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REVIEW

Profiling the Genetic and Molecular Characteristics of Glanzmann Thrombasthenia: Can It Guide Current and Future Therapies?

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Abstract: Glanzmann thrombasthenia (GT) is the most widely studied inherited disease of platelet function. Platelets fail to aggregate due to a defect in platelet-to-platelet attachment. The hemostatic plug fails to form and a moderate to severe bleeding diathesis results. Classically of autosomal recessive inheritance, GT is caused by defects within the *ITGA2B* and *ITGB3* genes that encode the αIIbβ3 integrin expressed at high density on the platelet surface and also in intracellular pools. Activated αIIbβ3 acts as a receptor for fibrinogen and other adhesive proteins that hold platelets together in a thrombus. Over 50 years of careful clinical and biological investigation have provided important advances that have improved not only the quality of life of the patients but which have also contributed to an understanding of how αIIbβ3 functions. Despite major improvements in our knowledge of GT and its genetic causes, extensive biological and clinical variability with respect to the severity and intensity of bleeding remains poorly understood. I now scan the repertoire of *ITGA2B* and *ITGB3* gene defects and highlight the wide genetic and biological heterogeneity within the type II and variant subgroups especially with regard to bleeding, clot retraction, the internal platelet Fg storage pool and the nature of the mutations causing the disease. I underline the continued importance of gene profiling and biological studies and emphasize the multifactorial etiology of the clinical expression of the disease. This is done in a manner to provide guidelines for future studies and future treatments of a disease that has not only aided research on rare diseases but also contributed to advances in antithrombotic therapy.

Keywords: Glanzmann thrombasthenia, inherited platelet disorder, bleeding syndrome, integrin, gene profiling, mutation analysis

Introduction

In Glanzmann thrombasthenia (GT) (OMIM#273,800) platelets fail to aggregate when stimulated by physiologic agonists while clot retraction is often defective.[1] Platelets interact with exposed subendothelium but a platelet-rich hemostatic plug fails to form. Spontaneous or trauma-dependent mucocutaneous bleeding is usually observed from birth. Epistaxis, gum bleeding, easy bruising, ecchymoses and petechiae are frequent and gastro-intestinal (GI) bleeding is a major problem particularly in older patients.[2,3] Menorrhagia is critical for women while pregnancy and childbirth present severe hemorrhagic risks. Surgery including tooth extraction requires preventative measures. The molecular basis of GT was clarified when, in Paris, I located a deficit of two major platelet membrane glycoproteins (GPs) in this disease.[4] As the complexity of the platelet surface topography became apparent

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these were named GPIIb and GPIIIa; it was quickly realized that they form a Ca^{2+}-dependent complex in platelets.[5–7]

The inability of GPIIb-IIIa to bind fibrinogen (Fg) or other adhesive proteins accounts for the lack of aggregation in GT. Clinical and biological heterogeneity led to the definition of three subgroups (type I, type II and variant forms) largely depending on the ability of platelets to retract a clot, store Fg and the level of GPIIb-IIIa expression (Table 1). A major advance came when GPIIb-IIIa was revealed as αIIbβ3, a member of the integrin superfamily of cellular receptors (reviewed by Coller and Shattil[8]). Electron microscopy initially showed GPIIb-IIIa to have a head and two legs; later crystallography strikingly revealed αIIbβ3 in a bent conformation that on activation straightened as it took on its ligand-binding conformation.[8–10] Precision crystallography and modeling showed αIIb and β3 to have precisely defined subdomains (shown in Figure 1).

ITGA2B (OMIM # 607,759) with 26 exons and *ITGB3* (OMIM # 173,470) with 15 exons encode αIIb and β3; both genes localizing to a 260-kb segment on the long arm of chromosome 17.[11,12] In pioneering work, Newman et al showed that GT was caused by defects in either gene with in Israeli-Arabs a founder *ITGA2B* mutation (c.IVS3(−3)-418del + frameshift (Fs)) while in Iraqi-Jewish patients there was a c.2031–2041del/premature termination in *ITGB3*.[13] Over the years, several hundred mutations with AR inheritance have been identified in GT including small deletions and insertions and splice site variants causing a Fs as well as abundant nonsense and missense mutations sometimes also associated with mRNA instability.[14–16] While repeated mutations may identify gene hotspots, others within ethnic groups are clearly founder mutations; yet for most families they remain private. Large deletions are rare.[17] In addition to αIIbβ3, platelets contain trace amounts of αvβ3 mostly lacking when the genetic lesion affects *ITGB3* but persisting and even in increased density

Table 1 Glanzmann Thrombasthenia in All Its Forms

Disease Description	Comments
Type I Subgroup	
- Absence of platelet aggregation and little or no clot retraction. Levels of αIIbβ3 <5% or absent. Platelet Fg storage pool lacking or negligible. AR inheritance.	- The most common type of GT, given by defects in *ITGA2B* and *ITGB3 genes*. With *ITGA2B* defects αvβ3 may still be present and functional. Patients susceptible to form isoantibodies reactive with αIIbβ3 and/or αvβ3 after blood transfusion or pregnancy.
Type II Subgroup	
- Absence of platelet aggregation but clot retraction can be partial or normal. Residual αIIbβ3 historically defined as 5–15% of normal levels. Platelet Fg pool can be substantial. AR inheritance.	- Frequency variable within populations but usually less than 20% of the patients. Given by defects in *ITGA2B* and *ITGB3*. Clot retraction defects and the platelet Fg storage capacity are mutation dependent.
Variant Forms	
- Absence of platelet aggregation but clot retraction and Fg storage highly variable. Residual αIIbβ3 mainly >50% or even normal but non-functional with little or no activation-dependent Fg binding as also shown by a lack of PAC-1 binding. AR inheritance.	- Rare. Can be given by defects in *ITGA2B* but mostly by *ITGB3* variants. Extracellular mutations directly or indirectly abrogate Fg-binding sites. Intracellular mutations stop signals for αIIbβ3 activation. Clot retraction and Fg storage are mutation dependent. Can be confused with defects in *FERMT3* and *RASGRP2* that prevent kindlin-3 (LAD-III disease) and CalDAG-GEFI signaling.
Upregulated αIIbβ3 and Macrothrombocytopenia (MTP)	
- Much reduced platelet aggregation with clot retraction and Fg storage again variable. Residual αIIbβ3 normally >30% but with spontaneous binding of PAC-1 (but rarely Fg). MTP mostly moderate with subpopulations of enlarged even giant platelets. AD inheritance.	- Rare. Patients with up-regulated αIIbβ3 interfering with megakaryocyte maturation and platelet biogenesis with enlarged platelets in variable numbers. Bleeding mostly due to defective αIIbβ3 function. Single allele mutations on *ITGA2B* but mostly *ITGB3*. Often these affect cytoplasmic domains.

Notes: The above criteria are basic for each subtype, but there is much overlap between them and clear boundaries do not exist. PAC-1 is an activation dependent IgM monoclonal antibody to αIIbβ3.
Abbreviations: AR, autosomal recessive; AD, autosomal dominant; LAD-III, leukocyte adhesion deficiency syndrome type III.

