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Inherited thrombocytopenias: history, advances and perspectives

Alan T. Nurden and Paquita Nurden

Institut Hospitalo-Universitaire LIRYC, Pessac, France

ABSTRACT

Over the last 100 years the role of platelets in hemostatic events and their production by megakaryocytes have gradually been defined. Progressively, thrombocytopenia was recognized as a cause of bleeding, first through an acquired immune disorder; then, since 1948, when Bernard-Soulier syndrome was first described, inherited thrombocytopenia became a fascinating example of Mendelian disease. The platelet count is often severely decreased and platelet size variable; associated platelet function defects frequently aggravate bleeding. Macrothrombocytopenia with variable proportions of enlarged platelets is common. The number of circulating platelets will depend on platelet production, consumption and lifespan. The bulk of macrothrombocytopenias arise from defects in megakaryopoiesis with causal variants in transcription factor genes giving rise to altered stem cell differentiation and changes in early megakaryocyte development and maturation. Genes encoding surface receptors, cytoskeletal and signaling proteins also feature prominently and Sanger sequencing associated with careful phenotyping has allowed their early classification. It quickly became apparent that many inherited thrombocytopenias are syndromic while others are linked to an increased risk of hematologic malignancies. In the last decade, the application of next-generation sequencing, including whole exome sequencing, and the use of gene platforms for rapid testing have greatly accelerated the discovery of causal genes and extended the list of variants in more common disorders. Genes linked to an increased platelet turnover and apoptosis have also been identified. The current challenges are now to use next-generation sequencing in first-step screening and to define bleeding risk and treatment better.

Correspondence:

ALAN NURDEN
nurdenat@gmail.com

History

In the late 19th century improvements to the light microscope led to anucleate platelets being visualized in great numbers in human blood. Early pioneers in the field of platelet research included the Canadian William Osler, a Paris hematologist, George Hayem, who performed the first accurate platelet count, and the Italian Giulio Bizzozero.[1] In 1906, James Homer Wright confirmed that platelets were produced by bone marrow megakaryocytes.[2] When, in 1951, Harrington *et al.*[3] observed purpura in a child of a mother with immune thrombocytopenic purpura, a maternal factor was said to be destroying the platelets. Anti-platelet antibodies were identified as the cause and platelet transfusions, immunosuppressive therapy and splenectomy became standard treatments. Acquired thrombocytopenia, often with defective platelet function, has many causes. For example, it may be an immune response linked to blood transfusions and drugs, a direct consequence of viral or bacterial infections, be the result of other hematologic disorders or be secondary to many major illnesses. However, some thrombocytopenias are inherited and Professors Jean Bernard and Jean-Pierre Soulier in Paris were pioneers in this domain, describing in 1948 what they called in French *"dystrophie thrombocytaire hémorragipare congénitale"*, later re-named the Bernard-Soulier syndrome (BSS).[4] Strikingly, many platelets were enlarged and some were giant. A key to the molecular defect was the platelet deficit of sialic acid, a negatively charged monosaccharide terminating many of the oligosaccharides of platelet glycoproteins (GP) and

some glycolipids.[5] The discovery that a major component of the platelet surface, first identified as "GPI", was absent from platelets in BSS began the long road to defining the molecular landscape of inherited thrombocytopenias.[6] This centenary review will take a concise look at this journey emphasizing major steps in the current understanding of these conditions and highlighting recent advances in what is an intriguing subset of Mendelian diseases.

Platelet production and lifespan

The platelet count is the result of a balance between the biogenesis, senescence and consumption of these cells. Briefly, CD34+ hematopoietic stem cells (HSC) first give rise to megakaryocyte-erythrocyte progenitors, a proportion of which will develop into colonies that mature by endomitosis to polylobulated megakaryocytes with a high DNA content (Figure 1). Megakaryocyte differentiation is regulated by transcription factors as well as by interactions with chemokines, cytokines and constituents of the extracellular matrix.[7] Thrombopoietin, acting through its receptor Mpl, is a key factor.[8] Mostly produced in the liver, thrombopoietin levels in blood are governed by the platelet number; feedback mechanisms stimulate HSC proliferation and megakaryopoiesis. The demarcation

membrane system of mature megakaryocytes is a membrane reservoir for platelet biogenesis. Migrating to the endothelial cell sinusoid barrier, mature megakaryocytes extend long, branched, protrusions, termed proplatelets, into the blood where under flow they release large numbers of platelets from their ends.[9,10] This whole process is highly dependent on cytoskeletal proteins including myosin IIA, actin filaments and the tubulins that provide tracks for the transport of organelles with a Cdc42/RhoA regulatory circuit guiding transendothelial platelet biogenesis.[11] Type I collagen in the extracellular matrix is a negative regulator, preventing premature proplatelet formation. Early reports also insisted that megakaryocytes can migrate directly into the blood and accumulate in the lungs. Using two-photon microscopy, *in vivo* lineage tracing technologies and a series of lung transplants in mouse models, Lefrancais *et al.*[12] have confirmed that the lung is indeed a site for platelet biogenesis. In conditions of acute need, direct megakaryocyte rupture is an alternative pathway for platelet production.[13]

If unused in hemostatic events or pathological processes, human platelets circulate for 7-10 days (Figure 2). Platelet clearance due to senescence occurs in the liver. Loss of sialic acid through the action of neuraminidase during platelet aging exposes galactose or N-acetyl-galactosamine, residues that mediate platelet binding to the
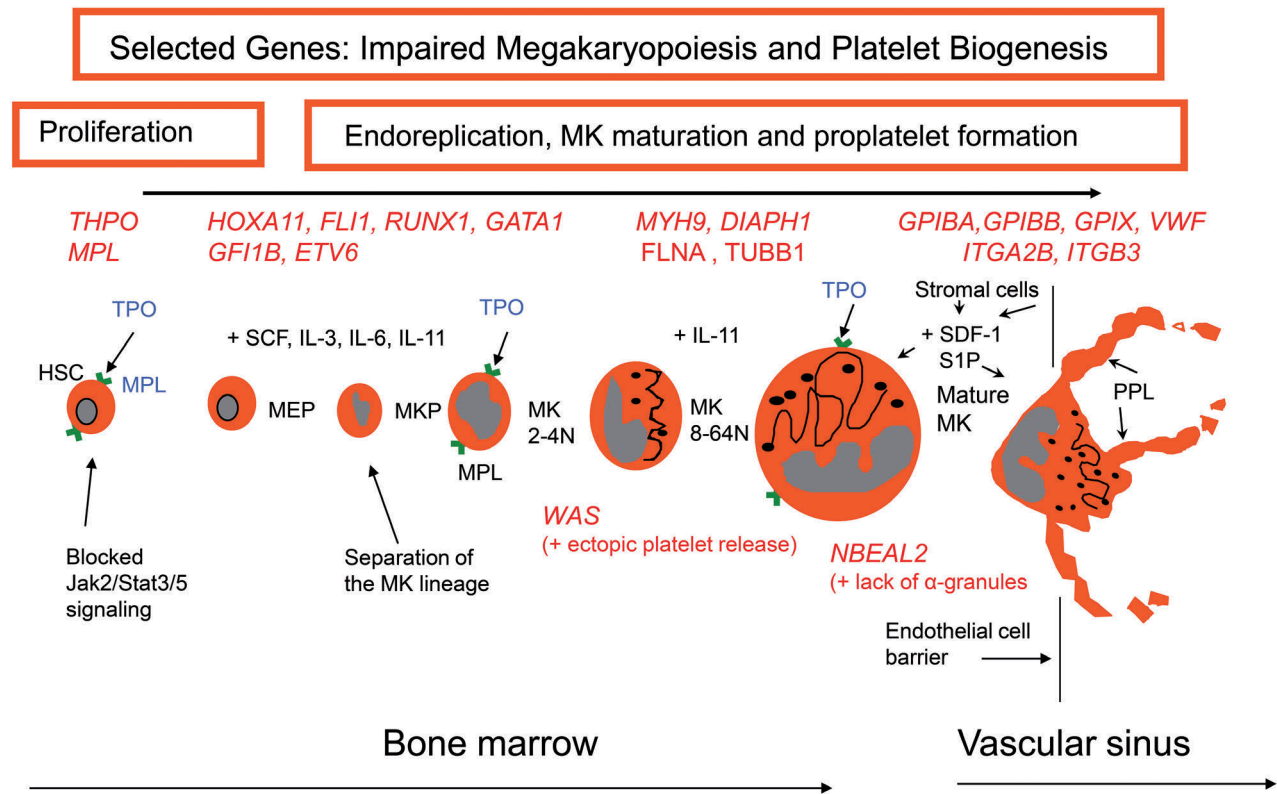

