table 1. Classification of inherited thrombocytopenias according to platelet size.

Inherited thrombocytopenias	*Abbreviation*	*OMIM[a]*	*S/NS[b]*	*Inherited Pattern[c]*	*Gene*	*Gene Localization*
Small Platelets						
Wiskott-Aldrich syndrome	WAS	301000	S	X-L	WAS	Xp11
X-linked thrombocytopenia	XLT	313900	NS			
Normal-sized platelets						
Familial platelet disorder with predisposition to acute myelogenous leukemia	FPD/AML	601399	S	AD	CBFA2	21q22
Amegakaryocytic thrombocytopenia	CAMT	604498	NS	AR	c-mpl	1p34
Amegakaryocytic thrombocytopenia with radio-ulnar synostosis	CTRUS	605432	S	AD	HOXA11	7p15-14
Thrombocytopenia with absent radii	TAR	274000	S	AR	n.d.	n.d.
Other thrombocytopenias	THC2	188000	NS	AD	n.d.	10p12 Heterogeneity
Large platelets						
Bernard-Soulier syndrome	BSS	231200	NS	AD	GPIbα GPIbβ GPIX	 17p13 22q11 3q21
Velocardiofacial syndrome	VCFS	192430	S	AD	CGS[d] GPIbβ	 22q11
Platelet-type or pseudo von Willebrand's disease	PTvWD	177820	NS	AD	GPIbα	17p12
Benign Mediterranean macrothrombocytopenia	n.d.	153670	NS	AD	n.d.	n.d.
X-linked thrombocytopenia and dyserythropoiesis with or without anemia	XLTT	300367 314050	S	X-L	GATA-1	Xp11
Paris-Trousseau type thrombocytopenia	TCPT	188025/600588				
Jacobsen's syndrome	JBS	147791	S	AD	CGS[d] Fli-1 Ets-1	11q23
MYH9-related disease						
May-Hegglin anomaly	MHA	155100	NS	AD	MYH9	22q12-13
Sebastian syndrome	SBS	605249	NS			
Fechtner syndrome	FTNS	153640	S			
Epstein syndrome	EPTS	153650	S			
Gray platelet syndrome	GPS	139090	NS	AD	n.d.	n.d.
Montreal platelet syndrome	MPS	n.d.	NS	AD	n.d.	n.d.
Macrothrombocytopenia with platelet expression of glycophorin A	n.d.	n.d.	S	AD	n.d.	n.d.

[a]On line Mendelian inheritance in man; [b]S:syndromic form; NS:non-syndromic form; [c]A.D.:autosomal dominant; A.R.:autosomal recessive; X-L:X-linked; [d]contiguous gene syndrome.

required (*see below Diagnostic difficulties*). However, this classification is one of the best because it does at least help clinicians and researchers in diagnostic approaches.

Inherited thrombocytopenias with reduced platelet size

A reduced mean platelet volume (MPV) is associated only with Wiskott-Aldrich syndrome and X-linked thrombocytopenia, two variants of a single disorder due to mutations in the WAS gene.

Wiskott-Aldrich syndrome (WAS) and X-linked thrombocytopenia (XLT)

Definition. WAS and XLT are X-linked diseases characterized by small platelets and thrombocytopenia. WAS also includes a severe immune dysregulation responsible for recurrent infections, allergy, autoimmune diseases and lymphoreticular malignancies. Only minimal symptoms of immunodeficiency occur in XLT patients. WAS is a rare disease with an incidence of approximately 1 case in 250,000 people in the European population. The

frequency of XLT, although unknown, is lower than that of WAS.

Pathogenesis. WAS and XLT are caused by mutations on a gene located on the short arm of chromosome X that encodes a 502 amino acid protein (WASp).[1,2] Although classical WAS has been reported in one young girl,[3] female carriers usually have no clinical signs because of the preferential inactivation of the mutated X chromosome in hematopoietic cells.[4-6] Over 100 different mutations, mainly nucleotide substitutions, have been identified. No evident genotype-phenotype correlation has been revealed, although nonsense and frameshift mutations are more frequently associated with severe immunodeficiency, while amino acid substitutions are more common in XLT.[7,8]

WASp is a proline-rich intracellular protein expressed exclusively in hematopoietic stem cell-derived lineages. It belongs to a widely expressed family of proteins involved in transduction of signals from receptors to the actin cytoskeleton. One of the most important functions of WASp is to act as an effector for CDC42. This molecule is a member of the Rho family of small GTP-binding proteins, and is known to induce the actin-dependent formation of focal adhesion complexes and filopodial extensions.[9] In the absence of WASp, cellular processes directly related to re-organization of cytoskeletal architecture, such as those deriving from cell activation and directing cell mobility and phagocytosis, are defective.[10]

Thrombocytopenia and reduced platelet volume have a complex origin. *In vitro* studies showed that cultured WAS and XLT stem cells were able to differentiate into megakaryocytes with normal morphology. Moreover, these megakaryocytes formed normal pseudopodia that progressively elongated to produce a normal amount of detached platelets with normal size.[11] Consistent with the observation that splenectomy improves platelet count and increases platelet size,[12] it is likely that the reduction of platelet size and number occurs in the blood circulation as a consequence of the abnormal organization of the platelet cytoskeleton deriving from the WASp deficiency.[13]

The progressive *decrease in T-cell number and function* is the most important determinant of immune dysregulation. Following activation, normal lymphocytes undergo an asymmetric assembly of receptors and signaling molecules on the cell surface, known as the cap, and begin to proliferate. T-cells from WAS patients and mutant mice showed abnormalities of both antigen receptor-induced proliferation and antigen receptor cap formation.[14] In contrast, murine B-cells had normal proliferation and normal cap formation, suggesting that there may be a functional redundancy in this cell type.[15]

Abnormalities of macrophages and dendritic cells contribute to the immune pathology of WAS patients. These cells do not respond to chemical stimuli in a directional manner and are dysmotile when adherent to surfaces, indicating that CDC42-WASp-mediated filopodia formation is essential for chemotaxis.[9] Moreover, consistent abrogation of Fc-mediated phagocytosis associated with reduction in local actin polymerization has been reported.[16]

Clinical aspects. According to the classical definitions, patients with XLT suffer from birth from an isolated bleeding tendency, while patients with WAS present the additional finding of a severe immunodeficiency that worsens during childhood. However, this clear-cut distinction is scholastic, in that transitional forms with thrombocytopenia and mild immune defects have been reported. Moreover, the severity of the immune defect may vary within the affected members of a single family. The *bleeding tendency* ranges from minor purpura to life-threatening intracranial or gastrointestinal hemorrhage. Clinical manifestations of the *immune dysfunction* include susceptibility to infections, eczema, autoimmune phenomena, vasculitis, inflammatory polyarthritis, inflammatory bowel disease, and an increased incidence of lymphoproliferative disorders. The median survival of WAS patients is about 15 years. The causes of death are infection (44%), bleeding (23%), and malignancies (26%).[17]

Laboratory features and diagnosis. The most invariable laboratory abnormality is thrombocytopenia (44% of patients have fewer than 20×10^9 platelets/L) with a small platelet volume (MPV usually less than 5 fL). The bleeding time is generally prolonged to a greater extent than would be expected from the platelet count. Functional, biochemical and ultrastructural studies usually do not identify gross defects apart from a moderate storage pool deficiency.[18] A progressive decrease in the numbers and function of T-lymphocytes during childhood is associated with defects in proliferative responses of these cells, deficient antibody responses to polysaccharide and protein antigens, and low or absent levels of isohemagglutinins. Serum levels of IgG are usually normal, whereas those of IgM are typically depressed, and IgA and IgE elevated.[9]

Inherited thrombocytopenias with normal platelet size

The platelets in these inherited thrombocytopenias show no gross morphologic, biochemical or functional abnormalities. Several forms were identified on the basis of the associated features, such as skeletal defects or propensity to develop acute leukemia. Molecular biology techniques are revealing that most of the inherited thrombocytopenias with normal MPV derive from abnormalities in the complex regulatory mechanisms of megakaryocytopoiesis.

Familial platelet disorder with predisposition to myeloid malignancy (FPD/AML)

FPD/AML is an autosomal dominant disorder characterized by thrombocytopenia with a prolonged bleeding time, an *aspirin-like* functional platelet defect and a predisposition to acute myeloid leukemia (AML).[19] Twelve families have been reported. The defective gene was first localized on chromosome 21q22.1-22.2 by linkage analysis[20, 21] and then identified by mutational screening. Nonsense and missense mutations or intragenic deletions in one allele of the CBFA2 gene (also called AML1, RUNX1, PEBP2α B) were detected in FPD/AML pedigrees.[22-24] CBFA2, which together with CBFB constitutes a hematopoietic transcription regulation complex, is highly expressed in thymus, bone marrow and peripheral blood and appears to have a role in the development of normal hematopoiesis. Bone marrow or peripheral blood cell cultures from FPD/AML patients showed a decrease in megakaryocyte colonies, which were also smaller in size. Haploinsufficiency of the CBFA2/CBFB complex may contribute to a quantitative and qualitative platelet defect due to inappropriate levels of expression of downstream genes involved in megakaryocyte differentiation. Although it is not clear how mutations contribute to the tumors, CBFA2 is implicated in the pathogenesis of AML, being either involved in acquired chromosomal translocations, the most frequent of which is t(8;21), or affected by point mutations.[25] Therefore, haploinsufficiency and/or loss of function of the residual CBFA2 allele could be responsible for AML in FPD/AML kindred.