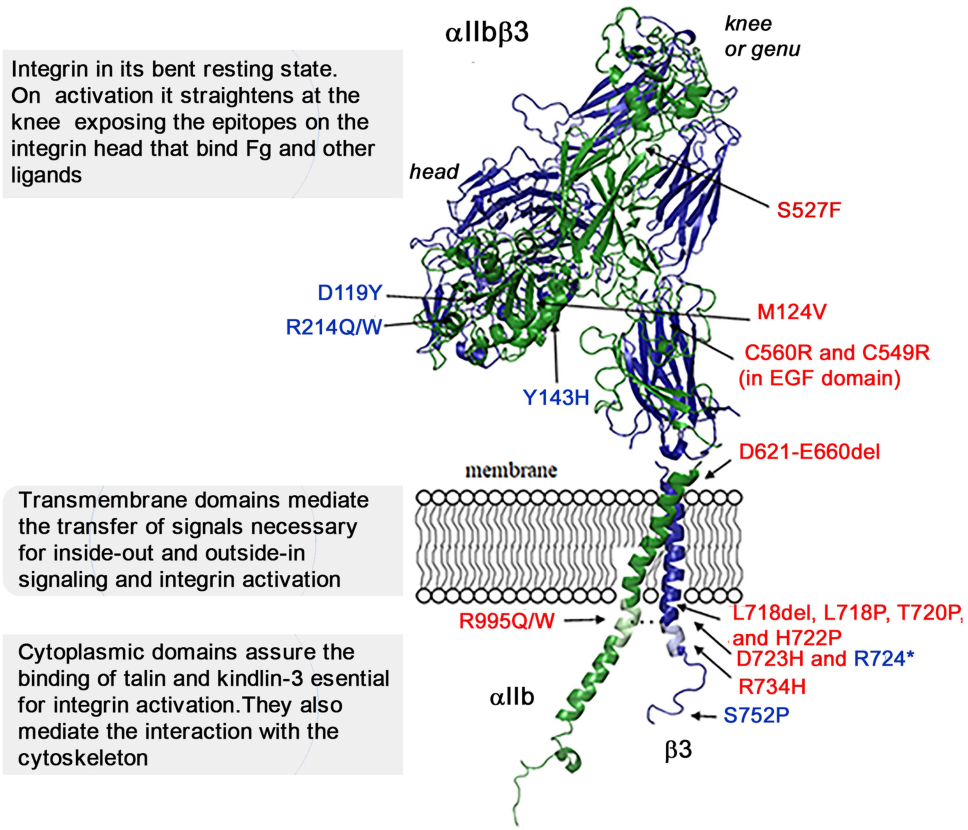

Figure 1 Structural representation of αIIbβ3 in its bent conformation showing the mutations that give rise to selected variant forms of GT or to related phenotypes. This model is based on the crystal structure of αIIbβ3; it was constructed using the PyMol Molecular Graphics System, version 1.3 Schrödinger, LLC and 3fcs and 2knc pdb files as described.[16] The αIIb subunit is in green and β3 is in blue. Precision crystallography and modeling showed that αIIb has 4 major extracellular domains (β-propeller, thigh, calf-1 and calf-2) whereas β3 has more (β-I or β-A, hybrid, plexin-semaphorin-integrin (PSI), 4 epidermal growth factor (EGF) and the β-tail domain).[8,10] Loss-of-function mutations (in blue) in the β3 extracellular head prevent binding of Fg or other adhesive proteins to the opened integrin headpiece following platelet activation, while those in the β3 cytoplasmic tail prevent binding of kindlin-3 and/or talin, and block steps essential for integrin activation. Gain-of-function mutations (in red) lead to at least partial activation of αIIbβ3 and often associated with MTP accompanied by a variable loss of αIIbβ3 function. All mutations are detailed and referenced in the text.

when *ITGA2B* mutations are the cause.[18] While αIIbβ3 is more or less specific for MKs and platelets, αvβ3 through the more promiscuous translation of *ITGB3* is widespread but without influencing the bleeding phenotype in GT.[19]

An objective of this review is to question whether in 2021 it is valid to classify GT into distinct subgroups. In so doing, I ask 1) what are the genetic causes allowing the expression of residual αIIbβ3, 2) whether this residual αIIbβ3 is functional, and 3) whether type II and variant GT give rise to a milder form of GT. Likewise, I ask how the advent of high-throughput sequencing procedures can be used to better advantage in determining patient care.

Biological Testing Within the Type I and Type II Subgroups

Diagnosis of GT is straightforward given the clinical characteristics and phenotype as revealed by platelet function and biological testing. Platelets fail to form large aggregates in response to physiologic agonists in a platelet aggregometer although small clusters were noted by microscopy.[1] Using flow cytometry (FC), others showed how GT platelets formed small aggregates when interacting with collagen through the α2β1 receptor.[20] Ristocetin-induced platelet agglutination (RIPA) mediated through the binding of VWF to GPIb is mostly normal in GT but can be reversible or occur in cycles. Clot retraction provides useful information on the type II and variant forms while measuring the closure time in the point-of-care Platelet Function Analyzer-100 (PFA-100) has largely replaced the bleeding time. Highly recommended for patient management is the International Society of Thrombosis and Haemostasis-Bleeding Assessment Tool (ISTH-BAT) that uses a standardized questionnaire to enable the clinician to establish a comprehensive clinical file.[21]

An inherent difficulty in characterizing type II and variant GT is accurately measuring αIIbβ3 expression on platelets. Currently, the binding of monoclonal antibodies (MoAbs) is assessed directly or indirectly by FC. However, copy number can be influenced by bivalent IgG MoAbs cross-linking adjacent αIIbβ3 complexes; a less likely occurrence at a low surface density of αIIbβ3 and although the use of Fab fragments is advisable their use is rare. The genetic variants causative of GT can also influence MoAb binding, either directly or through long-range allosteric effects. Thus, measures are approximate and many groups report results for a combination of MoAbs to different epitopes as well as performing Western blotting (WB) but as this review will underline the results are frequently disparate. Overlap of αIIbβ3 density between heterozygotes and normal donors confuses family studies. Key to understanding the heterogeneity within the type II and variant subgroups is to determine the degree to which the residual αIIbβ3 is functional (Table 1).

Ethnic Groups

While an estimate of 1 per million is the often given frequency of GT, it is more common in certain ethnic groups where consanguinity prevails. These include Jewish and Arab groups in Israel, French Manouche gypsies, and rural communities in India and Iran.[13,22–24] Studies on the French gypsies initially concerned the Strasbourg area in France; focussing on *ITGA2B* in view of trace amounts of β3 in WB, the authors identified a c.1544+1G>A substitution at the 5′ splice donor site of intron 15.[22] The result was an 8-bp deletion at the 3′-end of exon 15, a premature stop codon and a severely truncated αIIb. Analysis of family members revealed a strong association between a haplotype of five polymorphic loci covering a 4-cm region and the mutation suggesting a founder effect dating back to 300–400 years.[25] More recently, Zhou et al[26] screened 93 families with GT within the Chinese Han population with a lower level of consanguinity (18%) than other ethnic groups where GT is prevalent. Significantly, 74% of the patients had type I GT, 24% type II and only 2% had a variant form. A total of 43 genetic variants were identified. As had been noted earlier, patients with the same genotype sometimes presented with markedly different bleeding severities.[2,17]

Missense Mutations and Biological Heterogeneity of Type II GT

The type II subgroup is highly heterogeneous with respect to the amount of residual αIIbβ3, the genetic cause, the functionality of the residual integrin, and bleeding severity. The selected case reports detailed and summarized in Suppl Table I reflect this. The results show how the mechanism by which αIIbβ3 binds Fg after activation and its capture and storage in α-granules is clearly different. Neither is there a clear relationship between the ability of platelets to capture Fg and retract a clot, contradicting historical reports.[2,3] Also, variable is adhesion to surface-bound Fg, mostly studied using transfected cells.