Figure 1. A schema of the major steps of megakaryopoiesis highlighting how inherited defects of selected genes cause thrombocytopenia. Megakaryocytes arise from hematopoietic stem cells that proliferate to first form megakaryocyte-erythrocyte progenitors, a proportion of which give rise to colony-forming megakaryocyte progenitors. These events require the interaction of thrombopoietin with its receptor and are under the influence of various growth factors (e.g. stem cell factor, cytokines and interleukins). The developing megakaryocytes undergo endomitosis to increase chromosome number; they then mature before migrating to the vascular sinus where they extend proplatelets or migrate themselves across the endothelial cell barrier into the vascular sinus. This latter step is under the influence of many factors including stromal derived growth factor-1 and sphingosine-1-phosphate. Selected genes with variants causing inherited thrombocytopenias are shown in red. HSC: hematopoietic stem cells; TPO: thrombopoietin; MPL: thrombopoietin receptor; MEP: megakaryocyte-erythrocyte progenitor; SCF: stem cell factor; IL: interleukin; MKP: megakaryocyte progenitor, MK: megakaryocyte; SDF-1: stromal derived growth factor-1; S1P: sphingosine-1-phosphate; PPL: proplatelet.

hepatocyte Ashwell-Morell receptor, an act that stimulates the synthesis of thrombopoietin.[14] Subsequent studies using a mouse model additionally pointed to clearance by Kupffer cells in the liver sinusoids; here N-acetyl-galactosamine was recognized by the lectin, CLEC4A.[15] State-of-the-art microscopy then pinpointed how Küpffer cells also recognized β-galactose on aged mouse or human platelets with a galactose-binding lectin mediating the phagocytosis.[16] Most importantly, Küpffer cells were shown to continually scan circulating platelets with touch-and-go interactions as these latter pass through sinusoidal endothelial fenestrae.

In megakaryocytes, the role of apoptosis during pro-platelet formation and platelet biogenesis is controversial and largely unproven although it may intervene after platelet release or during stress situations such as infections and inflammation.[17] In a classic paper, the pro-survival protein, Bcl-X_L, was proposed to constrain the pro-death Bak in platelets; the balance acting as a molecular clock for platelet survival.[18] Others have shown how apoptotic pathways and necrosis engage pro-death proteins triggering mitochondrial membrane permeabilization, cytochrome C release and caspase activation leading to Ca^{2+}-dependent exposure of phosphatidylserine, which is not only recognized by phagocytic cells but also leads to procoagulant activity.[19]

The following sections of this review will describe familial defects of platelet production and megakaryopoiesis; affected genes will be shown to interfere with HSC proliferation, megakaryocyte maturation and migration and the alteration of platelet lifespan. Many are accompanied by an increased platelet size (Figure 2) while others have a normal platelet size or even small platelets (Figure 3). Platelet function is variably affected. While some thrombocytopenias are isolated, many are syndromic and/or associated with other conditions that may be of major clinical importance. Bleeding is mostly muco-cutaneous, severe in some disorders, but infrequent or even absent in others, particularly when the decrease in platelet count is modest. A summary of the genes involved and the principle characteristics of each disorder are presented in *Online Supplementary Table S1*.

Classic inherited thrombocytopenias

We begin with a series of named inherited thrombocytopenias whose characterization has marked the history of inherited platelet disorders. In many of these syndromes, thrombocytopenia, defined as a platelet count <150x10⁹/L, is accompanied by platelet function defects that aggravate bleeding. In early studies genotyping mostly involved candidate gene sequencing and linkage studies.

Bernard-Soulier syndrome

The "GPI" lesion in BSS platelets was quickly shown to involve a complex of four distinct subunits each encoded by a distinct gene. The large, heavily glycosylated GPIbα is attached by a disulfide to the small GPIbβ, while GPIX and GPV are non-covalently associated, all in a 2:2:2:1 stoichiometry. Bleeding in BSS patients is disproportionate to

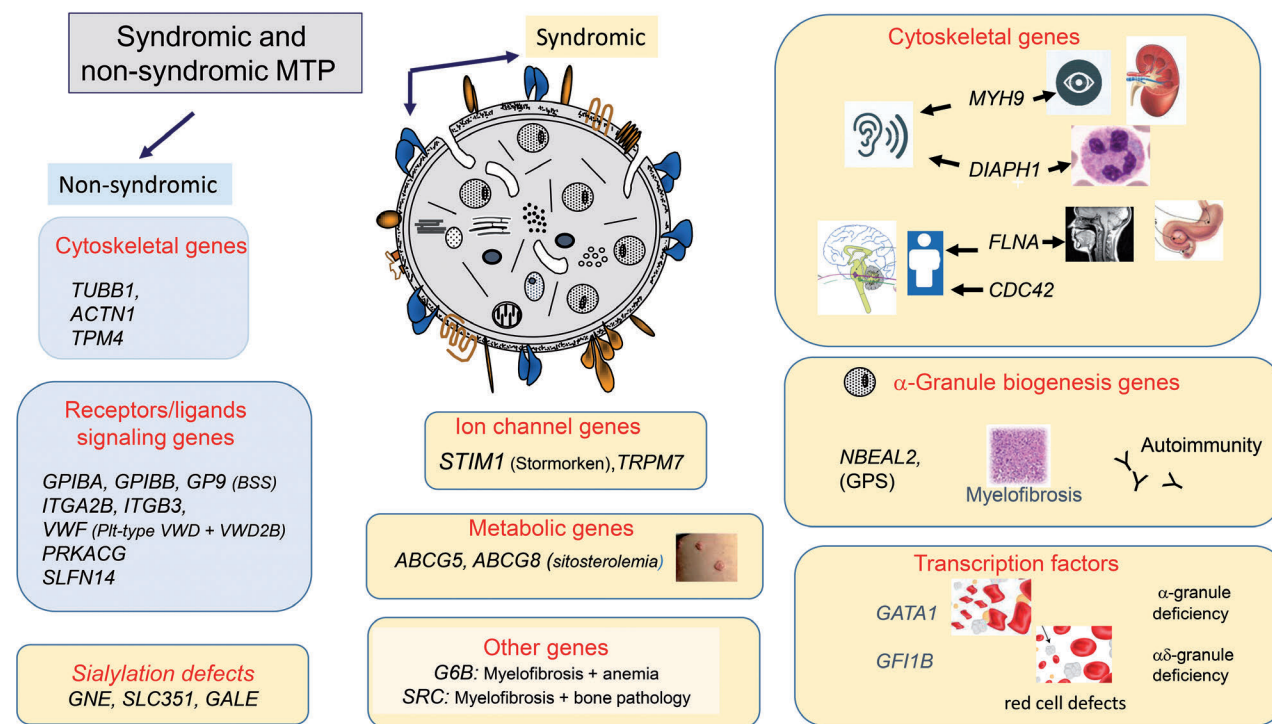**Figure 2. A cartoon showing genes causing non-syndromic and syndromic macrothrombocytopenias.** The causative genes are grouped according to the nature of the encoded protein and/or the secondary condition(s) that may accompany the macrothrombocytopenia. MTP: macrothrombocytopenias; BSS: Bernard-Soulier syndrome; pl-type VWD: platelet-type von Willebrand syndrome; VWD2B: von Willebrand disease type 2B; GPS: gray platelet syndrome.

the degree of thrombocytopenia because the absence of GPIbα prevents the shear-dependent tethering of platelets to von Willebrand factor (VWF) in vascular lesions, an abnormality mimicked by the loss of ristocetin-induced platelet agglutination in a diagnostic test.[20,21] The search for mutations first targeted *GP1BA,* the gene encoding GPIbα, but mutations in *GP1BB* and *GP9* were also quickly shown to cause BSS by preventing the surface expression of GPIbα.[22] The generation and rescue of BSS in a mouse model confirmed the link between GPIbα loss and the appearance of giant platelets and, therefore, macrothrombocytopenia.[23] Typical findings in BSS and the mouse models are aberrant formation of the demarcation membrane system in megakaryocytes while fewer proplatelets protrude into the vascular sinus and these proplatelets are thicker with larger heads. While the loss of megakaryocyte interactions with extracellular proteins remains a plausible molecular basis of BSS, the absence of mechanical stabilizing interactions between GPIb, cytoskeletal proteins and internal membranes is another likely factor.