Amegakaryocytic thrombocytopenias

This group of inherited thrombocytopenias comprises three forms deriving from defective megakaryocytic differentiation: congenital amegakaryocytic thrombocytopenia (CAMT), thrombocytopenia with absent radii (TAR), and congenital amegakaryocytic thrombocytopenia with radioulnar synostosis (CTRUS). The molecular bases of these disorders are associated with abnormalities either in the thrombopoietin (TPO) signaling pathway or in the function of homeobox genes. TPO and its receptor, c-mpl, directly promote the entire process of megakaryocytopoiesis and thrombopoiesis.[26] Several pieces of evidence demonstrate that the transduction signal pathway triggered by TPO/c-mpl binding is involved in the commitment of hematopoietic stem cells to megakaryocytopoiesis, proliferation of megakaryocyte progenitor cells, differentiation of megakaryoblasts, and platelet production from megakaryocytes. *In vivo* studies showed that TPO induces a marked increase in platelet count in animals, and TPO knock-out mice have severe thrombocytopenia.[27] In humans, administration of recombinant TPO raises the platelet count and platelet yield in volunteer apheresis donors, and raises the platelet count in cancer patients both prior to and following myelosuppressive chemotherapy.[28] Taken together, these data clearly indicate that TPO and c-mpl are central to the control of platelet production in humans and are both strong candidates for being involved in the pathogenesis of amegakaryocytic thrombocytopenias. As matter of fact, mutations of the c-mpl gene or defects of the TPO signaling pathway have been found in patients affected by CAMT and TAR, respectively. Several homeobox genes are expressed in hematopoietic cells and some are also co-expressed in the developing forelimb, suggesting their possible role in these syndromes. Although the relationship between the molecular and cellular defects is unclear, mutations in the HOXA11 gene were recently identified in two unrelated CTRUS families.

Congenital amegakaryocytic thrombocytopenia (CAMT)

CAMT is an autosomal recessive bone marrow failure syndrome characterized by isolated hypomegakaryocytic thrombocytopenia during the first years of life developing into a bone marrow aplasia in later childhood. Fifteen families have been reported so far. The molecular cause is a deficiency in expression or function of the thrombopoietin receptor, c-mpl. Frameshift or nonsense, as well as missense, mutations in the c-mpl gene have been identified.[29-32] Consistent with these data, patients with CAMT show a defective response to TPO in megakaryocyte colony formation. Since CAMT patients have decreased numbers of erythroid and myeloid progenitors and are prone to develop pancytopenia, TPO and its receptor are likely to be vital

for hematopoietic stem cell function. This conclusion is consistent with the clinical features of c-mpl deficient mice, which have defects in all hematopoietic progenitors leading to pancytopenia.

Congenital amegakaryocytic thrombocytopenia with radio-ulnar synostosis (CTRUS)

CTRUS is an autosomal dominant disorder characterized by congenital amegakaryocytic thrombocytopenia, aplastic anemia, proximal radial ulnar synostosis, clinodactyly, syndactyly, hip dysplasia and sensorineural hearing loss, as recently reported in two unrelated families.[33] Patients were found to be heterozygous for a single base-pair deletion resulting in a truncated Hoxa11 protein.[34] The Hoxa11 gene belongs to the family of homeobox genes, which encode regulatory proteins that are central to bone morphology as well as hematopoietic differentiation and proliferation. Target destruction of mouse Hoxa11, both alone and in combination with other Hox genes, established that their principal effect on skeletal development is localized to the forearm.[35] Limb malformations were seen to varying degrees in both homozygous and heterozygous Hoxa11-mutant mice. However, published studies of the Hoxa11-mutant mice did not include data on hematologic findings, thus leaving the role of the gene in this aspect of the disorder unclear.

Thrombocytopenia with absent radii (TAR)

TAR syndrome is an autosomal recessive disease characterized by hypomegakaryocytic thrombocytopenia and bilateral radial aplasia. Relevant heterogeneity regarding additional congenital malformations and clinical evolution has been documented.[36] It is the most common of the amegakaryocytic thrombocytopenias, with more than 50 families having been reported. Thrombocytopenia is extremely severe only during the first years of life since it progressively improves, with the platelet count returning to within normal values in adulthood. As expected in amegakaryocytic thrombocytopenia, TPO was found to be elevated in the serum of TAR patients, who showed no *in vitro* reactivity of platelets to recombinant TPO, thus indicating abnormalities in the TPO/c-mpl signaling axis. However, the c-mpl receptor is expressed on the surface of platelets, and TPO and c-mpl genes do not carry mutations as demonstrated by linkage and mutational analysis.[37-39] Moreover, a putative implication of HOX genes was also excluded at least for HOXA10, HOXA11, and HOXD11.[40] Further analysis of signal transduction of the TPO-c-mpl system, as well as other pathways involved in platelet production and limb development, are all important in order to clarify the pathogenesis of this disease.

Other thrombocytopenias with normal platelet size

Once all the disorders listed above have been excluded, several patients with inherited thrombocytopenia and normal platelet volume remain without a definite diagnosis. Iolascon *et al.*[41] recently studied a large pedigree with seventeen individuals from an Italian family with an undefined form of thrombocytopenia. Platelet counts ranged from 31 to 109×10^9/L and showed normal aggregation tests and normal response to TPO. Bone marrow examination revealed normal megakaryocyte number with evident dysmegakaryocytopoietic findings, such as micromegakaryocytes and megakaryocytes with a single nucleus and/or a delayed cytoplasmic maturation. The gene responsible for thrombocytopenia was mapped on chromosome 10p12.1,[42] but the gene of this disorder (THC2) has not been cloned yet. The localization of THC2 has recently been confirmed by linkage analysis in a family from North America.[43] Not all the families with this form of thrombocytopenia have a defect mapping to 10p12.1, suggesting genetic heterogeneity with at least another gene responsible for normal platelet production (*Savoia, personal communication*).

Inherited thrombocytopenias with increased platelet size

Macrothrombocytopenias are the most frequent inherited forms and comprise a group of heterogeneous diseases characterized by a reduction of the number but an increase of the volume of platelets. Bernard-Soulier syndrome together with all its variants and MYH9-related diseases have been defined at the molecular level, the deficiencies having been identified in the platelet membrane complex formed by glycoproteins (GP) Ib/IX/V and the heavy chain of non-muscle myosin, respectively. Transcription factors regulating specific genes expressed in megakaryocytic lineage are also implicated in the pathogenesis of different forms, such as X-linked thrombocytopenia and dyserythropoiesis with or without anemia, Paris-Trousseau type thrombocytopenia and Jacobsen's syndrome. The molecular defects of other macrothrombocytopenias are unknown and their diagnosis requires the recognition of associated features, including a-granule deficiency (gray platelet syndrome),

platelet expression of glycophorin A, and spontaneous in vitro aggregation of platelets (Montreal platelet syndrome). All these disorders do not exhaust the chapter of inherited macrothrombocytopenias, because other forms with no or mild clinical symptoms, such as Mediterranean macrothrombocytopenia, have not yet been defined at clinical and molecular levels.

Bernard-Soulier syndrome (BSS)

Definition. In 1948 Bernard and Soulier[44] described a patient with a severe bleeding tendency, mild thrombocytopenia, and giant platelets in peripheral blood smears. At present, BSS is defined as a macrothrombocytopenia with quantitative and/or qualitative defects of the GPIb/IX/V complex of platelet membranes. Based on data from European, North American, and Japanese populations, the frequency of homozygous BSS has been estimated to be approximately 1 case in 1 million people,[45] and, according to the Hardy-Weinberg law, the frequency of heterozygotes is supposed to be 1 in 500.

Inheritance. BSS is classically described as a recessive disorder, and heterozygous subjects are expected to be asymptomatic carriers. However, a careful search in literature revealed that many heterozygous relatives of BSS patients with a mutation of either GPIbα, GPIbβ or GPIX had a moderate bleeding diathesis, mild macrothrombocytopenia and reduced amount of platelet GPIb/IX/V complex.[46] Moreover, a mild form of BSS transmitted as an autosomal dominant trait was reported in one Caucasian pedigree.[47] Furthermore, heterozygous missense mutations of GPIbα have been found in families affected by an autosomal dominant mild macrothrombocytopenia. After a genome wide search in Italian families that localized the defective gene on chromosome 17p, a heterozygous Val156Ala substitution (Bolzano mutation) of GPIbα was identified.[46] Bolzano patients showed a typical platelet GP profile defined by a reduced binding (about 50% of control) of antibodies against the epitope destroyed by the mutation. Even in GPIbβ, a heterozygous missense mutation was found to cause an isolated giant platelet thrombocytopenia.[48] In this case, the expression of the GPIb/IX/V complex was reduced and the amount of GPIbβ was 66% of the normal value. On the basis of these pieces of evidence, the classification of BSS as a recessive disease does not allow the recognition of heterozygous symptomatic patients, and BSS would be better defined as an autosomal dominant macrothrombocytopenia with incomplete penetrance, in which the rare homozygous patients have a more severely abnormal phenotype than do the heterozygous ones.