Patients with ITGA2B Defects

The first patients genotyped for type II GT were independently described and concerned a homozygous p.R327H substitution in αIIb.[27,28] A twofold difference in residual platelet αIIbβ3 content between the probands suggests an influence of other undefined factors. The maturation or trafficking of pro-αIIbβ3 was shown to be impaired in transfected COS-7 or Chinese Hamster Ovary (CHO) cells. A homogeneous *ITGA2B* p.L183P mutation in a man with severe bleeding illustrates the difficulties of evaluating αIIbβ3 expression in some cases.[29] While complex-dependent MoAbs bound minimally, WB revealed 30–35% of each subunit with αIIb showing signs of proteolysis. Platelet αvβ3 was somewhat increased. Expressed in CHO cells the surface expression of the mutated αIIbβ3 was 60% as measured using subunit-specific MoAbs, whereas complex-dependent MoAbs or the activation-dependent MoAb, PAC-1, bound minimally. Both of the above mutations concerned the αIIb β-propeller.

Ambo et al[30] reported two Japanese women with platelets having residual αIIbβ3 who were homozygous for p.Q747P affecting the αIIb calf-2 domain. Platelets attached to surface-bound Fg at intermediate levels; clot retraction was near normal. In fact, p.Q747P is a founder mutation for type II GT in Japan. While other homozygous patients had a similar phenotype, compound heterozygosity combining p.Q747P and a splice site mutation leading to exon 18 skipping gave a lower 4–8% αIIbβ3 expression.[31] In CHO cells αIIbP747β3 bound Fg in the presence of an activating MoAb. Pulse-chase labeling with (35S)-methionine showed that its maturation was impaired. Next to be published was a young Spanish girl with

lifelong bleeding; her platelets had 10% residual αIIbβ3, low platelet Fg, but supported a much-reduced clot retraction.[32] She was compound heterozygous for a paternal splice site variant IVS5(+2)C->A transversion predicting a truncated protein and highly unstable mRNA, and a maternal p.C674R substitution that disrupted a disulfide bridge. Her heterozygous mother had platelets with only 30% of normal levels suggesting that her mutation had a dominant-negative effect. Expression of αIIbR674β3 in CHO cells confirmed the platelet phenotype with intracellular retention of pro-αIIbR674 by the chaperone BiP explaining a reduced transport to the Golgi apparatus and then to the surface membrane.[33]

Basani et al[34] reported type II GT linked to an *ITGA2B* p.P145L mutation reoccurring in families of American, Dutch and Chinese origins with mild to severe bleeding. Two Mennonite siblings had platelets with residual αIIbβ3 unable to bind PAC-1 and with a much-reduced Fg storage pool. Expression of αIIbβ3 was lower or absent in the Dutch and Chinese families with compound heterozygosity (5% and <1%, respectively,) again illustrating how second allele mutations help program αIIbβ3 density. For the Chinese patient, p.P145L was given by a different nucleotide change. Mutation scanning of αIIbP145 in transfected COS-1 cells showed that only p.P145K abrogated αIIb synthesis, while other mutants including p. P145L allowed pro-αIIbβ3 formation but interfered with maturation. Highly conserved, P145 locates to the upper surface of the β-propeller and is adjacent to the W3:4.1 loop identified as a potential ligand-binding site by Kamata et al[35] Mitchell et al[36] reported a boy with parents from Puerto Rico and Canada and with platelets containing 3–6% αIIbβ3 and an Italian/Sicilian family where MoAb binding showed 7–8% αIIbβ3 for four family members. As few clinical and biological details were given these cases are not included in Suppl Table I. For both families, αvβ3 was unaffected and WB confirmed residual mature αIIb and β3 in their platelets. The American proband was compound heterozygous for p.V298F near the second Ca^{2+}-binding domain in blade 5 of the αIIb β-propeller, and p.Y380* that prevents αIIb expression. The Sicilian family combined p.C674R in the αIIb calf-1 domain and a p.I374T missense mutation within the third Ca^{2+}-binding domain in blade 6 of the β-propeller. Interestingly, his father was homozygous for p.C674R while his children were compound heterozygous for both mutations. When the two β-propeller missense mutations were expressed in recombinant αIIbβ3 in 293T cells pulse-chase experiments showed much of the mutated pro-αIIb to be retained in the ER. Jayo et al[37] described a 2-year-old girl of Chinese nationality with lifelong bleeding, no platelet aggregation or clot retraction but with platelet Fg in the normal range. Her platelets expressed <10% residual αIIbβ3 but αvβ3 was normally present. Residual Fg binding indicated a qualitative defect. Sequencing of reverse-transcribed αIIb mRNA revealed compound heterozygosity: a c. C2829T transition giving a p.P912L substitution within the αIIb light chain and a c.C1750T transition in exon 17 of *ITGA2B* that gave p.R553* predicting truncated αIIb but also nonsense-mediated decay. The object of multiple reports in Asian patients, p.R553* is clearly a mutational hotspot. Transfection in CHO cells showed that αIIbβ3L912 reaches the surface but with a fivefold reduction in the rate of expression. Enigmatically, exontrap analysis of the mutant αIIb alleles in CHO cells showed that c.C2829T was also forcing skipping of exon 28; nonetheless, despite mRNA lacking exon 28 being present in platelets, the residual platelet αIIbL912β3 only concerned the full-length transcript.

More recent studies using in silico analysis and molecular dynamics simulations of variants affecting the αIIb calf-1 domain showed dynamic allosteric effects and that were mostly long range.[38] But of the seven variants studied, only the above-mentioned p.C674R mutation gave type II GT. Pillois et al[39] also reviewed structural modifications in and around the αIIb genu, a region that is the fulcrum of the bent "resting" state of αIIbβ3. This literature survey covered mutations extending from the lower part of the β-propeller through the thigh and upper calf-1 domains identifying 37 cases involving 16 missense mutations all causal of type I GT with only p.A446P (within the seventh blade of the β-propeller) and p.C674R allowing αIIbβ3 expression and type II GT. Static in silico modeling confirmed how modifications of structuring H-bonds were the major cause of GT in the thigh domain, whereas in calf-1 long-range effects predominated.