While classic BSS has autosomal recessive (AR) inheritance, mono-allelic forms with autosomal dominant (AD) transmission are a frequent cause of mild macrothrombocytopenia in Europe. The initial example was the Bolzano (p.A172V) mutation affecting GPIbα in Italian families, said to be responsible for Mediterranean macrothrombocytopenia, although other variants of *GP1BA* have since been described. More recently a series of single allele variants of *GPIBB* have been identified by whole exome sequencing (WES) in patients with mild macrothrombocytopenia.[24] The difference in phenotype given by AD single

allelic forms of BSS compared with heterozygosity for bi-allelic BSS has yet to be fully explained. A 1.5 to 3.0-Mb hemizygous mostly somatic deletion on chromosome 22q11.2 including *GPIBB* is seen in the Di-George and velocardiofacial syndromes in which multiple developmental defects are often accompanied by mild to moderate macrothrombocytopenia.[21]

Platelet-type von Willebrand disease and type 2B von Willebrand disease

GPIbα has seven leucine-rich repeats and flanking regions near its N-terminus; the mucin-like domain follows with the many negatively charged O-linked oligosaccharides that provide rigidity. In platelet-type von Willebrand disease, AD gain-of-function missense mutations within the leucine-rich repeats (and 1 deletion outside the repeats) promote spontaneous binding of large VWF multimers.[25] As a result, the higher molecular weight multimers are decreased or absent in plasma. Cross-linking of platelets by VWF favors platelet clumping and a high sensitivity to ristocetin-induced platelet agglutination. In culture, spontaneous binding of VWF multimers to maturing megakaryocytes inappropriately activates intracellular signaling pathways; as a result, there are fewer proplatelets and they have enlarged tips.[26] Furthermore, VWF-bound platelets are rapidly removed from the circulation in a process that is enhanced when aggregates are present. Bleeding is accentuated under conditions of stress, such as pregnancy when circulating large *VWF* multimers are elevated. Mention should also be made of type 2B von Willebrand disease, also with AD inheritance. In this condition single allele
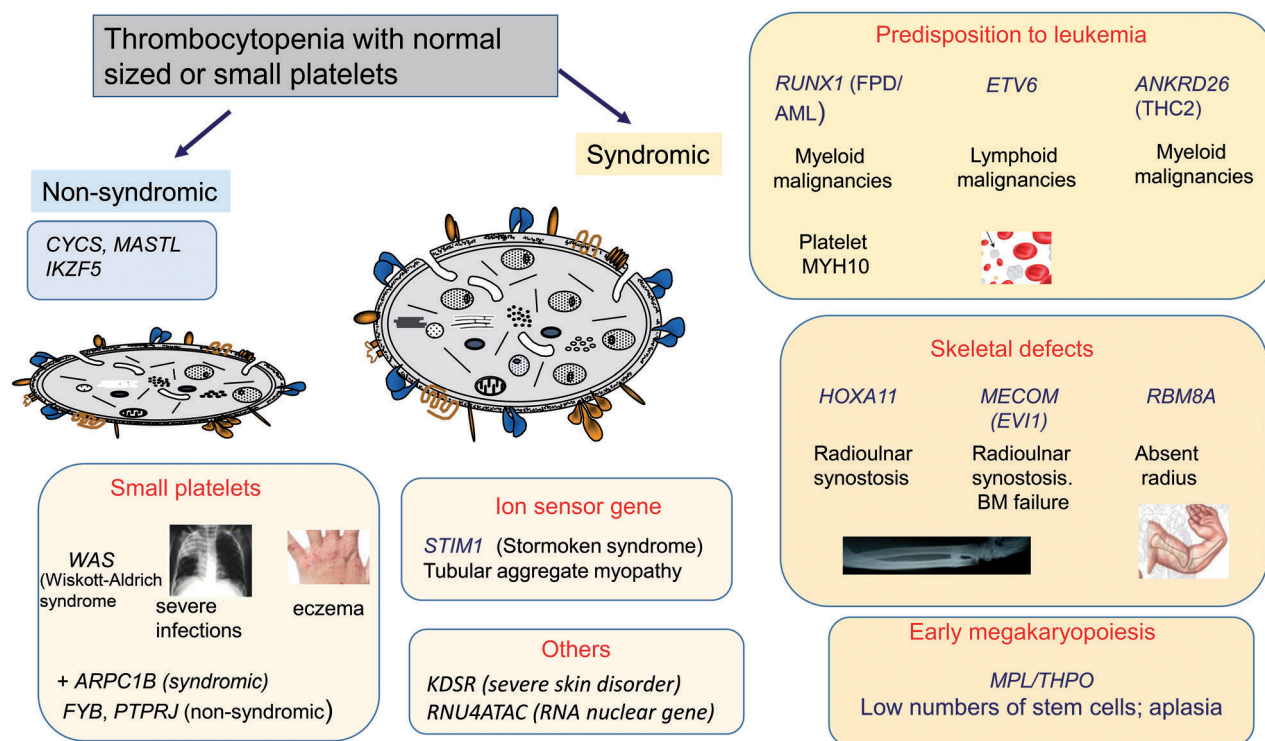

Figure 3. A cartoon showing genes causing non-syndromic and syndromic thrombocytopenias with normal sized or small platelets. The genes are grouped according to the nature of the encoded abnormal protein and to the accompanying secondary condition(s) that define the syndrome. BM: bone marrow.

gain-of-function mutations within exon 28 of VWF result in multimers that bind spontaneously to GIbα.[27] Enlarged platelets and thrombocytopenia are features and the cross-linking of platelets by adsorbed VWF multimers leads to platelet clumping in many patients. Megakaryocytes in culture appear to bind VWF multimers leaking from their own cells; the mature megakaryocytes have larger platelet territories while proplatelet-like protrusions are fewer, shorter and the swellings larger.[28] Patients of French-Canadian descent with what was called the "Montreal platelet syndrome", an inherited macrothrombocytopenia, in fact possessed the *VWF* p.V1316M mutation. Recent studies on both a mouse model and platelets from a patient with *VWF* V1316M have shown that platelet aggregation is also reduced due to the loss of essential signaling requirements for αIIbβ3 activation.[29] With regards to a possible loss of sialic acid from platelets of these patients, the same group showed that while endogenous neuraminidase translocated to the platelet surface, the desialylation threshold required for thrombocytopenia *in vivo* was not reached; intriguingly, N-glycans on αIIb and β3 and not GPIbα, were most targeted.[30] But both platelet-type von Willebrand disease and type 2B von Willebrand disease are disorders in which increased platelet turnover contributes to the thrombocytopenia.

MYH9-related disease

Myosin, heavy chain 9 (MYH9), a subunit of myosin IIA, mediates intracellular contractile forces generated through ATP hydrolysis and the actin cytoskeleton. AD-inherited *MYH9* variants are a common cause of moderate to severe macrothrombocytopenia and platelets can be very large.[31] Cytochemical or immunofluorescence detection of inclusions (Döhle-like bodies) in leukocytes on blood smears facilitates the diagnosis.[32] Myosin IIA is expressed in many tissues and *MYH9*-related disease will often become syndromic with age; symptoms include glomerulonephritis, potentially leading to renal failure (requiring dialysis or transplantation), sensorial hearing loss, early cataracts and elevated circulating liver enzymes indicating hepatic damage. Historically, combinations of macrothrombocytopenia with these conditions were known as May-Hegglin anomaly, and Epstein, Fechtner and Sebastian syndromes but all were later linked to mutations in *MYH9*.[33] Disease-causing variants are mostly missense but variants of all types have been described and affect both the N-terminal head or motor domain and C-terminal tail (responsible for dimerization and containing phosphorylation sites).[33,34] Some residues are mutational hotspots, for example S96 and R702 in the head and R1165 and D1424 in the tail and genotype-phenotype correlations have been linked to disease evolution in *MYH9*-related disease. Notwithstanding the AD inheritance, some of the mutations are sporadic and somatic germinal mosaicism has been reported. Lacking contractile force, affected megakaryocytes show reduced migration to the vascular sinus and, together with altered proplatelet formation, this results in ectopic but reduced platelet release in the marrow.[35] Treatment includes slowing the extra-hematologic effects with cochlear or kidney transplantation and renin-angiotensin inhibitors in some cases.

Gray platelet syndrome and related disorders.

A qualitative disorder characterized by moderate macrothrombocytopenia with "gray-colored" platelets on a stained blood smear, gray platelet syndrome has mostly AR inheritance. Patients have enlarged platelets lacking α-granules and their storage pool of proteins.[36] Thrombocytopenia is often progressive and bleeding highly variable. Enlarged spleens, myelofibrosis, high serum vitamin B12 levels and reduced platelet function help define the phenotype.[37] In 2011, three groups using different approaches including next-generation WES and RNA profiling showed that mutations in *NBEAL2* caused gray platelet syndrome in large, but distinct cohorts of patients (data reviewed by Chen *et al.*[38]). NBEAL2 is a scaffolding protein involved in α-granule ontogeny. Mouse *Nbeal2^-/-* models recapitulated the gray platelet syndrome phenotype and showed how the lack of the secretory pool of biologically active proteins affects wound healing, and has consequences for thrombosis and inflammation.[39] Cultured megakaryocytes from patients with *NBEAL2* mutations interacted abnormally with extracellular matrix proteins, including type I collagen, with reduced pro-platelet formation and branching.[40] Extensive emperipolesis of neutrophils by the megakaryocytes is another feature of gray platelet syndrome. Although altered neutrophil structure and increased infections were reported in early studies on this syndrome, only recently was a role for NBEAL2 in immunity confirmed.[41] New avenues for research on autoimmunity and inflammation in gray platelet syndrome have now opened.