Pathogenesis. Both the thrombocytopenia and the GPIb/IX/V defect lead to a tendency to bleed. The GPIb/IX/V complex is composed of membrane leucine-rich motif glycoproteins, GPIbα, GPIbβ, GPIX, and GPV, with a stoichiometry of 2:2:2:1, respectively. GPIbs are disulfide linked subunits whereas GPIX binds non-covalently to GPIbβ. GPV also associates with the complex non-covalently (Figure 1). The genes for GPIbα and GPIbβ are on chromosome 17 and 22, respectively, and GPIX and GPV are both on chromosome 3.[45] GPIb/IX/V complex is essential for normal hemostasis. The first response to vascular injury is the adhesion of platelets to exposed subendothelium by the interaction of von Willebrand factor (vWF) with the GPIb/IX/V complex and collagen with GPIa/IIa. Adhesion to the vessel wall activates platelets that change shape, activate the fibrinogen receptor (the GPIIb/IIIa complex), and undergo the release reaction (release α and dense granule constituents). Upon activation, platelets synthesize and release thromboxane A2 and platelet activating factor, which are potent platelet aggregating agonists and vasoconstrictors. GPIb/IX/V-vWF binding must play a key role in promoting all these events, but its specific contribution is not yet fully understood. Although there are no evident GPIb/IX/V motifs known to interact with signaling proteins, proteins associated with GP Ib/IX/V are potential mediators.[56] GPIbα is linked to a network of short submembranous actin filaments through an actin-binding protein. Moreover, cytoplasmic motifs of both GPIbs interact with the ζ isoform of 14-3-3, which is likely to regulate the activity and assembly of key signaling molecules. Calmodulin, another protein of the cytoskeleton that binds cytoplasmic tails of GPIbβ and GPV, could also be involved in the process of platelet activation.

The extracellular domain of GPIbα interacts not only with vWF but also with thrombin.[45] The functional significance of the thrombin-GPIb interaction has not been established, since it does not directly activate platelets. However, some evidence suggests that thrombin bound to GPIb more efficiently activates one or more of the other thrombin receptors. As a matter of fact, platelet aggregation in response to low-dose thrombin is reduced in BSS.

The pivotal role of GPIb/IX/V in both platelet adhesion and platelet activation explains why BSS patients have a more severe tendency to bleed than

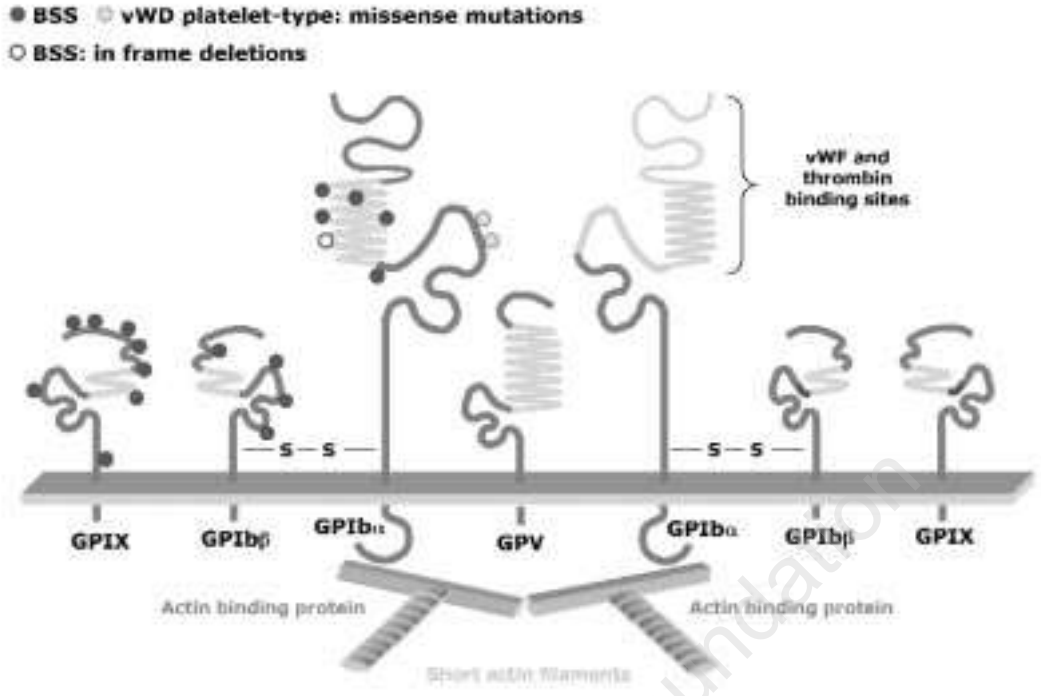

Figure 1. Mutations in the GPIb/IX/V complex. Schematic representation of the GPIb/IX/V complex, which is composed of 4 distinct transmembrane polypeptide subunits, GPIbα, GPIbβ, GPIV, and GPV with a stoichiometry of 2:2:2:1, respectively. At the N-terminus, each member contains a single or tandem leucine-rich repeat sequence (blue). GPIbα is disulfide linked to GPIbβ. The domains implicated in the binding of vWF and thrombin are also indicated (green). Nineteen different mutations have been identified in GPIbα. Most cause truncated proteins and do not allow the assembly of the complex on the platelet surface. There are missense mutations and one in-frame deletion compatible with the presence of the complex, which is however no longer able to bind vWF. Mutations of the GPIbα, Leu129Pro, Leu57Phe, Cys65Arg, Ala156Val (Bolzano variant), Leu 179 del (Nancy I variant), and Cys209Ser are located in the leucine-rich repeat.[45,49] Met230Val and Gly233Val are the mutations responsible for autosomal dominant PTvWD.[50,51] Of GPIbβ, 4 out of 6 are missense mutations, Arg17Cys, Pro74Arg, Tyr88Cys, and Ala108Pro, and one alters the GATA-1 binding site in the promoter region.[45,48,52] In patients with VCFS and BSS, one GPIbβ allele is deleted. All but one of the GPIX mutations are amino acid substitutions, Cys8Arg, Asp21Gly, Leu40Pro, Asn45Ser, Phe55Ser, Cys73Tyr, Cys97Tyr, Ala140Thr.[45,53-55] No mutation has so far been detected in the GPV gene.

would be expected on the basis of their platelet counts. On the other hand, it is not clear how the defect of GPIb/IX/V complex determines macrothrombocytopenia. Since a decreased density or function of GPIb/IX/V always correlates with platelet macrocytosis (*see below "X-linked thrombocytopenia and dyserythropoiesis with or without anemia' and 'MYH9-related disorders"*), it is tempting to speculate that the complex regulates normal platelet production and morphology, for instance, through the membrane cytoskeleton and actin filament binding. The evidence that the demarcation membrane system of megakaryocytes is similarly disordered and vacuolated in both BBS patients and a murine model in which the GPIbα gene was knocked out[57] seems to support this hypothesis.

Patients with classical BSS are homozygous or compound heterozygous for mutations in the GPIα, GPIbβ, or GPIX genes.[45,53-55,58,60-69] The BSS defects have been identified mainly as point mutations that produce premature stop signals and unstable polypeptides, but also as missense mutations affecting functional domains (Figure 1). In the majority of cases, the disorder is due to a quantitative defect of GPIb/IX/V, which is reduced or undetectable on the platelet surface. Because co-ordinated association of all four polypeptides after their synthesis and insertion into the membrane of the endoplasmic reticulum is required for the maintenance and stability of the complex, haploinsufficiency of either gene results in a decreased expression of the complex. However, in a few BSS patients the amount of platelet GPIb/IX/V complex is normal or only slightly reduced, but its function is severely defective. A typical example is the Bolzano variant of BSS. In this form, all the constituents of the GPIb/IX/V complex are expressed on the platelet surface, but a mutation affecting the leucine-rich repeat region

of GPIbα prevents its interaction with vWF. Interestingly, the frequency of the Bolzano allele in the Italian population is high, since it has been identified in 9 out of the 16 BSS alleles so far studied at the molecular level.[46,53,61,70] The effect of other amino acid substitutions in the GPIb and GPIX genes are briefly described in Figure 1 and extensively discussed by Lopez and co-authors.[45]

Clinical aspects. In *homozygous* BSS the bleeding tendency is usually evident from early childhood, but the severity of symptoms may change during puberty and adult life. Moreover, there is a considerable variability in symptoms among patients, even within a single family. Epistaxis is the most common symptom, with ecchymoses, menometrorrhagia, gingival hemorrhage, and gastrointestinal bleeding also being common. Most severe bleeding episodes are associated with surgery, dental extraction, menses, delivery, or accidents. Fatal hemorrhages are rare, although the majority of patients require transfusion at some time. As reported above, even heterozygous patients may show a mild to moderate bleeding tendency.