Patients with ITGB3 Defects

The first reported β3 mutation in a type II patient concerned a Chinese girl from a consanguineous family with a lifelong history of severe bleeding.[40] Clot retraction was absent, but platelet Fg was 36% of normal. Platelet αIIbβ3 expression was reduced to 6–14% and αvβ3 was likewise reduced. Sequencing of PCR-amplified cDNA identified a homozygous p.C374Y mutation in *ITGB3*. Transient transfection in CHO cells confirmed a 85–90% reduction in the

surface expression of the mutated αIIbβ3 yet the cells retained an ability to attach to surface-bound Fg. Novel integrin β3 subunit missense mutations were then reported for unrelated Japanese patients with type II GT and lifelong mild bleeding.[41] For all patients, platelet aggregation was absent but clot retraction was in the normal range. Platelet expression of αIIbβ3 ranged from 7.5% to 20%. The first patient, an elderly woman, was homozygous for p.H280P while the second and third patients, both male, were compound heterozygous for the same mutation combined with p.G579S and p.C560F, respectively, both in the EGF-3 domain. When expressed in CHO cells there was a significantly reduced αIIbβ3 expression for p.H280P and p.G579S but surprisingly a near normal expression for p.C560F. The expression of αvβ3 in the CHO cells was also affected. Preliminary information was provided that p.C560F and p.G579S in the EGF-3 domain were activating mutations, but their contribution to the residual αIIbβ3 on the patients' platelets is unknown. Other cases in Japan have since been reported to have the p.H280P variant which may represent a founder mutation.

A girl whose mother was diagnosed with von Willebrand disease (VWD) had lifelong bleeding but normal plasma VWF levels and platelets that agglutinated with ristocetin but which failed to aggregate with physiologic agonists.[42] Her platelets bound subnormal amounts of subunit-specific MoAbs to αIIb and β3 yet bound neither AP-2, a complex-specific MoAb nor on activation did they bind Fg. Western blotting confirmed about 10% of control platelet levels of normally migrating αIIb and β3. Notwithstanding, her platelets mediated a normal clot retraction. The patient was compound heterozygous for mutations in *ITGB3* thereby showing how two inherited platelet disorders (IPDs) can occur in the same family. A paternal c.G867C868 dinucleotide deletion was predicted to give a Fs and a stop codon at p.Q267; a 50% expression of normally sized αIIbβ3 in her father's platelets confirmed the non-expression of the truncated β3. Her second mutation was a missense p.L262P substitution; the introduction of a proline (P) was predicted to introduce a β-turn and to alter β3 conformation. In transfected cells, αIIbβ3P262 was unstable and only small amounts reached the surface. Pulse-chase experiments confirmed that αIIbβ3P262 formed in the ER, but that maturation was markedly delayed. The cells retracted a fibrin clot but failed to bind to immobilized Fg although normally attaching to and spreading on Fn and Vn. Nair et al[43] described a young boy with bleeding since birth. His platelets bound only trace amounts of MoAbs to αIIbβ3, but in WB αIIb and β3 were 10% to 30% of normal, unusually his β3 migrated as a high molecular weight band, thought to be β3 dimer as its migration normalized after disulfide reduction. Platelet Fg was <20% of normal. He was homozygous for a p.C506Y mutation in β3; this created an unpaired cysteine in the EGF-2 region. Different models proposed that C506 could link with C495 or C501 in β3.

An elderly Afghanistan woman from a consanguineous family had a lifelong history of mucocutaneous bleeding.[44] Platelet aggregation was absent and clot retraction only 10% of normal; platelet adhesion to immobilized Fg was much reduced with the residual interaction blocked by an αIIbβ3 antagonist. Her platelets expressed αIIbβ3 at 24% of normal levels by FC but failed to bind Fg or PAC-1 after activation with ADP. Thus, once more, the residual αIIbβ3 had both quantitative and qualitative defects. Platelet Fg was virtually absent. Sequencing of cDNA revealed a homozygous p.T176I mutation in *ITGA2B*. Interestingly, αIIbI176 locates to the blade 3 1:2 loop of the αIIb β-propeller suggesting that it induces conformation changes through allosteric effects rather than direct interference. Expression of the mutated αIIbβ3 in COS-7 or CHO cells was confirmed as being much reduced.

Morel-Kopp et al[45] reported a homozygous p.L196P β3 variant in a French woman with type II GT and mild bleeding. Her platelets expressed only 4100 copies of αIIbβ3 yet her intra-platelet pool of Fg was as high as 50%. A low expression of αIIbβ3P196 was confirmed in transfected CHO cells with increased cytoplasmic pro-αIIb suggesting altered pro-αIIbβ3 processing. Intriguingly, cells expressing αvβ3P196 failed to attach to surface-bound Fg, spread, form focal contacts, phosphorylate FAK (focal adhesion kinase) or retract a fibrin clot; events that were normal for cells with αIIbβ3P196. Nevertheless, αIIbβ3P196 failed to bind soluble Fg or PAC-1 when incubated with an activating MoAb. A second French family heterozygously expressing p.L196P and followed by us in Bordeaux had a similar phenotype. The proband, an elderly man with lifelong bleeding, had platelets with 8% αIIbβ3 but WB again revealed a substantial pool of Fg a finding suggestive of αIIbβ3 recycling. His mother, a sister and son, all heterozygous for β3 p.L196P had platelets with intermediate levels of αIIbβ3. A predicted second mutation in the proband was confirmed when later DNA sequencing revealed a p.C598Y substitution.[15,17] Previously reported

for a French type II patient, p.C598Y was said to be partially activated.[46] Thus, the Bordeaux patient is an example of type II GT given by compound heterozygosity for two contrasting mutations each of which contributes to residual αIIbβ3 expression.

Splice Site and Other Mutations in Type II GT

Mutations in the calf-1 and calf-2 domains of αIIb are not directly involved in ligand binding, but small discontinuous contacts link them to the EGF-3, EGF-4 and β-tail domains of β3.[47,48] A del-insert in exon 25 localizing to calf-2, and a transition in the acceptor splice site of intron 19 leading to in-frame skipping of exon 20 and affecting calf-1, resulted in type II GT.[49] In each case, the mutated pro-αIIb complexed with β3 in transfected BHK cells, but little mature αIIb resulted. Golgi mediated complex mannose glycosylation was not seen and immunolocalization confirmed that mutated αIIbβ3 was mostly retained in the ER. A homozygous c.2348+5G>C transversion in intron 23 of *ITGA2B* in an elderly male with type II GT (case 8 in the series reported by Nurden et al[17]) retained attention. Skipping of exon 23 was confirmed using a hybrid minigene transfection assay. Real-time PCR and specific primers showed major changes in spliced mRNAs together with a residual full transcript leading to the continued presence of 8–10% functional αIIbβ3 in his platelets. Although his platelets failed to aggregate, clot retraction was partial and platelet Fg was abundant and suggestive of αIIbβ3 recycling. Despite his residual αIIbβ3, he had severe bleeding when young receiving arm-to-arm transfusions in wartime. Much later, when elderly, he had severe GI bleeding. A Moroccan girl from a consanguineous marriage with frequent petechia, bruising and mucocutaneous hemorrhages possessed platelets with about 10% αIIbβ3 that bound Fg in small amounts when stimulated.[50] A homozygous c.G188A substitution at the splice donor site of intron 1 led to the use of alternative intronic donor sites leading to both a stop codon and nonsense-mediated decay and accounting for the low expression of αIIbβ3 in her platelets.