The absence of platelet α-granules is also a characteristic of children with arthrogryposis, renal dysfunction and cholestasis (ARC) syndrome, a severe AR multisystem disorder especially affecting the kidneys and linked to mutations in *VPS33B* and *VPS16B*, genes encoding proteins involved in α-granule biogenesis; however, platelet count and size are often normal and this syndrome is not included in *Online Supplementary Table S1*.[38] A paucity of α-granules in platelets of patients with X-linked mutations in *GATA1* is explained by its long-distance regulation of *NBEAL2*.[42] The transcription factor, GATA1, in complex with its cofactor FOG1 acts as a gene repressor in hematopoietic cell lineages including megakaryocytes and red blood cells. It was first identified as a cause of macrothrombocytopenia with moderate to severe bleeding by Nichols *et al.*, who found a p.V205M mutation in siblings with severe thrombocytopenia and dyserythropoietic anemia.[43] Most *GATA1* mutations result in dysmegakaryopoiesis with the marrow often containing an abundance of small megakaryocytes; the red blood cell abnormalities include features that can be observed in β-thalassemia and also congenital erythropoietic porphyria.[21] Phenotypic variation is considerable and the condition often improves with age. A loss of collagen-induced platelet aggregation in some patients remains unexplained. Macrothrombocytopenia, an absence of platelet α-granules and red blood cell defects variably characterize patients with AD germline mutations in *GFI1B*, a transcriptional repressor and key regulator of hematopoiesis.[44,45] The macrothrombocytopenia can be accompanied by platelet function defects and myelofibrosis; bleeding is mostly mild or even absent but may be severe after trauma. Abnormal megakaryocyte maturation and proplatelet formation contribute to the macrothrombocytopenia. The phenotype depends on the site and nature of the mutation and the *GFI1B* isoform affected.[46] Haploinsufficiency or non-functioning of GATA1 or GFI1B will change the activation of a number of genes coding for proteins maintaining platelet function.

Congenital amegakaryocytic thrombocytopenia

Born with severe thrombocytopenia but normal sized platelets, patients with congenital amegakaryocytic thrombocytopenia lack mature megakaryocytes in the bone marrow. Evolution to critical multi-lineage aplasia occurs before adulthood.[47,48] In the majority of cases, AR mutations in the *MPL* gene make the cells incapable of binding thrombopoietin; as a result circulating thrombopoietin levels are very high.[47] Nonsense mutations with a complete loss of Mpl give rise to a severe form (type I) while missense mutations and the presence of residual Mpl may give a milder form of the disease (type II). The mutations lead to a loss of thrombopoietin binding or on rare occasions, altered receptor recycling. In culture, megakaryocytes from the patients with congenital amegakaryocytic thrombocytopenia fail to form colonies. The mutations can also affect stem cell maintenance. Disease severity depends on the extent of the interference with JAK2/STAT and MAPK signaling pathways, with there being residual signaling in type II disease. HSC transplantation has been the treatment of choice.[48] Progression to aplastic anemia or myelodysplastic syndrome is a risk. Rare homozygous mutations in the *THPO* gene with loss of thrombopoietin production or an inability to bind to Mpl can also lead to bone marrow aplasia. Note that because of the largely hepatic origin of thrombopoietin, these patients are unsuitable for bone marrow transplantation.[48] Conversely, both *MPL* and *THPO* gain-of-function mutations are a source of familial thrombocytosis.

Wiskott-Aldrich syndrome

Wiskott-Aldrich syndrome (WAS) is an X-linked disorder described by Alfred Wiskott in 1937 and Robert Aldrich in 1954. The syndrome is characterized by severe immunodeficiency, thrombocytopenia and small platelets. The clinical spectrum includes eczema, infections, autoimmune hemolytic anemia and cancer; bleeding is mostly mild but can be severe if the platelet count is very low and can prove fatal.[49] Hemizygous mutations in the *WAS* gene abrogate expression or alter function of WAS protein: the protein has multiple roles in the early stages of actin polymerization and in signal transduction with virtually all hematopoietic lineages affected.[50,51] Megakaryocytes of WAS patients form proplatelets prematurely and shed platelets ectopically in the marrow.[52] There is also a peripheral component to the thrombocytopenia with circulating autoantibodies and platelet destruction in the spleen making WAS a rare inherited thrombocytopenia in which splenectomy is still performed. Patients lacking WAS protein have a worse phenotype than those with residual amounts; milder forms are known as X-linked thrombocytopenia. Allogeneic HSC transplantation, first performed for WAS over 40 years ago, has a good prognosis for severe cases with a high risk of bleeding and also benefits immunodeficiency.[53] In the absence of matched donors gene therapy using a lentiviral vector encoding functional WAS protein is a promising alternative, even if full normalization of the platelet count is not achieved.[54]

Familial platelet disorders with predisposition to myeloid or lymphoid malignancies

Defects of transcription factors are a major source of inherited thrombocytopenias (Figure 1). Those that interfere with the early stages of megakaryocyte maturation are often accompanied by a risk of developing myeloid or lymphoid malignancies and this was first shown for AD thrombocytopenia caused by mutations of *RUNX1*.[55-57] The thrombocytopenia is mild and platelet volume increases are modest, but platelet function is defective and a reduced dense granule secretion contributes to the mostly mild bleeding. Disease-causing variants occur across the gene with emphasis on the Runt homology domain that mediates DNA-binding and dimerization with the core binding factor (CBFAβ), a complex essential for regulated proliferation and differentiation of HSC and normal megakaryopoiesis as well as suppression of erythroid gene expression. *RUNX1* mutations interfere with ploidy and prolong the expression of the cytoskeletal protein MYH10, whose natural loss triggers the switch from mitosis to endomitosis; the result is an abundance of immature megakaryocytes that fail to differentiate properly.[21] Furthermore, RUNX1 directly or indirectly regulates the synthesis of a number of platelet proteins thereby altering the function of those platelets that are affected. Decreased DNA repair, a pro-inflammatory environment and the prolonged life of HSC predispose patients to acute myeloid leukemia and related conditions. Most of the genetic variants act through haploinsufficiency but, significantly, those that act in a dominant negative manner have a higher probability of causing malignancy.[57]

Myeloid malignancies and myelodysplastic syndromes are also a risk for patients with mutations in the 5' untranslated region of *ANKRD26*, which are associated with mostly moderate thrombocytopenia, mild bleeding and normal sized platelets (a condition also known as thrombocytopenia 2, THC2).[58-60] Unexplained findings in some patients are a low density of α2β1 integrin, decreased numbers of platelet α-granules and large, particulate structures formed of ubiquitinated proteins or proteasomes. As for mutations of *RUNX1*, patients with *ANKRD26* mutations have downregulated megakaryocyte maturation leading to an abundance of cells with hypolobulated nuclei. Mechanistically, mutations of the 5' untranslated region of *ANKRD26* prevent downregulation of this gene by RUNX1 and FLI1, leading to increased ANKRD26 protein with a lack of repression in the terminal stages of megakaryopoiesis.[60] Extramedullary hematopoiesis, a characteristic of inherited hemolytic anemia, and myeloproliferative and myelodysplastic syndromes, was reported in a case with *ANKRD26*-related thrombocytopenia.[61] It should be noted that THC2 also includes the Ser/Thr kinase *MASTL* without a tendency for malignancy, dealt with in a later section.

Mutations of *ETV6* lead to AD thrombocytopenia and a predisposition to acute lymphoblastic leukemia.[62] *ETV6* is a tumor repressor gene with close links with *FLI1*. Red cell macrocytosis is said to accompany the thrombocytopenia in some families. An early study involving 130 families with suspected inherited thrombocytopenias identified *ETV6* gene variants in seven of them; four of the family members had developed B-cell acute lymphoblastic leukemia during childhood.[63] Thrombocytopenia was moderate as was the bleeding tendency; platelet size was normal but platelet spreading on fibrinogen was defective. Megakaryocytes in culture produced fewer proplatelets. Mutations within the consensus ETS DNA binding sites of *ETV6* resulted in reduced nuclear localization and transcriptional repression with cytoskeletal genes especially

affected. One mutation (p.R369W) involved a residue previously described as a somatic mutation in different forms of leukemia and cancer.[64]

Linkage with skeletal disorders

Genetic defects responsible for inherited thrombocytopenias can be syndromic and involve a wide range of other organs and tissues as we have already seen for *MYH9*-related disease and WAS. Here we provide four examples in which the disease includes well-defined skeletal defects. The Paris-Trousseau syndrome combines macrothrombocytopenia and a panoply of developmental defects including dysmorphogenesis of the face and digits, possible pancytopenia, cardiac abnormalities and mental retardation, which are all characteristic of Jacobsen syndrome.[21] The inheritance is largely AD with single allele expression of a small deletion in chromosome 11q23 that includes *FLI1*, an ETS transcription factor family member and a main regulator of megakaryopoiesis.[65] Genes targeted by *FLI1* include *MPL*, *GPIBA*, and *ITGA2* and a lack of transactivation early in megakaryocyte development due to a transient hemizygous loss of the affected allele is responsible for the pathology.[65] Dual populations of normal and immature megakaryocytes are generated, the latter with reduced polyploidization and persistence of the cytoskeletal protein, MYH10 (also see the section on RUNX1). Some of the α-granules in the enlarged platelets appear fused and giant.