Laboratory features and diagnosis. In homozygous BSS the platelet count ranges from 10 to 280 $\times 10^9$/L,[45] indicating that thrombocytopenia is a variable feature of this condition. In contrast, platelet macrocytosis is always present, with more than one-third of platelets being larger than half a red cell and a few others larger than lymphocytes (Figure 2). Bone marrow examination has no diagnostic value. The bleeding time is often prolonged, with different levels of severity. Homozygous BSS platelets fail to aggregate *in vitro* in response to ristocetin or botrocetin, and also respond slowly to low doses of thrombin. This defect can be corrected in von Willebrand's disease, but not in BSS, by the addition of normal plasma. Platelet aggregation independent of the GPIb/IX/V-vWF interaction, such as that induced by collagen, ADP or epinephrine, is usually within the normal range. Flow cytometry and/or electrophoretic techniques are required to confirm the GPIb/IX/V defect. Variant-type BSS can be discriminated by conformational-dependent monoclonal antibodies against GPIb.[46]

In *heterozygous* BSS patients the platelet count ranges from very low (20×10^9/L) to normal values. *In vitro* platelet aggregation induced by ristocetin is usually normal because the residual amount of normal GPIb/IX/V complex is sufficient to support platelet-vWF interaction. However, the constant presence of a variable percentage of large platelets is a good hallmark of the condition (Figure 2, B). Flow cytometry of platelets is required to demonstrate the partial GPIb/IX/V defect: the absolute value of GPIb/IX/V per platelet may be within the normal range because of the increased volume of the platelets, but the GPIb/IX/V-GPIIb/IIIa ratio is always decreased to about half of normal values. As in homozygous forms, conformation-dependent monoclonal antibodies against GPIb are required to recognize heterozygous subjects with variant-type BSS.

Velocardiofacial syndrome (VCFS)

Macrothrombocytopenia is also a feature of velocardiofacial syndrome, a congenital disorder associated with hemizygous 22q11 deletions. The syndrome is characterized by cleft palate, cardiac anomalies, typical facies, and learning disabilities.[71] In most patients, there is no bleeding diathesis or only a mild one, and *in vitro* platelet function is normal. Since the 22q11 deletions include GPIbβ, one of the three genes defective in BSS, VCFS/DGS individuals are heterozygous BSS patients.[72] A few patients had a diminished response to ristocetin and suffered from serious hemorrhagic episodes, thus exhibiting a clinical spectrum compatible with a diagnosis of homozygous BSS.[50,73] In these cases, the haplo-insufficiency due to the cytogenetic abnormality is likely to have revealed a mutant BSS allele inherited from a parent. In a patient with BSS and a deletion in the VCFS chromosomal region 22q11.2, the GPIbβ protein was completely absent in platelets, suggesting that a mutation affected the undeleted allele of the GPIbβ gene.[73]

Platelet-type or pseudo von Willebrand's disease (PTvWD)

PTvWD is a rare autosomal dominant platelet disorder characterized by macrothrombocytopenia and a bleeding tendency with clinical and laboratory features similar to those of von Willebrand's disease (vWD) type 2B.[74] Two mutations (Gly233Val or Met239Val) in the carboxy terminal flanking sequence of the GPIbα leucine-rich repeats are implicated in this disorder.[51,75] Both amino acid substitutions are gain-of-function mutations that increase the affinity of GPIb/IX/V for vWF. As a consequence, spontaneous binding of circulating vWF to platelet occurs, and the deriving *in vivo* platelet clumping shortens platelet survival and induces thrombocytopenia. Moreover, circulating platelets with vWF already bound to their surface adhere less efficiently to subendothelial vWF when the vessel wall is damaged. Why most patients present variably enlarged platelets remains unknown. The bleeding time is often prolonged, and patients suffer from mild to moderate mucocutaneous hemor-

rhage. PTvWD can be diagnosed by laboratory findings that include enhanced platelet aggregation in response to ristocetin or botrocetin, variable spontaneous aggregation of platelets stirred in platelet-rich-plasma, and mild reduction of vWF levels in plasma, with a disproportionate reduction of the largest multimers. The same abnormalities are also present in patients with vWD type 2B, in which the increased affinity of vWF for platelets is due to mutations in the vWF gene. Several assays may differentiate between vWD type 2B and PTvWD: 1) normal vWF (or normal plasma) induces *in vitro* platelet agglutination in PTvWD, but not in vWD type 2B; 2) vWF (or plasma) from vWD type 2B patients lowers the amount of ristocetin necessary to induce platelet agglutination in platelet-rich plasma from healthy subjects, whereas vWF from PTvWD exerts the opposite effect; 3) washed PTvWD platelets maintain their functional abnormalities when resuspended in normal plasma, while similarly treated platelets from vWD type 2B subjects function normally.

Benign Mediterranean macrothrombocytopenia

Most of the *undefined* macrothrombocytopenias described in literature are characterized by autosomal dominant transmission and ineffective thrombopoiesis. In 1975, von Behrens[76] examined platelet count and MPV in 145 apparently healthy subjects from Italy and the Balkan peninsula. Since many of these subjects were affected by an undefined macrothrombocytopenia not observed in controls from North Europe, this condition was named *Mediterranean macrothrombocytopenia*. A retrospective study of thrombocytopenic patients undergoing platelet kinetic analysis isolated 54 cases with chronic macrothrombocytopenia of unknown origin.[77] The condition, characterized by mild or no clinical manifestations, normal bone marrow megakaryocytosis and platelet survival, and normal in vitro platelet function, was reported as *genetic thrombocytopenia with autosomal dominant transmission*. More recently, 47 similar patients (belonging to 13 families) have been reported as affected by *chronic isolated hereditary macrothrombocytopenia*.[78]

As reported above (*see Bernard-Soulier syndrome*), it has been recently shown that many patients with this poorly defined form of autosomal dominant macrothrombocytopenia had a defect of platelet GPIb/IX/V complex due to heterozygous mutations of the genes for GPIbα or GPIbβ, and had, therefore, to be classified as having heterozygous BSS.[46,48] However, other patients with the same defect had no mutations of GPIbα, GPIbβ, GPV, or GPIX,[79] (*Savoia, personal communication*), thus suggesting that the GPIb/IX/V content on platelet membrane depends on other factors that might be responsible also for the macrothrombocytopenia. Components of the cytoskeleton involved in the anchorage of GPIb/IX/V or proteins controlling the expression of megakaryocytic specific genes are plausible candidates. Consistent with this hypothesis, a reduction of GPIb/IX/V complex was observed both in patients affected by MHA and SBS, which are due to mutations of non-muscle myosin heavy chain IIA (NMMHC-IIA) (see MYH9-related disorders), and in a patient carrying a mutation in the hematopoietic transcription factor GATA-1.[80] Finally, there are forms of Mediterranean macrothrombocytopenia with a normal GPIb/IX/V complex, the pathogenesis of which remains to be clarified.[46]

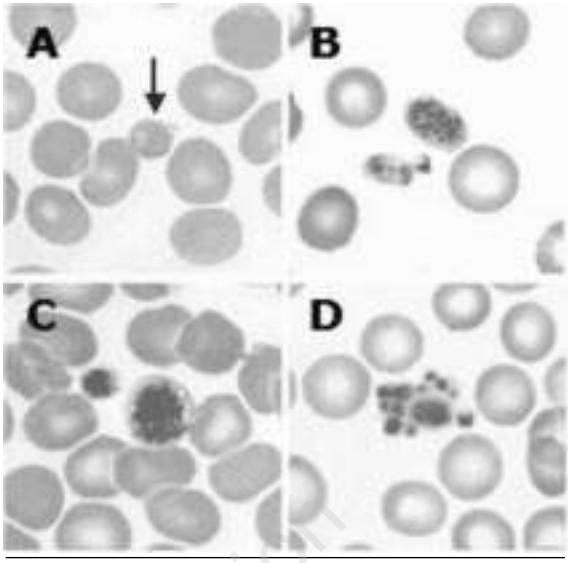

Figure 2. Morphologic examination of platelets on May-Grünwald-Giemsa stained peripheral blood films. The presence of large platelets that look gray due to the absence of granules (A) strongly suggests a diagnosis of gray platelet syndrome. Platelet anisocytosis with some platelets the same size as or larger than a red cell indicates homozygous BSS or MYH9-related disorders (B and C, respectively). In heterozygous BSS (D), as in other macrothrombocytopenias, platelets larger than half a red cell are observed among normal-sized platelets.

Macrothrombocytopenias with defetcs in hematopoietic transcription factors

Hematopoiesis is a complex process modulated by genes involved in differentiation, proliferation and apoptosis. Recent studies have indicated the impor-

tance of some transcription factors, such as GATA-1, FOG-1, Fli-1, and CBFA2 in regulating early and midway stages of thrombopoiesis.[81] The study of rare inherited thrombocytopenic syndromes in man and mice has provided insight into the molecular mechanisms regulating megakaryocyte maturation. As described above, patients with FDP/AML carry germline mutations of the CBFA2 gene. Instead, mutations in the GATA-1 gene have been found in families affected by X-linked thrombocytopenia and dyserythropoiesis with or without anemia.[80,82,83] GATA-1 is a member of the GATA family of zinc-finger proteins, which activate transcription by DNA binding in the *cis*-regulatory elements in specific lineages, including erythroid, megakaryocytic, eosinophilic and mast cells. GATA-1 contains two zinc fingers, one for sequence-specific direct DNA binding and the other for both stabilization of the DNA binding and interaction with a zinc finger protein, *Friend of GATA-1* (FOG1), which together with GATA-1 operates synergistically to regulate transcription during both erythroid and megakaryocytic cell differentiation. All characterized erythroid- and megakaryocytic-specific genes contain GATA motifs in critical *cis*-regulatory elements. Not all the patients with the inherited thrombocytopenias described below had platelet macrocytosis or were studied in this respect. However, most of them had larger than normal platelets and for this reason they are described in the section dedicated to macrothrombocytopenias.