Mutations Reducing αIIbβ3 Expression and Overlapping That of Variant Forms

The classification of type I; type II and variant GT on αIIbβ3 numbers alone is purely arbitrary for borderline cases. For example, Jackson et al[51] described a girl with platelets unable to bind Fg or aggregate when stimulated but with a normal clot retraction. Her platelets expressed 27% αIIb, 16% β3 with subunit-specific MoAbs, but with minimal binding of MoAbs to complex-dependent epitopes. In WB, her platelets contained about 30% of the normal levels of each subunit. Her αIIbβ3 was unstable and failed to express LIBS-binding sites when challenged. She had a homozygous p.S162L substitution in β3 with a destabilizing effect on αIIbβ3. Highly conserved, S162 lies between the metal ion-dependent adhesion site (MIDAS) domain and a ligand regulatory loop near the so-called synergistic metal ion-binding site (SyMBS) of β3. Expression of mutated αIIbβ3L162 in COS-7 cells led to pro-αIIbβ3 synthesis but delayed maturation with only small amounts of mature αIIbβ3 and a rapid proteolysis of both subunits. This patient nicely emphasizes how the αIIbβ3 determinants responsible for platelet aggregation and clot retraction are distinct. Platelets of a young Japanese woman (Osaka-12) with moderate mucocutaneous bleeding also sustained a residual clot retraction despite failing to aggregate.[52] Her platelets expressed 36–41% αIIb and β3 as assessed using MoAbs to the subunits but bound only 13% of a complex-dependent MoAb. Her αIIbβ3 failed to bind PAC-1 when activated and aberrantly expressed LIBS epitopes. She was compound heterozygous for a p.Y143H substitution in the W3 4–1 loop of the αIIb β-propeller and a null allele; the latter largely accounting for the low αIIbβ3 density. Transfected HEK cells normally expressed αIIbH143β3 but failed to bind PAC-1 or Fg in the presence of an activating MoAb. Identical results obtained for αIIbA143β3 suggested that tyrosine is essential at position 143. Her phenotype strongly resembled that of the so-called KO variant with an Arg-Thr insertion between 160 and 161 of αIIb (Figure 1). Notwithstanding, cells transfected with αIIbH143β3 underwent a partial clot retraction, whereas the KO variant abrogated it.

Variant Forms of GT and αIIbβ3 Functioning

Activation of αIIbβ3 by "inside-out" signaling involves conformational changes in the subunit tails that when transmitted to the extracellular domains enable ligand binding.[8] This and/or clustering of αIIbβ3 initiate "outside-in" signaling and responses such as platelet spreading on Fg and clot retraction where αIIbβ3 transmits forces generated by actin and myosin. Important is

phosphorylation of the β3-cytoplasmic tail and c-Src and RhoA activities with retraction regulated by a molecular switch involving β3 dephosphorylation and cleavage at Y759.[53] Variants that mostly possess 50% or more αIIbβ3 in their platelets show loss of function and may concern one or both signaling pathways.

Mutations Affecting Extracellular Domains

Loss-of-function mutations that block the ability of αIIbβ3 to bind Fg when stimulated (Figure 1) have largely contributed to our understanding of how ligand binding occurs. The CAM variant was the first genotyped GT variant, a homozygous p.D119Y substitution causing loss of a divalent cation structured Fg-binding site within the MIDAS domain of β3.[54] Also of major importance were homozygous p.R214Q or W substitutions within the ADMIDAS (adjacent to MIDAS) domain of β3.[55,56] Here, αIIbβ3 was hypersensitive to divalent cation chelation and unable to bind Fg. In all cases, clot retraction and the platelet Fg storage pool were severely reduced. Yet treatment of αIIbβ3 with dithiothreitol restored the Fg-binding capacity. These extracellular variant forms occurred in patients with clinically severe GT. While mostly the mutations affect β3, the Japanese KO variant (also associated with severe bleeding) is an exception.[57] Here, platelets fully expressed αIIbβ3 that is unable to bind Fg or PAC-1 when stimulated and clot retraction is subnormal. In addition, αIIbβ3 was refractory to the activating MoAb PT25. A homozygous p.R,T160-161 insert on the upper face of the αIIb β-propeller localized to the C146-C167 loop; a purported ligand-binding site. Alanine substitution of oxygenated residues within this loop and expression in 293 cells revealed that only D163A abolished ligand-binding function.

Cytoplasmic Domain Mutations

Platelets of an elderly Argentinian man with a mild bleeding syndrome failed to bind Fg or aggregate with ADP despite 44% of normal levels of αIIbβ3.[58] Uniquely, he experienced severe limb deep vein thrombosis (DVT) after a long air flight.[3,19] His αIIbβ3 was predominantly in an internal platelet pool. Possessing a heterozygous β3 p.S752P cytoplasmic domain mutation his platelet Fg and clot retraction were near normal. While platelet aggregation and soluble Fg-binding remained minimal, α-granule Fg was secreted attached to αIIbβ3 of internal membranes after platelets were challenged with thrombin.[59] An RGDS-affinity column retained detergent solubilized αIIbβ3 from his platelets confirming an intrinsic Fg-binding capacity.[58] As his daughter's platelets aggregated normally while retaining 50% αIIbβ3 expression, the father was hypothesized to have a "nul" allele. A key to the effect of this mutation is loss of binding of kindlin-3, an essential protein for αIIbβ3 activation.[8,15] A similar phenotype in a black American girl with severe bleeding and platelets expressing intermediate levels of non-functional αIIbβ3 was given by compound heterozygosity associating a null allele and a c.2268C>T, p.R724* variant giving a truncated protein missing the final 39 amino acids of the β3 cytoplasmic domain.[60] This patient lacked not only the kindlin-3 binding site but also a talin-binding domain essential for αIIbβ3 signaling.[61] CHO cells expressing αIIbβ3R724* failed to spread on Fg associated with a loss of FAK phosphorylation.

Extracellular Activating Mutations in αIIbβ3

Activating mutations within extracellular domains mostly concern cysteine residues, and β3 is rich in conserved disulfides of which 31 localize to the EGF domains. Pionering was a homozygous p.C560R substitution in the β3 EGF-3 domain of a Frenchman whose platelets expressed 20% αIIbβ3 (Figure 1).[62] He had a mild form of GT despite a long bleeding time and mild thrombocytopenia (100 to 150×10^9 platelets/L). Although platelet anisotropy was noted, platelet size changes were not striking. The patient underwent a kidney transplant when middle-aged and required multiple transfusions. The residual integrin on his platelets spontaneously bound anti-LIBS MoAbs and PAC-1. Strikingly, his circulating platelets had significant amounts of surface-bound Fg organized in clusters.[62] Yet his platelets showed no visual signs of being activated and secreted normally with thrombin when a delayed but residual platelet aggregation occurred. Clot retraction was severely reduced, but platelet Fg was normal. A stable CHO cell line expressing αIIbβ3R560 mimicked the properties of the patient's platelets, and spontaneously bound PAC-1. The cells attached readily to surface-bound Fg spreading with increased velocity. Interestingly, the patient died when elderly from a thrombotic event. Mor-Cohen et al[63] identified a founder p.C549R variant in β3 in consanguineous Jordanian Arab families with type II GT and severe

bleeding. Platelet aggregation was markedly impaired and clot retraction reduced. Transfected BHK cells showed that surface αIIbβ3 spontaneously and maximally bound PAC-1, while WB showed a higher than normal presence of pro-αIIb much of which was retained in the ER. The affected C549 forms a highly conserved disulfide with C558 in EGF-3. Kamata et al[64] investigated the role of β3 disulfides by systematically substituting cysteine with serine followed by the expression of the recombinant αIIbβ3 in CHO cells. Disruption of a single disulfide in the EGF domains commonly led to activated integrin, while disruption of only 2 of 13 disulfides outside this region led to activation.