In the thrombocytopenia with absent radius (TAR) syndrome, bilateral radius aplasia and various other developmental defects are accompanied at birth by severe thrombocytopenia but normal sized platelets. Initial studies identified a chromosome 1.21.1 heterozygous microdeletion in patients with TAR syndrome, but the genetics was only fully resolved when WES showed the necessity of a combination of the microdeletion including the *RBM8A* gene on one allele with one of two rare single nucleotide polymorphisms in regulatory regions of *RBM8A* on the second allele.[66] In fact, *RBM8A* codes for Y14, a nuclear and cytoplasmic protein that interacts with mRNA produced by splicing; how it relates to the TAR phenotype remains largely unknown. Hematopoietic progenitors have a reduced response to thrombopoietin and fail to differentiate into megakaryocytes in the marrow. Other hematologic defects include leukocytosis in some patients; enigmatically, the platelet count may correct with age.[67] TAR syndrome is to be distinguished from radio-ulnar synostosis with amegakaryocytic thrombocytopenia caused by a heterozygous truncating *HOXA11* mutation (RUSAT1) or, as determined by WES screening, by *de-novo* missense mutations in *MECOM* (RUSAT2) encoding oncoprotein EVI1 leading to altered activator protein-1 and transforming growth factor-β-mediated transcriptional responses.[68,69] Both *HOXA11* and *MECOM* mutations cause defects in the early phase of megakaryopoiesis and the risk of trilineage marrow aplasia makes the patients candidates for HSC transplantation.

Defects of platelet biogenesis due to cytoskeletal protein defects

We have already highlighted how mutations affecting the myosin IIA heavy chain are a frequent cause of macrothrombocytopenia in *MYH9*-related disease. This prompted research for mutations of genes encoding other cytoskeletal proteins in patients with congenital thrombocytopenia. X-linked mutations in *FLNA*, encoding filamin-A (FLNA), cause a syndromic disorder combined with periventricular nodular heterotopia/otopalatodigital syndromes and other developmental defects.[21] It can also be associated with Ehlers-Danlos syndrome. We first showed that three patients in France also had macrothrombocytopenia and that bleeding could be severe.[70] Characterization of their genotype was aided by reports that mice with FlnA-null megakaryocytes prematurely release large and fragile platelets that are rapidly removed from the circulation.[71] Curiously, platelets of some patients had giant α-granules. Cultured megakaryocytes from patients extended proplatelets with large swellings and released giant platelets.[71] Filamin A promotes branching of actin filaments as well as anchoring membrane receptors and *FLNA* mutations associate with a loss of platelet adhesive function. Interestingly, a rare gain-of-function missense mutation caused isolated macrothrombocytopenia.[71] Initially, *FLNA* mutations were speculated to cause macrothrombocytopenia through loss of the GPIb-FLNA interaction and involvement of RhoA (a GTPase); more recently an alternative mechanism has been proposed based on the loss of αIIbβ3-filamin A signaling.[11,72]

Hearing loss and macrothrombocytopenia with moderate bleeding were reported in patients from two unrelated families with a heterozygous gain-of-function truncating mutation in *DIAPH1*; a variant identified by WES followed by crosschecking variants against a database of genes causing deafness.[73] DIAPH1 is a member of the formin family of cytoskeletal proteins and is a regulator of actin filament formation. The above gain-of-function mutation resulted in cytoskeletal dysregulation and megakaryocytes with reduced but premature proplatelet formation similar to that seen in *MYH9*-related disease. The defect was linked to a loss of autoinhibition within the diaphanous autoregulatory domain and was distinguished from a frameshift modification that produced hearing loss in a group of families in South America but without hematologic problems.[73] Brief mention should also be made of moderate macrothrombocytopenia associated with a cluster of missense mutations detected by next-generation sequencing (NGS) in five out of 13 cases with AD missense mutations in *CDC42*.[74] CDC42 is a small GTPase and a Rho family member and the mutations were said to affect the change between the active and inactive states of the molecule. The disease is syndromic and is associated with various developmental defects including intellectual disability, brain defects, muscle tone abnormalities, facial dysmorphism, and cardiac abnormalities although these were somewhat less apparent for patients with mutations causing macrothrombocytopenia. So far, few platelet function studies appear to have been performed but, on the basis of mouse models, both megakaryocyte maturation and platelet function are likely to be affected.[11] So far, bleeding has not been reported as a problem for the patients.

Mutations of *TUBB1* encoding β1-tubulin cause macrothrombocytopenia through altered association with α-tubulin and defective microtubule assembly.[75,76] The defect leads to altered megakaryocyte maturation with the production of fewer proplatelets with large tips but there is a high degree of phenotypic and genotypic variability and while the causal effect of some variants is clear,

other predicted pathological variants have little effect on platelet count or size. Interestingly, TUBB1 dysfunction may lead to genome instability; co-segregation with myeloid malignancy has been reported, but this tendency needs confirmation as *TUBB1* mutations are relatively common. Thyroid dysgenesis has also been linked to *TUBB1* mutations in some patients. Variants identified by WES in exons 5 and 6 of *ACTB* encoding β-cytoplasmic actin cause syndromic thrombocytopenia by compromising microtubule organization during the final stages of megakaryocyte maturation, but bleeding has not been reported.[77] The defect has been located in five families and is accompanied by platelet size variability including large forms; associated and highly variable clinical features are given in *Online Supplementary Table S1*. The defect intervenes in the final stages of platelet biogenesis and pre-platelet (a purported intermediate platelet precursor) fragmentation but no defects were seen in megakaryocyte proplatelet formation in culture.[77] Interestingly, variants in exons 2 to 4 give rise to a severe developmental disorder, Baraitser-Winter cerebrofrontofacial syndrome, but no thrombocytopenia.[77]

Single allele missense mutations in *ACTN1* encoding α-actinin were first identified in Japanese patients with moderate thrombocytopenia and in a large French pedigree by WES and by genome-wide linkage analysis.[78,79] Their presence was later revealed as quite common in a non-syndromic form of thrombocytopenia with mild or no bleeding although blood loss can be a problem after surgery. α-Actinin is a dimeric protein that cross-links actin filaments into bundles; mutations affecting functional domains act in a dominant-negative manner to disrupt actin filament organization and proplatelet structure in megakaryocytes. Platelet size changes are variable between patients. It is a quite common form of mild inherited thrombocytopenia.[78-80]

A final cytoskeletal gene identified by WES in two human pedigrees with macrothrombocytopenia is *TPM4* encoding tropomyosin 4, another actin-binding protein.[81] A premature stop codon on a single allele segregated with platelet size increases and a low platelet count. Gene identification was helped by phenotype comparison with a mouse conditional knockout model and confirmed by short hairpin RNA knockdown of *TPM4* in normal human megakaryocytes. Platelet function was affected although bleeding was mild. Megakaryocytes in culture produced abundant proplatelets but with fewer branches and enlarged tips, the latter being a standard finding in patients with defects of genes encoding for cytoskeletal proteins, which results in a dynamic re-organization of the cytoskeleton.

Quite remarkably, all of the eight genes encoding cytoskeletal proteins and which give rise to macrothrombocytopenia do so with AD inheritance (*Online Supplementary Table S1*).

Abnormal integrin interactions with extracellular matrix proteins

The final steps of megakaryocyte maturation and the migration of these cells towards the vascular sinus depend not only on cytoskeletal proteins, but also on interactions between surface receptors and extracellular matrix con-

stituents. Glanzmann thrombasthenia is the classic disorder of platelet function; with AR inheritance; bleeding occurs because thrombus formation and platelet aggregation fail in the absence or non-functioning of integrin αIIbβ3. Caused by AR mutations in *ITGA2B* and *ITGB3*, the platelet count is normal in the absence of major bleeding.[82] However, rare gain-of-function mutations affecting the structure of αIIb or β3 can give rise to macrothrombocytopenia. Our group made the initial report in 1998, describing a single allele p.R995Q mutation in the αIIb cytoplasmic domain; this was later shown to be associated with a null allele.[83] Platelet aggregation was much decreased with surface αIIbβ3 at 18%, the macrothrombocytopenia was modest and bleeding infrequent. The affected residue forms an intracellular salt bridge with D723 of β3, itself mutated in a variant of Glanzmann thrombasthenia with macrothrombocytopenia (detailed in Nurden *et al.*[82]). Breaking this bond leads to conformational changes that are transmitted to the extracellular domains with a "partial" activation of αIIbβ3. Since these reports, macrothrombocytopenia with AD inheritance has been shown for other intracytoplasmic or membrane proximal gain-of-function variants affecting αIIbβ3.[84,85] One hypothesis is that the partially activated integrin on megakaryocytes modifies megakaryocyte contact with extracellular matrix proteins in the marrow. This may promote cytoskeletal re-organization with proplatelets having fewer branches and enlarged tips. Interestingly, a gain-of-function mutation affecting αIIbβ3 had a dominant phenotypic effect with respect to a loss-of-function variant.[86]

Next-generation sequencing is expanding the landscape of inherited thrombocytopenias

Careful phenotyping and the use of NGS have led to the identification of a number of new genes responsible for inherited thrombocytopenia with or without large platelets or accompanying platelet function defects. Some have been discussed in preceding sections, others, confined to a limited number of families or of unusual phenotype and with links to signaling pathways, are presented below. All of the upwards of 40 genes now recognized as causative of inherited thrombocytopenia are included in *Online Supplementary Table S1*.