X-linked thrombocytopenia and dyserythropoiesis with or without anemia (XLTT)

In 1977, Thompson *et al.*[84] described a family suffering from X-linked thrombocytopenia with thalassemia. Members of the family had splenomegaly and petechiae, a prolonged bleeding time due to platelet dysfunction, reticulocytosis and unbalanced (hemo)globin chain synthesis resembling that of β-thalassemia minor. Minor defects (reticulocytosis, globin synthesis imbalance) were found in some females. In this family, a R216Q mutation of the GATA-1 gene, localized on Xp11-12, caused a reduced interaction of the transcription factor to the DNA binding site.[85] Another four different missense mutations were then identified. Three substitutions lead to macrothrombocytopenia associated with either mild dyserythropoiesis (G208S and D218G) or anemia (D218Y).[80,83,86] Another family with X-linked dyserythropoietic anemia and thrombocytopenia carried a V205M mutation, which, as D218G, D218Y, and G208S, reduces the affinity between GATA-1 and FOG-1. Platelet size in these patients has not been reported, although severe abnormalities of platelet ultrastructure have been described.

A defective interaction between GATA-1 and FOG1, as well as between GATA-1 and DNA, dysregulates the transcription of genes containing the GATA motif on their promoter. Attempts to correlate genotype and phenotype revealed that R216Q is the only mutation associated with thalassemia and normal platelet morphology. At the molecular level this mutation is the only one that interferes with the DNA binding affinity of GATA-1. Mutations leading to a defective interaction with FOG-1 are associated with additional findings, such as macrothrombocytopenia. The different clinical and hematologic features depend strictly on defects of either GATA-1 interactions or the transcriptional GATA-1-FOG-1 complex.

Megakaryocyte-restricted specific genes are expected to be expressed at lower levels in patients with GATA-1 defects. As mentioned above an extremely low transcription of the GATA-1 target genes, such as GPIb and GPIX, was revealed in the family affected by macrothrombocytopenia with mild dyserythropoiesis.[80] Flow cytometric analysis of patients' platelets confirmed the existence of a platelet population with an abnormal size distribution and reduced GPIb complex levels. This report also showed the presence of very immature platelets lacking almost all the membrane glycoproteins studied (GPIbα, GPIbβ, GPIIIa, GPIX, and GPV). The patients' platelets showed weak ristocetin-induced agglutination, compatible with the disturbed GPIb complex. Accordingly, electron microscopy of the patients' platelets revealed giant platelets with cytoplasmic clusters consisting of smooth endoplasmic reticulum and abnormal membrane complexes.

Consistent with clinical and molecular findings in human pathology, GATA-/- mice develop thrombocytopenia and an increased number of megakaryocytes characterized by ultrastructural abnormalities. A significant proportion of megakaryocytes express markedly lower levels of lineage specific genes, including GPIbα, GPIbβ, platelet factor 4, c-mpl, and NF-E2,[87] supporting the critical role of GATA-1 in megakaryocytopoiesis.

Paris-Trousseau type of thrombocytopenia (TCPT) and Jacobsen's syndrome (JBS)

The Ets-family of transcription factors is implicated in a wide range of physiologic and pathologic processes. Two members, the proto-oncogenes Fli-1 and Ets-1, display distinct and/or overlapping

functions in angiogenesis and hematopoiesis. They map on the long arm of chromosome 11, where deletions with breakpoints in 11q23.3-11q24.2 are typical in patients with JBS and TCPT, two macrothrombocytopenic syndromes inherited in an autosomal dominant fashion. JBS is a contiguous gene disease characterized by thrombocytopenia, mental retardation and typical cardiac and facial anomalies.[88] Although TCPT deletions span those reported in JBS, TCPT patients show predominantly thrombocytopenia and have only a mild affected phenotype without major physical defects.[89,90] Bone marrow analysis revealed an increased number of megakaryocytes, including many micromegakaryocytes. On peripheral blood smears, 10% of platelets were larger than normal and 15% contained giant granules likely deriving from the fusion of α-granules. Platelet life span and *in vitro* aggregation were normal, although giant granules were unable to release their contents after thrombin stimulation. Recently, a JBS patient showed platelets typical of TCPT, suggesting that both syndromes are likely to be the variable expression of a single disorder.[91] Hemizygous loss of Fli-1 and/or Ets-1 might be responsible for dysmegakaryocytopoiesis as seen in mice, in which however the absence of Fli-1 is fatal because of vascular abnormalities.[92]

MYH9-related disorders

Definition. The term *MYH9-related disorders* indicates a group of autosomal dominant illnesses, known as May-Hegglin anomaly, and Sebastian, Fetchner and Epstein syndromes, caused by mutations of *MYH9*, the gene encoding for the heavy chain of non-muscle myosin IIA (NMMHC-IIA). Since the cloning of the gene, 20 different *MYH9* mutations (mostly missense but also one small in-frame deletion, one nonsense, and three frameshift mutations) have been observed in 65 unrelated families from all over the world.[93-98] At birth almost all affected subjects have platelet macrocytosis, thrombocytopenia and leukocyte inclusion bodies. In childhood or adult life, some of them also develop sensorineural hearing loss, cataracts and a glomerulonephritis that sometimes leads to end stage renal failure. Patients with macrothrombocytopenia and leukocyte inclusions only were in the past classified as having MHA or SBS, depending on subtle differences in the ultrastructure of inclusion bodies, while subjects with additional clinical findings, such as renal failure, deafness and cataracts were diagnosed as having FTNS or EPTS, on the basis of the presence or absence of leukocyte inclusions, respectively (Table 2). However, these criteria of classification are limiting since MHA-SBS and EPTS-SBS are often indistinguishable. As a matter of fact, careful clinical and laboratory investigations revealed microscopic hematuria, hearing loss and/or cataracts in many patients previously classified as having MHA-SBS, while they failed to detect kidney, hearing and visual abnormalities in some affected relatives of FTNS patients. Moreover, inclusion bodies have also been found in leukocytes of EPTS subjects (*Seri, personal communication*). Therefore, MHA, SBS, FTNS and EPTS do not represent distinct entities but rather a single disease with a heterogeneous clinical spectrum varying from a mild form with macrothrombocytopenia and leukocyte inclusions only to a severe form complicated by hearing loss, cataracts and/or microscopic hematuria, which can develop into severe renal failure. The new nosological entity *MHY9-related disorders* better interprets the recent knowledge in this field and identifies all patients at risk of developing renal, hearing or visual defects.

Table 2. Criteria previously used for the diagnosis of MHA, SBS, FTNS, and EPTS. Since this classification is unable to classify most of the patients, these disorders are now considered a single illness caused by various expressions of MYH9 mutations. Moreover, leukocyte inclusions are present in all patients, although in some of them they are small and difficult to identify.

	Macro-thrombocytopenia	*Hearing loss*	*Cataract*	*Renal defect*	*Leukocyte inclusions*[a]
MHA	Yes	No	No	No	Yes (type 1)
SBS	Yes	No	No	No	Yes (type 2)
FTNS	Yes	Yes	Yes	Yes	Yes (type 2)
EPTS	Yes	Yes	No	Yes	No

[a]*Ultrastructure of leukocyte inclusions: type 1, clusters of ribosomes aligned along parallel filaments; type 2, dispersed filaments and randomly distributed ribosomes.*

Pathogenesis. Non-muscle myosin IIA is a hexameric enzyme composed of two heavy chains and four light chains (Figure 3). Heavy chain dimerization yields a polar structure with two definite regions: at the N-terminus, the globular head with actin- and ATP-binding domains that is involved in motor activity, and at the C-terminus the alpha-helical coiled coil that plays a regulatory role.[99] All the MYH9 mutations so far identified (Figure 3) are expected to have a role in the correct assembly or stability of the quaternary myosin complex. Defects