But not all activating mutations involve cysteine residues (Figure 1). An intriguing patient was reported by Vanhoorelbeke et al[65] and involved a heterozygous p.S527F substitution in the I-EGF-3 domain of β3. The proband was a young Arab man with mild bleeding and much decreased platelet aggregation with ADP despite normal αIIbβ3 numbers. Most studies were performed using transfected CHO cells with the mutated integrin spontaneously binding Fg and PAC-1. Intriguingly, the cells formed aggregates when stirred with Fg. Anti-LIBS MoAbs bound spontaneously to αIIbβ3F527 although their binding increased when RGDS was present. β3F527 probably causes steric hindrance preventing the I-EGF-3 domain from entering a cleft necessary for the bent integrin resting conformation. A 6-month-old girl with mucocutaneous bleeding had platelets unable to aggregate or retract a clot although platelet Fg was present.[66] Amounts of αIIb and β3 were substantial although estimated as below 50%. ADP stimulated platelets failed to bind PAC-1 but strangely some Fg binding was noted. Sequencing revealed compound heterozygosity for p.D119Y and p.M124V missense mutations within the β3 MIDAS domain. Homozygous p.D119Y gives rise to the classic CAM variant (see the preceding section).[54] CHO cells transfected with αIIb and β3V124 showed increased adhesion and spreading on immobilized Fg despite a low surface expression of the mutated integrin. They also formed aggregates when agitated with soluble Fg, suggesting that αIIbβ3V124 was constitutively active. The failure of the patient's platelets to spontaneously aggregate was probably due to the dominance of the loss-of-function αIIbβ3Y119. About 50% of the recombinant αIIbβ3V124 was present as pro-αIIbβ3 in the transfected cells showing a delayed maturation.

Questions are raised as to why the platelets of patients with activating mutations do not obstruct the microcirculation. One possibility is that the Fg binds monovalently and thereby blocks platelet-to-platelet cohesion.

Macrothrombocytopenia (MTP) and a GT-Like State

The cases in the earlier section possess gain-of-function mutations with AR inheritance but without significant abnormalities in platelet count and size. In contrast, an increasingly noted group of patients associate single allele gain-of-function variants of αIIbβ3 with AD transmission and MTP (Table 1) (Figure 1). In the early 1990s, my colleagues and I reported an unique case with mild bleeding, mild thrombocytopenia (100–160 × 10^9/L), some enlarged platelets and defective platelet aggregation.[67,68] Although surface αIIbβ3 was 10–20% of normal, his platelets mediated clot retraction and stored Fg; a normal αIIbβ3 internal pool suggested defective recycling to the surface. Mutation screening first highlighted a heterozygous p.R995Q mutation within the highly conserved GFFKR sequence of the αIIb cytoplasmic tail. Expression of αIIbQ995β3 in Cos-7 and CHO cells confirmed a reduced transport to the cell surface. Although the mutated αIIbβ3 did not bind Fg spontaneously, there was weak binding of PAC-1. Significantly, αIIbR995 forms an intracellular salt bridge with β3D723 and weakening or breaking this link upgrades the activation status of αIIbβ3 (Figure 1).[69] Family studies showed that R995Q came from his father, but discrepancies in their αIIbβ3 expression predicted a non-identified second maternal mutation in the son. This was later identified as a pathogenic 13bp intronic deletion near the splicing acceptor site for exon 15 of *ITGA2B* (its mechanism is discussed later in the review).[17] Subsequently, patients in five Japanese families with MTP and AD inheritance were reported with a heterozygous p.R995W substitution.[70] With a - normal second allele platelet surface αIIbβ3 expression was 50% to 70% of normal. Spontaneous PAC-1 binding to platelets or transfected 293T cells confirmed that αIIbβ3 was in a partially activated conformation. Furthermore, when they were plated on Fg they spontaneously formed proplatelet-like protrusions – a finding previously reported for a patient with a heterozygous β3 p.D723H variant, D723 being the partner for R995 in the salt bridge linking the two cytoplasmic domains.[70,71] However, enigmatically this latter patient while having MTP did not have a significant platelet aggregation defect. Later, three

French families were reported with MTP, GT-like defects and heterozygous variants affecting the salt bridge.[72] Two families possessed p.R995W in αIIb and the third p. D723H in β3. In silico modeling confirmed that both variants created steric interference, weakening the intracytoplasmic ionic clasp but with secondary influences extending to other nearby amino acids. For all the above patients both the bleeding syndrome and the MTP were moderate. Nevertheless, their phenotypic variability was striking. Platelet αIIbβ3 expression ranged from 25% to 50%. Intriguingly, electron microscopy showed many enlarged round platelets often with a heterogeneous distribution of α-granules many of which showed signs of fusion. Early MK maturation was normal, but proplatelets were short and had enlarged tips. Recently, Morais et al[73] have reported more European cases with AD variants affecting the salt bridge and all with mild forms of MTP; again the authors emphasized the phenotypic variability of such cases and which extended to the degree of spontaneous activation of αIIbβ3.

Mutations in the single-pass transmembrane domains of αIIbβ3 can also give MTP. Members of two Italian families associated a moderate AD thrombocytopenia with large platelets, defective platelet function and moderate/severe bleeding.[74] The platelets associated a low surface αIIbβ3 expression with minimal spontaneous PAC-1 binding. Platelet spreading on Fg was impaired as was shear-dependent platelet adhesion to collagen. Yet clot retraction was normal. A novel heterozygous mutation in *ITGB3* led to a large in-frame deletion (p.D621-E660). Truncated β3 was noted in their platelets and a dominant negative effect was hypothesized. A heterozygous p. L718P mutation in β3 was reported in a Spanish woman with severe bleeding and MTP.[75] Platelet aggregation was much reduced and a secretion defect was found with little surface expression of P-selectin and CD63. Surface αIIbβ3 was selectively reduced. Her platelets spread poorly on Fg with defective lamellipodia. CHO cells expressing recombinant αIIbβ3P718 directly bound Fg and PAC-1 and spontaneously agglutinated in the presence of Fg. When plated on Fg, they formed long extensions often with a swelling at the tip. We have reported a heterozygous β3 p.L718del in a woman with a GT-like syndrome with MTP; her platelets did not spontaneously bind PAC-1.[76] Platelet anisotropy was again associated with enlarged "fused" α-granules. Recently, Morais et al[73] have added p.G976V in αIIb and p.T720P, p.H722P and p.R734H (p. R760H) in β3 to the list causing MTP with GT-like functional defects (Figure 1).