Microthrombocytopenia

The classic disorder with a low platelet count and small platelets is WAS, but microthrombocytopenia and a similar phenotype including eczema, leukocytoclastic vasculitis, eosinophilia and elevated IgA and IgE levels was recently linked in two unrelated patients by WES to AR mutations in *ARPC1B*, a gene that encodes a subunit of the human actin-related protein 2/3 complex (Arp2/3).[87] In fact, Arp2/3 acts with WAS protein in regulating the branching of actin filaments and plays a central role in cell migration, vesicular trafficking and cytokinesis. Platelets from the patients showed defective spreading. Loss of Arp2/3 function is syndromic in that it predisposed to inflammatory disease althoug many of the immunodeficiencies characteristic of WAS have so far not been observed. Microthrombocytopenia was also linked by NGS in two consanguineous families to a loss-of-function homozygous AR mutation in *FYB*, encoding the cytosolic

adaptor protein ADAP (SLAP-130) for the Fyn tyrosine kinase.[88] The bleeding syndrome in these families was mild. As discussed by the authors, studies using mouse Adap^{-/-} models showed that the small platelets had a short survival (also a feature of WAS) and defective αIIbβ3 activation, findings confirmed in the patients. Subsequent whole sternum three-dimensional confocal microscopy and intravital two-photon microscopy detected reduced megakaryocyte maturation in Adap^{-/-} mice with signs of ectopic release of platelet-like particles within the bone marrow.[89] Biallelic mutations in *PTPRJ*, encoding CD148, a master regulator of Src family kinases and the most abundant membrane-bound tyrosine phosphatase in platelets and megakaryocytes, were next shown to result in non-syndromic microthrombocytopenia in two siblings with mild bleeding.[90] Loss of CD148 was accompanied by platelet aggregation defects, particularly in relation to GPVI signaling. The thrombocytopenia was again linked to ectopic platelet release within the bone marrow, a characteristic of WAS.

Src, a universal tyrosine kinase

A gain-of-function, heterozygous E727K mutation was identified in *SRC* by WES in nine members of a large family; it was linked to thrombocytopenia, a low content of α-granules and reduced platelet function.[91] The thrombocytopenia was syndromic with myelofibrosis, the disease being accompanied by bone defects including mild facial dysmorphia. The marrow showed trilineage dysplasia with immature megakaryocytes poorly able to form proplatelets. Platelets were variable in size and morphologically abnormal; they responded poorly to collagen with reduced GPVI signaling. The gain-of-function mutation was suggested to lift autoinhibition with spontaneous Src activation and upgraded protein tyrosine phosphorylation. The abnormalities were reproduced in a zebrafish model. The mutation may have a feedback on Mpl function.

Mutations affecting ion gradients

Stormorken syndrome was described many years ago as a "multifaceted bleeding syndrome" in which an unusual form of macrothrombocytopenia was associated with reduced platelet survival and upgraded prothrombin consumption.[92] It was linked to a spontaneous expression of phosphatidylserine on platelets. Platelet function and thrombus formation under flow were affected. Much later it resurfaced as a syndromic macrothrombocytopenia with a range of clinical features including mild anemia, asplenia, myopathy with tubular aggregates, miosis, immune dysfunction, ichthyosis and dyslexia.[93,94] Bleeding is life-long but is mostly mild. Heterozygous gain-of-function AD variants in *STIM1* identified by WES are the cause of the disease. STIM1 is a Ca^{2+}-sensor within the endoplasmic reticulum; on Ca^{2+}-depletion conformational changes induce its coiled-coil 1 domain to elongate and interact with ORAI1, a surface membrane channel that mediates store-operated calcium entry into cells. ORAI1 can be affected in rare cases with miosis and non-progressive myopathy but without significant platelet abnormalities. The mutations make STIM1 (and secondarily ORAI1) constitutively active, favoring Ca^{2+} entry into platelets and presumably megakaryocytes. The York platelet syndrome, with large and morphologically abnormal platelets with giant opaque organelles also had gain-of-function mutations in *STIM1* thereby expanding the spectrum of Stormorken syndrome.[94] WES and comparison with mouse models led us to identify variants in the *TRPM7* gene in two French index cases with macrothrombocytopenia and with a possible link to atrial fibrillation in one family.[95] TRPM7 is both a kinase and an ion channel primarily linked to Mg^{2+} homeostasis; its absence in mice or disease-causing variants in man were both accompanied by cytoskeletal changes in megakaryocytes.

Other miscellaneous defects

Patients in three families with an often strong bleeding history, moderate thrombocytopenia and enlarged platelets were linked by WES to heterozygous mutations within *SLFN14* (Schlafen family member 14).[96] SLFN14 locates to the nucleus, where it acts as an endoribonuclease and is possibly involved in RNA surveillance. The variants in patients with macrothrombocytopenia localize to the ATPase-AAA-4 domain. The protein is known to associate with ribosomes where it regulates RNA degradation; SLFN14 expression was reduced in the patients and a dominant-negative effect was suggested.[97] Platelet dense granule content was low and platelet aggregation particularly reduced with collagen, modestly reduced and reversible with ADP but normal with arachidonic acid. In a separate study, severe macrothrombocytopenia but with AR inheritance was linked through WES to a homozygous missense mutation in the *PRKACG* gene encoding the gamma-catalytic subunit of the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A.[98] Protein kinase A phosphorylates multiple substrates in platelets and in its absence platelet activation and cytoskeletal organization (with extensive degradation of filamin A) were impaired. However the very low platelet count (<10x10⁹/L) precluded platelet aggregation testing. Significantly, megakaryocytes from this patient had a defect in proplatelet formation. Next in this section are AR mutations in *G6B* (*MPIG6B*) encoding the megakaryocyte and platelet inhibitory receptor, G6b-B, a critical regulator of hematopoietic lineage differentiation and megakaryocyte function and platelet production.[99] The mutations were detected by WES screening of four families with macrothrombocytopenia. One feature was the presence of focal myelofibrosis atypically even in children. Bleeding was moderate and accompanied by anemia and leukocytosis. In a mouse model megakaryocytes were abnormal with reduced proplatelet formation. G6b-B regulates the activity of the tyrosine phosphatases Shp1 and Shp2. Nonetheless, platelet aggregation to ADP was only minimally affected.

Severe thrombocytopenia observed in four Spanish families was associated with a disorder of ceramide biosynthesis with WES linking the disease to AR mutations in *KDSR* encoding 3-ketodihydrosphingosine reductase, a lipid enzyme that localizes to the endoplasmic reticulum.[100] Interestingly, in at least two of these cases the platelet count was normal at birth but fell rapidly to around 30x10⁹/L or lower. Impaired platelet biogenesis was speculated to be due to decreased synthesis of sphingosine-1 phosphate but formal proof of this is lacking. Another disorder in which the platelet count falls after birth is Roifman syndrome in which moderate thrombocytopenia accompanies a variable syndromic disorder characterized by growth retardation, spondylo-epiphyseal dysplasia, cognitive delay and hypogammaglobulinemia.[101] Interestingly, the disease is driven by AR variants in the small RNA nuclear gene *RNU4ATAC* that intervenes in intron splicing. The platelets

were unusually round and had altered tubulin and actin levels with *DIAPH1* being one of the affected genes.

Finally, mention must be made of *IKZF5* encoding a transcription factor known as Pegasus that was highlighted by whole genome sequencing of over 13,000 individuals enrolled in the National Institute for Health Research Bioresource and including 233 cases of isolated thrombocytopenia.[102] Various causal missense variants were detected in patients with isolated thrombocytopenia but mild or no bleeding; the mutations (sometimes *de novo*) were proposed to interfere with DNA binding and to selectively alter the expression of a series of proteins expressed in megakaryocytes and platelets. The thrombocytopenia was mild and platelet size was normal. The platelet α-granule content was low but the platelet aggregation response was reported as inconsistent. The megakaryocytes produced fewer proplatelets.