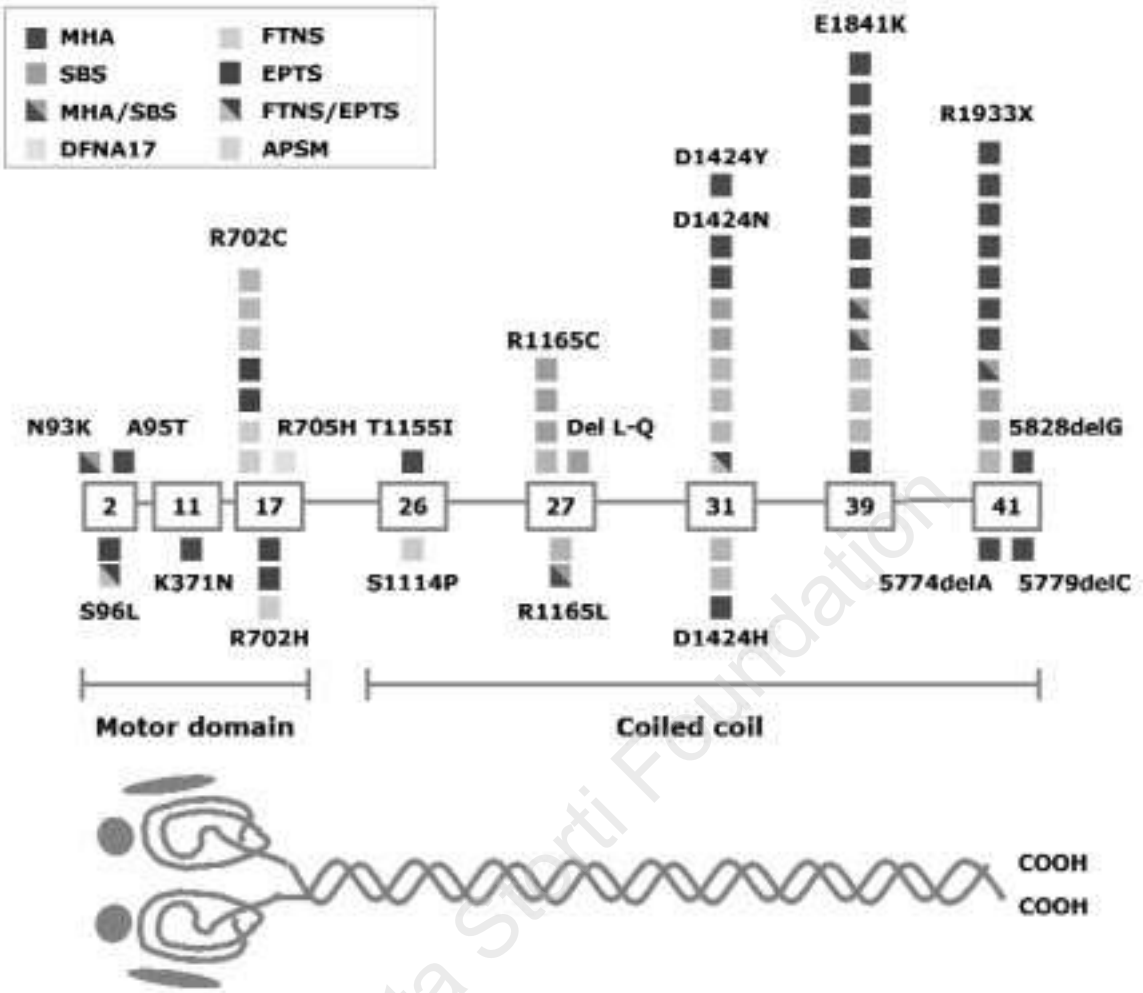

Figure 3. Mutations of the MYH9 gene. The genomic structure of MYH9 consists of 41 exons with the ATG of the open reading frame in exon 2 and the stop codon in exon 41. The gene encodes for the heavy chain of the non-muscle myosin of class IIA. Members of this class are hexameric enzymes composed of two identical heavy chains and two pairs of light chains. The myosin molecule consists of a long a-helical or tail domain and two separate globular domains. These two different regions mediate different functions: the tail is responsible for the spontaneous assembly of myosin molecules into thick filaments whereas the heads are involved in moving the molecules against adjacent actin filaments. Twenty different mutations have been identified in 65 families.[93-98] Each patient is indicated by a colored square based on his diagnosis of MHA, SBS, FTNS or EPTS. Differential diagnoses between MHA and SBS, as well as between FTNS and EPTS, were not always available so these are indicated with two-color symbols. The only non-syndromic DFN17 deafness family is also reported (yellow square). APSM patients have been reported as being affected autosomal dominant Alport syndrome without leukocyte inclusions, the clinical symptoms being macrothrombocytopenia, nephritis, deafness and cataracts.[91]

in the motor domain of NMMHC-IIA are more frequently associated with severe renal involvement, while abnormalities in the C-terminus of the coiled coil domain have been observed mainly in the families without renal failure. However, the same mutation has been found in patients with different clinical findings suggesting that the phenotype results from a complex interaction of altered MYH9 and modifying genes.

Thrombocytopenia derives from ineffective thrombopoiesis, since the amount of megakaryocytes and platelet survival are normal[100] and splenectomy does not improve the platelet count. Except for the increased size, platelet appearance is normal at both optical and ultrastructural microscopy. However, immunomorphologic analyses of platelets revealed that NMMHC-IIA was not uniformly distributed but that it was either absent

or clumped into a few spots. Moreover, α-tubulin was not organized in a circumferential band of microtubules at the cell periphery but was distributed unevenly. The basic defect of platelets in MYH9-related disorders is, therefore, related to a profound abnormality of the cytoskeleton. The bleeding tendency of patients with MYH9-related disorders is often more severe than would be expected on the basis of the platelet count, suggesting that a functional defect of platelets is coupled with the thrombocytopenia. *In vitro* platelet aggregation and release reactions are usually normal, but platelets fail to undergo shape-change, a process that requires correct functioning of NMMHC-IIA.[101] The recent observation of a significant reduction of GPIb/IX/V in the largest platelets[102] indicates that this defect could contribute to the bleeding diathesis.

Leukocyte inclusion bodies have been a puzzling feature since their first description by Hegglin in 1945.[103] In MYH9-related disorders they are observed in 25-75% of neutrophils, and much more rarely in eosinophils and monocytes. One cell usually contains one inclusion although up to three inclusions have been detected in a single neutrophil. These round or spindle-shaped inclusions of 2-7 μm in size are usually located in the cell periphery and appear sky-blue at May-Grünwald-Giemsa staining (Figure 4). Because of their similarity to Döhle bodies of infection, they have been named Döhle-like bodies. The inclusions, with no specific granules and limiting membranes, consist of ribosomes and microfilaments 7-10 nm in diameter. Two ultrastructural patterns of Döhle-like bodies have been described (Figure 5); there are clusters of ribosomes aligned along parallel filaments, as seen in MHA, or randomly distributed ribosomes within highly dispersed filaments typical of SBS and FTNS. In patients, NMMHC-IIA is clustered within Döhle-like bodies (Figure 3) whereas it is uniformly distributed in normal cells.[104] Moreover, specific antibodies recognized an abnormal clustered distribution of NMMHC-IIA also in those patients with apparently no inclusions on May-Grünwald-Giemsa stained blood smears (EPS). Ultrastructural immunocytochemistry showed that these microfilaments of Döhle-like bodies contain NMMHC-IIA, thus confirming that *MYH9* mutations are responsible for the pathologic formation of the inclusions.[105]

The pathogenesis of *renal failure* in patients with MYH9 mutations is being clarified. In patients with end-stage renal failure, NMMHC-IIA was abnormally distributed in both mesangial and tubular cells, and podocytes revealed focal and segmental fusion with no interpodocyte slit diaphragm.[105] Podocyte foot processes have a contractile structure composed of actin, myosin and other proteins, which modulate ultrafiltration in response to different factors and stresses. Therefore it is likely that anomalies of the podocyte cytoskeleton damage the glomerular filtration barrier leading to hematuria and, in the most severe cases, to renal failure.

The pathogenesis of the *hearing loss* and *cataracts* remains obscure because it is difficult to predict the role of NMMHC-IIA in the ear and eye, although it is likely to be correlated to the contractile role of the actin-myosin complex.

Clinical aspects. Bleeding diathesis, high tone hearing loss, renal involvement and cataracts are, in descending order of frequency, the clinical features of *MYH9*-related diseases. The bleeding diathesis is usually mild, although a few patients present life-threatening hemorrhages and others have no bleeding tendency. In symptomatic patients, bleeding first occurs during infancy and its severity does not change during life. Easy bruising, prolonged menstrual periods and epistaxis are the most common complaints. Kidney, hearing and visual defects may appear early in the infancy or later in adult life. Renal involvement ranges from microscopic hematuria to end stage renal failure.

Laboratory features and diagnosis. The only constant feature of patients with *MYH9*-related disorders is platelet macrocytosis (Figure 2). On blood smears, 5-40% of platelets are larger than red cells. Most patients have mild to severe thrombocytopenia, but a few affected subjects have normal platelet counts. As discussed below, electronic counters underestimate platelet count and mean platelet volume. In almost all patients, careful examination allows the identification of Döhle-like bodies in 25-75% of neutrophils. Immunocytochemistry with anti-NMMHC-IIA antibodies is more sensitive, showing a spotty myosin distribution in all neutrophils of all patients.[104] Initial signs of glomerulonephritis are microscopic hematuria and proteinuria. Hearing tests and eye examinations may discover hearing loss and cataracts before they become symptomatic.