These patients raise important questions. Quite clearly, the conformational status of αIIbβ3 influences MK reactivity with extracellular proteins in the bone marrow and affects platelet biogenesis, but the relationships between the changes caused by the above mutations and the step-by-step exposure of the determinants that allow soluble Fg binding remain to be determined. Furthermore, an argument can be made that "Glanzmann thrombasthenia-like macrothrombocytopenia" should define a distinct disease in the way that "platelet-like VWD" has common usage to define upregulated binding of VWF to GPIb.[77,78]

Large Series and Frequency of GT Subtypes

As sequencing capacity progressed, large cohorts of GT patients were genotyped and have considerably extended our insight into the mutation repertoire of this disease. Initially, Peretz et al[79] examined the molecular basis for GT in 40 families with a high degree of parental consanguinity from Southern India. Most patients had type I GT and 23 mutations (13 in *ITGA2B* and 10 in *ITGB3)* were identified. Founder effects were confirmed by haplotyping families with repeated p.L117W and p.Y281* mutations reflecting the closeness of the communities. Subgroups were not identified although a β3 p.R67Q variant had normal αIIbβ3 expression and clot retraction. Missense mutations affecting αIIb primarily concerned the β-propeller or thigh domains while those in β3 were distributed within the PSI, β-I and hybrid domains. Nelson et al[80] followed with a further 15 patients from the same communities. Significantly, 14 showed no αIIb in WB and 10 had no β3, while for 4 others, β3 was severely reduced. Platelet Fg was undetectable for 13 patients, severely reduced in 1; only 1 patient had normal platelet surface αIIbβ3 expression and normal Fg. Disease-causing mutations were identified for 11 patients with type I GT predominating.

Jallu et al[81] examined phenotype/genotype relationships in 24 Paris patients with 19 classed as type I and 3 as type II. They identified 29 mutations, their validation included expression in COS-7 cells, in silico analysis and mRNA processing. Noteworthy was a p.Q595H substitution that failed to change αIIbβ3 expression in COS-7 cells despite its absence from platelets; in fact, the causal c.1878G>T transversion led to altered mRNA splicing

and skipping of exon 18 in megakaryocytes. One type II patient was compound heterozygous for an *ITGA2B* c.3060+2T>C transition leading to deletion of exon 29. For another, only a heterozygous *ITGA2B* p.R946* nonsense mutation was detected with a predicted but non-identified second allele mutation allowing residual expression of αIIbβ3. Two type II patients had platelets with substantial pools of Fg (51% and 83%, respectively,). Sandrock-Lang et al[82] in Germany reported 19 GT patients including 11 type I and 2 type II. They found 27 mutations. One type II patient, a male Arab with severe bleeding, was compound heterozygous for the *ITGA2B* p.L183P missense mutation and a c.3092delT within exon 30 of *ITGA2B* introducing a Fs and a protein prolonged in the cytoplasmic tail. The second patient combined a p.I154M missense mutation and a known p.R597X stop codon within *ITGA2B*. Both missense mutations affected the αIIb β-propeller.

In Bordeaux, we led a large international consortium that genotyped members of 76 affected families.[17] Sequencing in a national sequencing center (Genoscope) identified 78 disease-causing variants; in parallel 4 large deletions or duplications were found using quantitative real-time PCR. Most families were from France; others were from Argentina, Canada, Morocco, Spain, Switzerland and the USA. Many had a history of blood transfusions or rFVIIa in response to bleeding or preventively. Occasionally bleeding was mild and wide variability was highlighted. Phenotyping assigned 58 families with type I GT, 9 with type II and 8 had variant forms. *ITGA2B* missense mutations mainly affected the β-propeller and caused type I GT but gave type II in three families. Of the calf-1 and calf-2 domain mutations, p.V903F reoccurred in two nonrelated French families with type II GT and compound heterozygosity; expression studies confirmed an altered maturation of pro-αIIbF903β3. An elderly French male with type II GT associated an *ITGA2B* p.G792E missense variant with a stop codon. Heterozygous p.C674R associated with a null allele in a patient with type II GT was first reported with homogeneous expression in Spanish patients with type II GT (Suppl Table I).[32] Noteworthy were missense p.Q595H and p.F160V mutations given by nucleotide transitions that also interfere with splicing as previously noted for p.Q595H by Jallu et al.[81] While homozygous β3 mutations mostly gave type I GT, a heterozygous p.R37C variant associated with p.R143* in a patient with type II GT. When expressed in COS-7 cells, intracellular pro-αIIbβ3C37 predominated. While most β-I domain mutations gave type I GT, the type II subgroup was occasionally seen. Four missense mutations localized to the disulfide-rich β3 EGF-3 and EGF-4 domains but gave type I GT. Multiple stop codons and splice site variations affected both genes. Particularly interesting was a French patient heterozygous for the Manouche gypsy mutation with 50% αIIbβ3 but no platelet aggregation and who was also homozygous for a *FERMT3* mutation.[83] *FERMT3* encodes kindlin-3 and its absence rendered the residual αIIbβ3 non-functional. Clinically, his severe bleeding phenotype was associated with mild immunodeficiency characteristic of leukocyte adhesion deficiency type III (LAD-III) syndrome given by mutations of *FERMT3*. A Jamaican patient with normal αIIbβ3 and no mutations in *ITGA2B* and *ITGB3* was later found by my group to be homozygous for a mutation in *RASGRP2* abrogating CaLDAG-GEFI function.[84] Careful phenotyping is needed to exclude *RASGRP2* and *FERMT3* defects in cases with variant GT (Table 1).

Exon skipping occurred in three families with *ITGA2B* variants and in five families with *ITGB3* variants. Enigmatic was a heterozygous 13-bp single allele deletion in intron 14 first reported by Jallu et al[81] and affecting seven families with type I GT and no obvious ancestral links. These included three Catalan family members confirming its inheritance. A hybrid minigene transfection assay in COS-7 cells showed that it invoked a new cryptic 5′ splice site causing a 2-bp deletion in the mRNA, a reading-frame shift, and a premature stop codon after 105 aberrant amino acids. The abnormal mRNA was also predicted to be a target for nonsense-mediated decay.

GT Platelets and Fibrin

While early studies confirmed a role for αIIbβ3 in fibrin-dependent clot retraction, the wide diversity in the mutation-dependent responses in type II and variant GT imply that Fg and fibrin interact with different sites on αIIbβ3. Podolnikova et al[85] showed how a cluster of newly exposed amino acids in the Fg γ370-381 sequence mediate the interaction of immobilised Fg or fibrin with αIIbβ3. Much later, the same group revealed how this cluster had several potential contact sites within the αIIb β-propeller.[86] These differ from the activation-dependent sites on αIIbβ3 that mediate binding of the γ404GAKQAGDV411 peptide or RGDS sequences of Fg and which respectively localize to the extremity of the αIIb β-propeller and to the β3 β-I domain.[8,35] The situation clarified when the GPVI collagen

receptor on platelets was also shown to recognize fibrin and surface-bound Fg.[87,88] Thus, not only does GPVI participate in platelet binding to the vessel wall, it also intervenes as a more generalized signaling receptor in thrombus formation. GPVI not only may explain the accumulation of platelets of type I GT patients on fibrin, it may also intervene in the microthrombus formation seen for some patients. Nonetheless, the genetic absence of GPVI does not abrogate clot retraction and recent studies show that GPVI and αIIbβ3 play complementary non-redundant roles.[89,90] GPVI may also have a physiologic role in inflammatory states, cancer and other acquired states in GT.[19,91]