Thrombocytopenia, apoptosis and deficient glycosylation

Early apoptosis with phagocytosis of platelets by macrophages in the spleen or glycosylation changes followed by platelet clearance in the liver are potential causes of inherited thrombocytopenia. Although evidence for such mechanisms remains incomplete, the identification of some mutations, largely by NGS, has revived interest, particularly in deficient glycosylation.

Apoptosis

Non-syndromic mild AD thrombocytopenia but with little or no bleeding and platelets of normal size is associated with mutations in the *CYCS* gene encoding mitochondrial cytochrome c, a disease first reported in New Zealand.[103,104] Although normal-looking megakaryocytes were present in the bone marrow, the number of naked nuclei was increased. Their presence as well as platelets in the marrow space was indicative of ectopic release. Electron microscopy revealed platelets lacking glycogen stores and the microtubule coil. Culture of megakaryocytes from the patients showed that abnormal and normal platelets were both released. Whether or not *CYCS* mutations, so far limited to three families, lead to loss of mitochondrial inner membrane potential and platelet phosphatidylserine expression remains speculative. Mention must also be made of a missense p.E167D mutation in *MASTL*, coding for a microtubule-associated Ser/Thr kinase, in a large family first reported many years previously. The mutation was linked to moderate thrombocytopenia (with normal sized platelets), incomplete differentiation of high ploidy megakaryocytes and a propensity to mild bleeding.[58,105] Recent studies on a mouse knock-in model suggest that this is a gain-of-function mutation leading to platelet survival changes and to platelets that extend unusually long pseudopods and contain hyper-stabilized microtubules.[106] In mice, Mastl inhibits protein phosphatase 2, an important negative regulator of several major platelet activation pathways. The result is an increased propensity of platelets to form aggregates, reduced platelet survival and increased signs of apoptosis.

Desialylation and other glycosylation changes

An early study showed macrothrombocytopenia and neutropenia in a patient with a specific defect in α-2,3-sialylation and rapid platelet clearance by the Ashwell-Morell receptor in the liver but the gene defect was not identified.[107] More recent studies using WES have linked macrothrombocytopenia with AR mutations of *GNE* encoding UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase, a bi-functional enzyme essential for N-acetylneuraminic acid synthesis (the principle sialic acid of human platelets).[108-110] Interestingly *GNE* mutations are also a cause of myopathy and in the above studies macrothrombocytopenia was either syndromic or isolated depending on the report. Severe menorrhagia and bleeding into the corpus luteum in affected women were noteworthy. Platelets were large and the numbers of reticulated (young) platelets often increased, features suggesting accelerated platelet clearance. Precise quantification of the platelet sialic acid deficiency and a direct measure of platelet survival have not, however, been made.

A necessary step in the synthesis of oligosaccharides is the transport of cytidine monophosphate-bound sialic acid to the Golgi apparatus. This requires a transporter protein SLC35A1, whose molecular deficiency was recently reported in two siblings with moderate thrombocytopenia, giant platelets, and mild bleeding.[111] The macrothrombocytopenia is syndromic, featuring delayed psychomotor development, epilepsy, ataxia, microcephaly and choreiform movements. Exome sequencing highlighted a homozygous missense mutation in *SLC35A1* in both siblings; their consanguineous parents were heterozygous. Lectin binding confirmed abnormally high β-galactose exposure on platelets from the patients and increased numbers of reticulated platelets. Bone marrow biopsies showed a high percentage of immature megakaryocytes, yet in culture megakaryocytes from the patients showed normal maturation and proplatelet formation. However quantification of platelet survival using a non-obese diabetic/severe combined immunodeficiency mouse model confirmed that the patients' platelets had a very short lifespan. In a separate study, severe thrombocytopenia with enlarged platelets and dysplastic megakaryocytes was the characteristic of six members of a consanguineous family in whom WES and homozygosity mapping revealed a homozygous mutation in *GALE*, encoding UDP-galactose-4-epimerase, an enzyme responsible for UDP-galactose inter-conversion with UDP-glucose and UDP-N-acetylgalactosamine inter-conversion with UDP-N-acetylglucosamine.[112] Some individuals also showed mild anemia and febrile neutropenia while galactosemia is a feature of mutations of this gene. With predicted protein instability, the enzyme retained 40% of its normal activity. Knockdown of GALE in hematopoietic cells slowed the proliferation of these cells in liquid culture; larger colonies possibly reflected an increased presence of immature cells.

Macrothrombocytopenia and sitosterolemia

An unusual inherited macrothrombocytopenia with epigenetic or environmental implications is sitosterolemia. In this condition, giant platelets associate with AR mutations in *ABCG5* and *ABCG8*, genes which code for proteins that act as cellular transporters of plant sterols, the latter accumulating at high levels in blood and tissues.[113] Xanthomas, premature atherosclerosis and hemolytic anemia are other

features; not all patients develop hematologic abnormalities while macrothrombocytopenia can also be isolated. Studies in mice show knock-on effects with sterol intercalation within the demarcation membrane system of megakaryocytes leading to release of hyper-reactive enlarged platelets with deregulation of multiple signaling pathways including GPIbα shedding.[114]

Diagnosis

The continuing difficulties in diagnosis of inherited thrombocytopenias should not be underestimated. While classic disorders such as BSS, *MYH9*-related disease and WAS are now relatively straightforward to identify, the majority are not and in the absence of a well-defined family history of bleeding, new cases can still be confused with immune thrombocytopenic purpura. Diagnostic algorithms with steps based on clinical data and laboratory testing aid the work-up and management of patients.[115-118] The widespread use of whole blood electronic counters to evaluate platelet count and size has helped, while assessing platelet function, distinguishing between syndromic or non-syndromic forms, identifying associated hematologic or marrow defects are important parts of any strategy. A successful diagnosis avoids unnecessary splenectomy and/or the use of drugs given for immune thrombocytopenic purpura. It helps to predict disease evolution, and is indispensable for selecting patients for HSC transplantation or gene therapy. Noteworthy advances have been the application of Human Phenotype Ontology terms and the identification of phenotype clusters of associated pathologies as an aid to establishing phenotype/genotype relationships.[119] Nonetheless preparing an extensive Human Phenotype Ontology listing is not always compatible with day-to-day hospital practice. Classic tests for evaluating platelet function, such as aggregometry (Born platelet aggregometer, impedance aggregometry or other methods), secretion assays, flow cytometry, western blotting and electron microscopy, are time consuming, expensive to perform and require trained personnel. Critically, many such tests are difficult or impossible to perform when the platelet count is low. Cytochemical or immunofluorescence staining of blood smears is a recognized first step for detecting enlarged platelets, and is particularly useful in third-world countries.[32,117] The evaluation of platelet spreading on single protein substrates provides more information.[120] The use of computer-based high-throughput testing of platelet function and thrombus formation under flow on extracellular matrix proteins on microchips is full of promise but has been little tested for thrombocytopenias.[121]

As highlighted throughout our review, applying NGS to inherited thrombocytopenias is greatly expanding the list of causative genes as well as increasing the number of variants implicated in the classic diseases. The initial success of WES in genotyping gray platelet syndrome and the TAR syndromes led to the BRIDGE-Bleeding and Platelet Disorders (BRIDGE-BPD) project, orchestrated by Professor W. Ouwehand, which combines the unique sequencing and bioinformatics resources of the Sanger Institute in Cambridge (UK).[122] The Genotyping and Platelet Phenotyping (GAPP) consortium led by Professor S. Watson in Birmingham, UK has been another major player.[123] As the number of causal genes for all forms of

inherited platelet disorders increased, BRIDGE-BPD and GAPP both put together platforms to test patients for potentially pathogenic variants against previously identified target genes concentrating on exomes, untranslated regions and selected intronic regions.[124-127] Indeed, GAPP designed a gene panel specific for inherited thrombocytopenias.[127] In contrast, the ThromboGenomics Consortium (Department of Haematology, University of Cambridge, UK) included a limited number of genes causal for other blood and thrombotic disorders; the panel was regularly updated and a large cohort of 2,396 patients screened.[125,126] The use of NGS and high-throughput procedures for diagnosing platelet disorders including thrombocytopenias has quickly expanded worldwide as is illustrated by reports from Italy, Japan, Spain, France, Holland and Scandinavia as well as North America.[46,60,77,78,80,87,108,113,128, 129]

The question now is not whether to apply NGS procedures in the mainstream of diagnosis but when and how.[128] Certainly, a strong argument can now be made to use them upfront: early identification of a causal mutation in a known gene will avoid much unnecessary biological characterization, as we have stated recently in this journal.[130] It is certainly necessary if prenatal diagnosis or HSC transplantation is on the agenda. Nonetheless, upwards of 200 potential gene variants can be located for each patient, so prioritizing and filtering the variants is key.[131] Variant selection includes the exclusion of synonymous variants and those with a minor allele frequency >0.01 in the normal population. Therefore, in the absence of a previously validated mutation in a known causal gene for inherited thrombocytopenia much care must be taken. Only for a limited number of cases can linkage in large families be performed. Data evaluation in terms of quality control, depth of coverage of each gene, the conservation of affected nucleotides or amino acids, the choice of *in silico* pathogenicity prediction software, and the classification of variants according to appropriate genetic guidelines have been nicely reviewed elsewhere.[125,131]

In taking account of these advances, the fundamentals for a systematic approach to the diagnosis and management of inherited thrombocytopenia remain largely those expertly outlined in a recent European Hematology Association consensus report on mild and moderate bleeding disorders, to which the reader is directed.[132] In the context of immune thrombocytopenia, a systematic approach includes careful primary tier assessment of the basic clinic-pathological information for each new patient prior to next stage investigations and, if needed, referral to a specialist center. The recommendations in this European Hematology Association report are very useful in emergency situations and for prevention or control of spontaneous bleeding, such as epistaxis or gum bleeding and at-risk situations, including dental and surgical procedures or traumatic events when immediate practical decisions must be made.