Gray platelet syndrome (GPS)

Definition. GPS is a rare inherited thrombocytopenia in which the platelets are large and have a decreased α-granule content.[106] Among the 50 cases described so far, there are families with autosomal dominant and recessive transmission and also sporadic cases.[107-111]

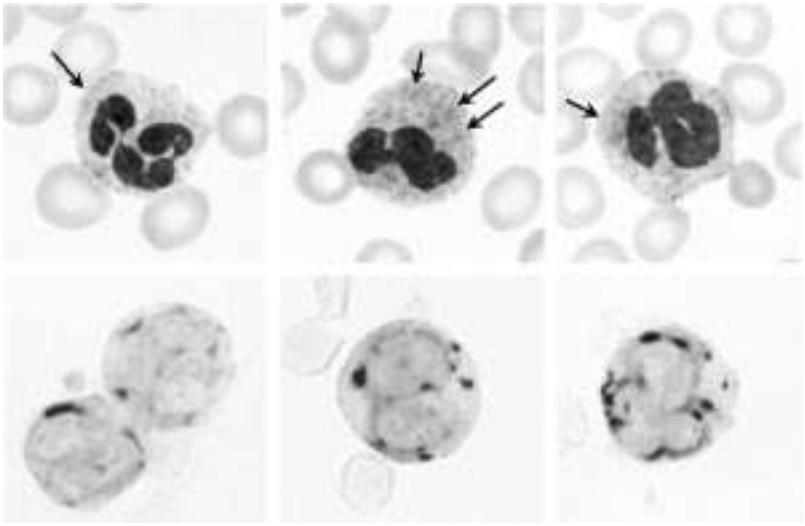

Figure 4. Döhle-like bodies in polymorphonuclear leukocytes are a distinguishing feature of MYH9-related disorders. On MGG stained peripheral blood smears (upper line) they appear as faint, light-blue, round or spindle-shaped amorphous inclusions located at the cell periphery or in the inner cytoplasm. Their identification requires experience. Immunocytochemisty (or immunofluorescence) with an antibody against NMMHC-IIA (lower line) shows that myosin is clustered within leukocyte inclusions (red spots) instead of being uniformly distributed throughout the entire cytoplasm. Immunocytochemistry is more sensitive than May-Grünwald-Giemsa staining because it also identifies very small inclusions.

Pathogenesis. GPS platelets appear gray on May-Grünwald-Giemsa stained blood films because of abnormalities of the α-granules (Figure 2), which are limited by a normal membrane but do not contain any components.[112] Although the gene responsible for GPS is unknown, the basic defect is the inability of megakaryocytes to pack endogenously synthesized secretory proteins into developing α-granules. These molecules (including growth factors) are misdirected into the lumen of the demarcation membrane system and then secreted into the extracellular space of bone marrow,[107] where they are responsible for the development of myelofibrosis.[113, 114] Since platelets do not release their hemostatic proteins, such as fibrinogen, vWF, thrombospondin and factor V, at the site of a vascular injury this defect probably contributes to the bleeding tendency. The cause of thrombocytopenia in GPS is the subject of debate, because it is controversial whether platelet survival is normal or reduced and whether splenectomy ameliorates the platelet count.[106-109,115] At least in some families, the granule defect is not confined only to platelets, but also involves polymorphonuclear neutrophils that have a reduction of secretory vesicles and secondary granules.[107] An animal model of GPS, the Wistar Furth rat, has been identified. In this animal macrothrombocytopenia and α-granule defect seem to derive from an abnormal organization of the cytoskeleton, which also hampers platelet adhesion and spreading. However, the primary molecular defect in Wistar Furth rats, as in GPS patients, is unknown.[116]

Clinical aspects. Most patients with GPS have a prolonged bleeding time and a bleeding diathesis that ranges from mild to severe, although no fatal bleeds have been reported. Hemorrhages may occur even in subjects with a nearly normal platelet count. A mild reticular fibrosis is generally observed in the bone marrow, but this does not appear to be progressive or to induce anemia.

Laboratory features and diagnosis. Platelets are not easily detectable on blood films because of their pale, ghost-like appearance. Although GPS is classified as a macrothrombocytopenia, platelet anisocytosis is present with small, normal-sized and large platelets. Several abnormalities of in vitro platelet aggregation have been reported, including an impaired response to thrombin.[117] Defective high-molecular-weight multimers of vWF and extensive emperipolesis have been described in one family.[118] GPS may be suspected on the basis of the characteristic morphologic abnormalities of platelets and confirmed by analysis of the proteins normally stored within the alpha granules using different approaches, such as Western blot and immunologic assays.

Montreal platelet syndrome (MPS)

The peculiar feature of Montreal platelet syndrome is spontaneous *in vitro* platelet aggregation. After the first description in 1963 of one pedigree with autosomal dominant transmission,[119] only one other family has been reported.[120-122] The bleeding time was prolonged and patients had a tendency to bruise and episodes of hemorrhage. Platelet counts were severely reduced (5-40×10^9/L) and platelet size increased

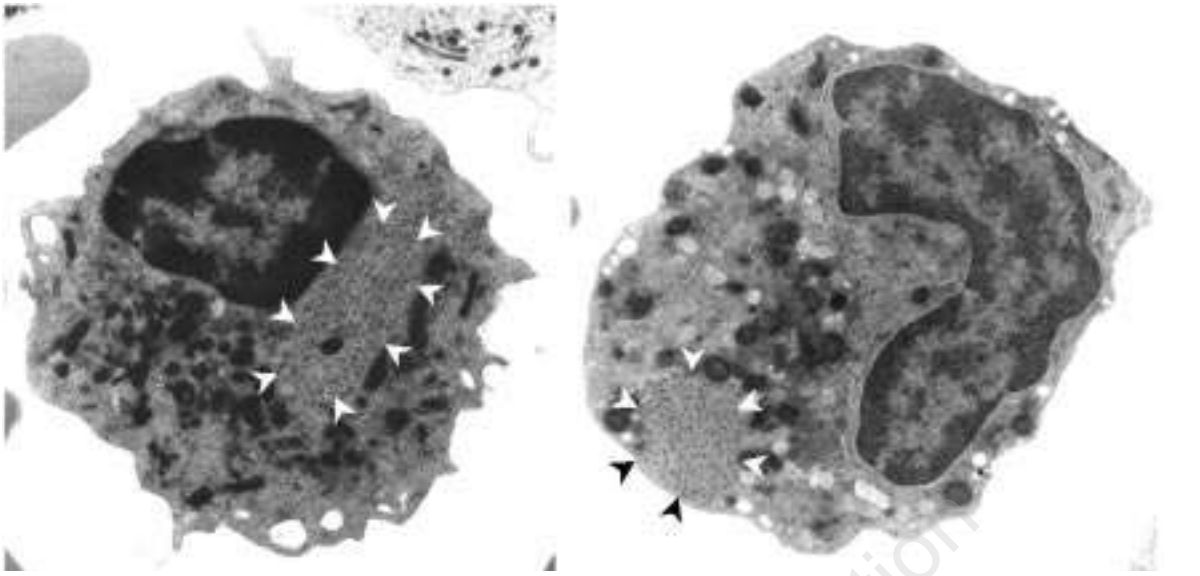

Figure 5. Ultrastructural features of Döhle-like bodies in MYH9-related disorders. In both the neutrophils, the leukocyte inclusion appears as an area free of specific granules (arrows) containing clusters of ribosomes. Ribosomes may be oriented along the axis of thin filaments (left panel) or randomly distributed (right panel). ***Courtesy of U. Magrini, Anatomic Pathology Department, IRCCS Policlinico San Matteo, University of Pavia, Italy.***

(median platelet diameter 3 mm). No ultrastructural abnormalities of platelets have been identified. Spontaneous aggregation occurs in anticoagulated whole blood, platelet-rich plasma and buffer solutions without calcium and fibrinogen. Platelet aggregation is further increased by either stirring or addition of ADP, epinephrine, collagen, arachidonic acid, ionophore A-23187 or ristocetin. Thrombin, in contrast, does not provide any evident reaction. A partial defect of calcium-activated neutral protease (calpain) has been identified.[121] This enzyme is involved in the cleavage of the cytoskeleton proteins, such as actin-binding protein and talin, suggesting that its deficiency may interfere with the expression of platelet binding sites for adhesive proteins. The pathogenesis of the platelet macrocytosis, as well as that of the thrombocytopenia, is unclear.

Hereditary macrothrombocytopenia with platelet expression of glycophorin A

This autosomal dominant disorder has been described in 13 members (three generations) of a single family with a mild bleeding tendency.[123] In 8 patients the macrothrombocytopenia was associated with a high-frequency hearing loss. Platelet counts varied between 50 and 120×10^9/L, and 30-40% of platelets were larger than 4 mm. Except for macrocytosis, platelet morphology was normal and leukocytes did not contain inclusions. No significant defect of in vitro platelet aggregation was observed. Flow cytometry revealed, as a differential feature, the presence of glycophorin A on the surface of large platelets. Although glycophorin A is an erythroid-specific protein, it has been described on several megakaryocytic leukemia cell lines, suggesting that its expression might be correlated to impaired megakaryocytopoiesis and release of immature thrombocytes.

Diagnostic difficulties

Differentiation between inherited and acquired thrombocytopenias

The recognition of the hereditary nature of a thrombocytopenia is often hampered by several difficulties. Particularly in the mild inherited forms, thrombocytopenia may be discovered incidentally late in infancy or in adult life, and other affected relatives may have normal or nearly normal platelet counts. Moreover, automated counters underestimate platelet counts in the case of platelet macrocytosis (*see below*). As a consequence, a mistaken diagnosis of severe idiopathic thrombocytopenic purpura is often made with the risk of patients being given undue immunosuppressive therapy or being subjected to splenectomy, as has been reported to have occurred in several cases.

To avoid this error, careful investigation of each patient and his relatives is essential, especially in those cases with no record of previously normal

platelet counts. Morphologic examination of peripheral blood smears is the most informative single examination because it can reveal qualitative platelet abnormalities, such as macrocytosis, in patients and other family members indicating an inherited disorder. The presence of inclusions in leukocyte cytoplasm is a hallmark of inherited *MYH9*-related disorders and therefore Döhle-like bodies should always be checked for in these cases.

Pitfalls of automated platelet counting in inherited thrombocytopenias

Present electronic counters measure platelets by enumerating particles within a specified volume window (e.g., 2-20 fL). On this basis, very large platelets, such as those of *MYH9*-related disorders or BSS, are not recognized and platelet count is underestimated. The larger the abnormality of platelet volume, the bigger the counter inaccuracy. In 8 of 15 patients with MHA, the counter estimated a platelet count 90% lower than the real value.[124] Platelet counters are, therefore, completely unreliable in subjects with inherited macrothrombocytopenias.

Cell counters that identify platelets bound to specific antibodies solve this problem. However these instruments are not available in most laboratories, and old methods based on microscopic observations of the whole blood are recommended to obtain a reliable count.[125,126] Due to the inability to recognize large platelets, counters also underestimate mean platelet volume. In the cited MHA patients, the mean platelet volume was 12.1±1.7 fL, but blood smears revealed that more than 50% of platelets were larger than half a red cell.[124]

Therapy

General measures

The most important aspect of management of inherited thrombocytopenias is to anticipate risks and to prevent bleeding. A major measure is to instruct patients to avoid drugs that impair platelet function (above all, aspirin). Regular dental care is essential to prevent gingival bleeding. Patients should be prepared for surgery and invasive procedures with platelet transfusions, desmopressin or antifibrinolytic drugs, according to the individual's bleeding tendency. Oral contraceptives should be used to prevent menorrhagia. When local bleeding occurs, it can often be treated by local measures, such as nasal packing in the case of epistaxis.

Platelet transfusions

Prophylactic platelet transfusions could seem the most appropriate measure for prevention of hemorrhages in inherited thrombocytopenias. However, in most cases the benefit of increasing the platelet count does not outweigh the risk deriving from exposure to allogeneic, manipulated and stored blood products. In fact, the thrombocytopenia and bleeding tendency are usually mild, and life-threatening bleeding episodes rarely occur even when the platelet count is very low. On the other hand, platelet transfusions are often responsible for transmission of infectious diseases, febrile reactions or development of alloimmunization and subsequent refractoriness to platelet infusions. Although alloimmunization is significantly reduced by leukodepletion of platelet concentrates, it still occurs in 10% of cases in the clinical setting of hematologic malignancies.[127] No study has been performed in inherited thrombocytopenias, but the rate of alloimmunization is expected be higher because these patients, differently from those with leukemia or lymphoma, do not receive immunosuppressive treatments.

The risk of *alloimmunization* (better defined in this case as isoimmunization) is even higher in the subjects with homozygous BSS and no GPIb/IX/V complex because they recognize these proteins in transfused platelets as foreign. On this basis, patients with inherited thrombocytopenias should receive platelet transfusions only to treat an active hemorrhage that cannot be otherwise managed and as prophylaxis prior to surgery or other major hemostatic stresses. When available, platelets from HLA-matched donors should be used to prevent or overcome alloimmunization.

A platelet count over 50×10^9/L is usually considered safe for the majority of surgical procedures, but platelet count is not a good reference parameter when a functional defect of platelets is also present. In these cases, the hemorrhagic risk evaluation relies on clinical history and the results of in vivo and *in vitro* tests of platelet function.

Desmopressin

Desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP) is a synthetic analog of the antidiuretic hormone, vasopressin, that was initially designed for the treatment of diabetes insipidus. When administered to healthy subjects or patients with mild hemophilia or vWD, DDAVP increases factor VIII and vWF transiently by releasing these molecules from storage sites into blood. Due to this property, desmopressin is currently used for the treatment of mild hemophilia A and vWD.[128] More recently, the clinical indications for DDAVP have been expanded to include congenital defects of

platelets. DDAVP has been reported to shorten bleeding time in patients with BSS, MHA and GPS.[53,124,129,130] The greatest experience has been gained in homozygous BSS. Among 14 patients treated with this drug, 8 showed a good response (normalization or halving of bleeding time), 4 had a minor response and two had no benefit.[53] After a dose of 0.3 mg per kg body weight, the peak response usually occurs within 60 min post-infusion. Since the bleeding time is not shortened in all patients but the response in each patient is constant on different occasions, a test dose is recommended in order to select, in advance, those patients who will benefit from this treatment during future bleeding episodes or as prevention of bleeding at the time of invasive procedures.

The reason why DDAVP ameliorates primary hemostasis in platelet disorders is still a matter of debate.[129]

DDAVP infusion may induce mild tachycardia, headache, and flushing. These symptoms derive from the vasodilatory effects of the drug and can be attenuated by slowing the rate of infusion. Due to its antidiuretic effect, DDAVP seldom induces hyponatremia and volume overload: fluid intake should, therefore, be restricted during treatment. Since myocardial infarction and stroke have been described in a few treated patients with hemophilia[131] and uremia,[132] this drug should be used with caution in elderly patients with cardiovascular disease. The pro-thrombotic effect is likely to be related to the release of ultralarge vWF multimers from the endothelial cells into the circulation. These multimers aggregate platelets directly in conditions of high shear stress, such as those occurring in stenotic arteries.[133]

DDAVP is contraindicated in PTvWD because the release of large VWF multimers from endothelial cells could induce *in vivo* agglutination of platelets and worsen the thrombocytopenia.

Activated factor VII

Activated factor VII (FVIIa) is hemostatically effective in the treatment of bleeding in hemophilic patients with inhibitors. More recently, a reduction in bleeding times has also been obtained in a few thrombocytopenic patients, as well as in patients with BSS, Glanzmann's thrombasthenia or acquired platelet dysfunction.[134] *In vitro* studies indicate that FVIIa increases the initial thrombin level, leading to faster platelet activation and thereby compensating for the thrombocytopenia.[135] Adverse reactions have been reported, such as the myocardial infarctions seen in hemophilia patients.[136] On this basis, FVIIa should be still regarded as an experimental drug in inherited thrombocytopenias.

Hematopoietic stem cell transplantation and gene therapy

Allogeneic hematopoietic stem cell transplantation is, in theory, an appealing therapy for inherited thrombocytopenias to restore normal megakaryocytopoiesis. However, in most cases the risk of such a procedure is still higher than that deriving from the bleeding tendency, and therefore little experience has been gained in this field. The only exception is the 134 transplants carried out in patients with WAS.[9] The procedure was successful in 80% of patients below the age of 5 years, but in < 50% above the age of 5 years old. In patients less than 5 years old there was no significant difference in survival between sibling and unrelated donor procedures. However, older patients have not fared well with unrelated transplants. Hematopoietic stem cell transplantation also cured 6 children affected by Glanzmann's thrombasthenia, (*Locatelli, personal communication*)[137-139] and two with BSS (*Locatelli, personal communication*) who had several life-threatening bleeding episodes. In all cases, i.e. the 7 transplanted from an HLA-identical sibling and the one from an HLA-matched unrelated donor, complete engraftment occurred and bleeding symptoms disappeared. Based on these results, transplantation should be considered in severe inherited platelet disorders, such as the amegakaryocytic forms, and when patients develop anti-platelet antibodies as a result of repeated transfusions.

Gene therapy could be an alternative to allogeneic stem cell transplantation, particularly in patients lacking an HLA-matched donor. It offers the hope of an ultimate cure for inherited disorders and mainly for those that affect the hematopoietic system, the possibility of harvesting and correcting autologous stem cells *in vitro* being attractive. However, the potential of this approach has yet to be demonstrated. Improvements in the efficiency of gene transfer and *in vivo* expansion of the corrected cells, as well as more information on biological functions of the defective gene, will be determinant for its success.

Splenectomy

Splenectomy has no effect in inherited thrombocytopenias except in WAS. In a case series of 39 WAS patients, splenectomy induced normalization of platelet count in almost all cases, although it increased the risk of infection. The median survival of splenectomized patients was 25 years, compared

with less than 5 years in unsplenectomized ones.[140] Splenectomy and daily prophylactic antibiotics are, therefore, indicated in those boys without a matched donor for hematopoietic stem cell transplantation. Splenectomy has also been performed in a few patients with BSS, GPS and *MYH9*-related disorders who were previously misdiagnosed as having idiopathic thrombocytopenic purpura. Although in some patients the platelet count increased soon after surgery, in the majority the amelioration was only transient.[53, 106, 115]

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