Treatment

As with all inherited platelet disorders, spontaneous bleeding includes epistaxis, gum bleeding, easy bruising and petechiae while gastrointestinal hemorrhage occurs in some severe cases; bleeding may be prolonged after cuts, trauma, or surgery, while for women, menorrhagia and childbirth are added risks.[133,134] For example, in a European

study of pregnancy, the risk of bleeding was greater when mothers had a severe bleeding history and a platelet count below 50x10⁹/L. Platelet count appeared more important as a parameter than the genetic cause of the inherited thrombocytopenia.[133] Clearly, studies support the classic theory that a primary role of platelets is to maintain vascular integrity.[135] Nevertheless, many subjects with new forms of inherited thrombocytopenias have modest reductions in platelet count and will bleed rarely or not at all in normal life. Bleeding in such patients depends not only on the extent of the fall in platelet count but also on the nature of accompanying functional defects. Furthermore, much evidence has accumulated on the non-hemostatic roles of platelets.[136] Using mouse models, Goerge *et al.* observed that thrombocytopenia rapidly leads to bleeding in inflamed skin and brain due to the loss of vascular integrity.[137] Much needs to be learned on how the genetic defects described in our review influence the non-hemostatic roles of platelets.

Managing bleeding in inherited thrombocytopenia is much the same as in all inherited platelet disorders, with platelet and red blood cell transfusions being the first options. The use of recombinant activated factor VII is especially recommended in BSS in which the absence of a major surface constituent (GPIb-IX-V) makes isoantibody formation likely and platelet transfusion ineffective.[138] Tranexamic acid or local measures are the most frequent options for mild bleeding and tranexamic acid is counseled prior to surgery or childbirth if the thrombocytopenia is severe, with platelet concentrates on standby. A quantitated bleeding score, such as the International Society of Thrombosis and Haemostasis Bleeding Assessment Tool, is useful for assessing disease severity but will not predict bleeding risk.[139-141] Curing the disease is still at its debut for inherited thrombocytopenia. Human stem cell and allogeneic bone marrow transplants have been used very successfully in children with WAS in whom the immunodeficiency is accompanied by a major bleeding risk.[53] It has also been used in congenital amegakaryocytic thrombocytopenia and more recently in gray platelet syndrome, in which a positive effect was noted on myelofibrosis, but each procedure has its complications in terms of donor matching and the choice of conditioning regime.[21,48,142] Lentivirus-based gene therapy is already a proven therapy in WAS when donor-matching for HSC transplantation is a problem, although restoration of the platelet count remains incomplete.[54] Clearly the gain *versus* risk profile for the patient must be evaluated case-by-case and current gene therapy procedures can only be considered when a patient's long-term survival is in question or perhaps when CRISPR-Cas gene editing becomes available. One highly promising, non-invasive approach to raising the platelet count is the use of the thrombopoietin-mimetics, eltrombopag or romiplostim.[143] Although bleeding is generally mild in *MYH9*-related disease, eltrombopag was first used successfully prior to surgery in a case with aggravated thrombocytopenia over 10 years ago.[144] Thrombopoietin-mimetics have more recently been used for a patient with a *DIAPH1* mutation prior to hip arthroplasty and as a "bridge" for a child with WAS and severe thrombocytopenia awaiting HSC transplantation.[145,146] The goal in such situations is to transiently increase the platelet count to >50x10⁹ platelets/L. Romiplostim has been successful in correcting the platelet count resulting from mutations in

THPO in congenital amegakaryocytic thrombocytopenia in which the patient's own thrombopoietin is absent or non-functional.[147] Overall, a careful and complete diagnosis is essential for the optimal management of patients, not only for those in need of special care but also to avoid over-reacting for patients with little risk of bleeding.

Perspectives

It is clear that we have entered a new era in the diagnosis of inherited thrombocytopenias with the arrival of WES, whole genome sequencing and selected high-throughput sequencing platforms. A feature of the reports so far is the large genetic heterogeneity. Crucial for the future will be a better understanding of regulatory DNA elements and untranslated regions, a new science that will include epigenomic profiling.[148] The challenges of whole genome sequencing are great but the potential to identify possible pathological traits hidden up to now, including difficult copy number variations and sequence variations deep within introns, is vast. The study of RNA sorting during platelet biogenesis and of the modulating influence of miRNA is also important.[149] Despite current technological advances, a high proportion of new cases of inherited thrombocytopenia remain without diagnosis; this proportion is often estimated to be around 50% but can be as high as 70% or more depending on the extent to which patients with easily identified disorders have been pre-screened.[127,128,150,151] Assigning a significance to the variants is key, because in the absence of confirmation with follow-up biological studies, including the use of mouse, zebrafish or Drosophila models or site-directed mutagenesis in megakaryocyte-related cells, the alternative is to use sophisticated bioinformatics; new variants are being variously graded as highly significant and likely pathogenic to those of unknown significance.[126,131] An early meta-analysis of genome-wide association studies identified 68 common single-nucleotide variants that influenced platelet count or platelet volume; genes already linked to inherited thrombocytopenia, such as *THPO*, *GPIBA*, *TUBB1* and the pro-survival gene, *BAK*, were included, but the majority involved genes with no known link to megakaryopoiesis.[152] Evaluating the sum of the effects of large combinations of single nucleotide variants and of novel gene variants of unknown significance has barely started yet and they may be disease-modulating, particularly if including heterozygous variants of known causal genes.[153] Their identification will require large study groups and high-powered bioinformatics beyond the scope of most individual laboratories.

A major challenge posed by inherited thrombocytopenia is identifying how patients, sometimes within the same family, with an identical genotype can vary so much clinically. In a GAPP study, there were descriptions of patients with excessive bleeding, mild thrombocytopenia and platelet dense granule secretion but with an enrichment of heterozygous *FLI1* and *RUNX1* hypomorphic mutations that were proposed to modulate phenotype.[154] On the basis of data from the ThromboGenomics project, we identified a disease-modifying *TUBB1* mutation in a patient with classic type I Glanzmann thrombasthenia who, confusingly, also had giant platelets and macrothrombocytopenia.[155] Such studies must be just the tip of the iceberg but the benefit of screening against large

gene panels is that some patients thought to have a monogenic condition may in fact be seen to have digenic or oligogenic pathologies. The extent of bleeding or predisposition to leukemia may crucially depend on gene variants that are different from those causing the primary disease. An early example is the *ARID5B* risk allele for leukemia in patients with *ETV6*-linked thrombocytopenias.[156] A future step for modern technologies is to identify variants and gene modulators able to influence phenotype and bleeding - either by protecting against or by exaggerating the primary disease. This will be key to improving prognosis and prevention.

The use of cost-effective NGS procedures, with the continued updating of gene panels, will require national networks with links to high-performance bioinformatics centers able to best analyze the data, perform variant filtering as well as the quality control of the sequencing procedures. As we have proposed elsewhere, this will be best done for Europe with a consensus[132] in which the European

Hematology Association can be a leader providing guidelines, as is already the case with its comprehensive roadmap for hematology research.[157] Early recognition of a germline mutation facilitates appropriate treatment, better monitoring for disease progression, proper donor selection for HSC transplantation (family members must also be tested to ensure that they do not carry the mutation), as well as optimal genetic counseling of the affected patients and their family. Quite clearly for many patients the associated pathologies are clinically more serious than the bleeding risk and for some there is an obvious age-dependence. Improving the quality of life of patients and, at the same time, avoiding their stigmatization requires comprehensive ethical considerations.[158] It is crucial to decide how to provide delicate genetic information concerning a potential risk for leukemia or cancer, especially when potentially causal variants are found in young children, or how to deal with the coincidental identification of gene defects likely to predict other major illnesses.

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