Platelet Proteome

Some 40 years ago, McGregor et al[92] studied platelet membrane glycoproteins in GT by associating carbohydrate-specific or protein-specific labeling procedures with high-resolution two-dimensional gel electrophoresis. Glycosylation defects of residual GPIIb and GPIIIa extended to other components of the platelet surface in type I and type II GT; suggesting an unexplored source of functional heterogeneity. Loroch et al[93] investigated platelet function and the platelet proteome in type I GT patients with homozygous Fs mutations in *ITGA2B* leading to premature stop codons. Mass spectroscopy validated about 3% αIIb and 5% β3 in platelets of the two patients studied. Signaling proteins such as kindlin-3, CalDAG-GEFI, and Src were all normally present as were αv, GPIb-IX and GPVI. Downregulated in platelets were Fg, FXIIIB, carboxypeptidase-B2 (a fibrinolysis inhibitor) and plasminogen despite normal plasma levels. FcγRIIA and laminin-α4 were upregulated. Megakaryocytes synthesize FXIIIA that is stored in platelets but FXIIIB is taken up – perhaps in association with Fg. Plasminogen interacts with carboxypeptidase B suggesting a common endocytic pathway. FcγRIIA increase was linked to increased dense granule secretion in response to immunoglobulin complexes. Recently, Blair et al[94] used state-of-the-art mass spectrometry (MS) and metal-tagged antibodies to study activation-dependent changes on GT platelets. They highlighted the elevated levels of CD9, CD42a and CD63 while the density of CD21, CD154 and GPVI was low. CD9 and CD63 are granule markers that were increased, whereas the normal expression of P-selectin, another granule membrane protein, was unexplained. But perhaps more significant is that the bulk of the platelet proteome appeared to be unchanged. It would be interesting to examine a wider range of patients including variant forms.

Diagnosis and High-Throughput Sequencing

The advent of high-throughput sequencing (HTS) for diagnosis of IPDs has greatly accelerated gene identification and mutation analysis as well as posing new sets of challenges.[77,78,95,96] Precise genotyping in GT is required for optimal clinical management. It also provides insight into αIIbβ3 structure–function relationships, facilitates family planning and assists in the development of new treatments including gene therapy. Targeted sequencing (TS), whole-exome sequencing (WES) or whole-genome sequencing (WGS) are all viable options with TS against a selected gene panel including *ITGA2B* and *ITGB3* the initial and most economic choice for GT. Potential users should consult the annually updated curated gene panel from the ISTH SSC.[95] The gene panel should also include *RASGRP2* encoding CaLDAG-GEFI and *FERMT3* encoding kindlin-3 whenever the clinical profile suggests GT variant-like platelet function. While WES can cover intronic flanking regions and splice sites its basic advantage is to allow the detection of new genes. A negative result in TS and WES should be followed by WGS when possible, but both WES and WGS require extensive computer-based facilities and technical back up.[96] Key is the classification of variants into those with known pathogenicity, likely pathogenic and those with uncertain pathogenicity, while copy number variations represent a separate challenge as do variants deep within introns (reviewed in Ver Donck et al[96]). Particularly interesting is that for some patients bleeding results from combinations of homozygous or single allele mutations on two or more different genes that may or may not involve *ITGB3* or *ITGA2B*. Variant analysis should also include those of modest pathogenicity that on their own do not cause a sufficient inhibition of platelet function to disturb hemostasis but which may nonetheless modulate disease severity. The identification of gene modifiers that influence disease pathogenesis and penetrance in GT is a major future challenge.

Extent That Mutations of *ITGA2B* and *ITGB3* are Found in the General Population

In a pioneering study, Buitrago et al[97] analysed next-generation sequencing data (WES and WGS strategies) from five international databases and assessed the frequency of missense mutations affecting αIIb and β3 in 16,108 normal individuals and compared their findings to

111 missense mutations reported in the literature as causing GT. Their survey identified 114 missense variants in *ITGA2B* (affecting 11% of the amino acids) and 68 in *ITGB3* (affecting 9% of the amino acids). Of these, 96% had minor allele frequencies (MAF) of <0.1% while a significant proportion were predicted to be deleterious. Strikingly, none of the missense mutations known to cause GT were present. This study showed that 1.3% of the population has a variant of *ITGA2B* or *ITGB3*. The authors concluded that on the basis of their rarity, the bulk of the amino acid substitutions had entered the population recently. We hypothesized that if this applied to the GT-causing mutations in our international cohort, then many patients would share non-damaging synonymous and non-synonymous SNPs in both genes.[17,98] We therefore quantified non-disease causing genetic variants including human platelet alloantigen (HPA) polymorphisms within the sequenced regions of *ITGA2B* and ITGB3.[98] Our results show that many damaging and GT-causing mutations share SNP haplotypes with little genetic variation in unrelated families of wide geographical origins: a result that agrees with many GT-causing mutations being of recent origin. It is probable that a high proportion of private mutations outside ethnic groups and closed communities will disappear due to natural selection and that the mutation profile in GT is constantly changing.

Treatment

If local measures fail, GT patients receive platelet transfusions or recombinant factor VIIa (rFVIIa) given with or without anti-fibrinolytics. George et al[3] evaluated treatment for 112 patients followed in Paris and reported that a majority had received such measures at least once. However, the extent of bleeding was difficult to predict and had no dependence on whether the patient had type I, type II or variant GT. This has been confirmed many times; bleeding may vary considerably in severity and frequency even among members of the same family.[17,26] One situation where treatment is adapted is in the presence of isoantibodies to αIIbβ3 with rFVIIa advised for patients with inhibitors and refractoriness.[99,100] Occasional adverse events linked to rFVIIa have mostly involved DVT.[19] Al-Battat et al[101] showed that platelets from type I patients compete with and hinder the hemostatic efficacy of transfused platelets and emphasized the importance of maintaining a high proportion of transfused platelets. But the major risk of platelet transfusion remains immune sensitization when the integrin is lacking in type I GT.[102,103] In this respect, genotyping and biological testing helps choice of treatment.

Pregnancy is rare in women with type I GT both through individual choice and the long-term use of hormonal therapy. Apart from bleeding risk, pregnancy may lead to immunization following passage of fetal cells into the mother's circulation.[103] Transplacental passage of maternal antibodies may cause thrombocytopenia in the fetus. Leticée et al[104] described pregnancy in a woman with type I GT and isoantibodies following blood transfusions. As the husband's platelets fully expressed αIIbβ3, the fetus was an obligate heterozygote. Fetal death occurred at 31 weeks due to intracranial hemorrhage. Although absent from platelets when αIIbβ3 and αvβ3 are lacking, HPA-systems on β3 are a factor on platelets and other cells that express αvβ3 when the gene defects affect *ITGA2B* as well as in type II GT and for variant GT. The risk of antibody formation will remain in the event of alloantigen incompatibility. Santoso et al[105] found that intracranial hemorrhage in mothers with fetal/neonatal alloimmune thrombocytopenia (FNAIT) caused by anti-HPA-1a antibodies, was predominately due to their reactivity with αvβ3 in endothelial cells. Evidence for anti-HPA-1 alloantibodies reactive with αvβ3 in GT was provided by Fiore et al[106] who detected them in polytransfused patients with the French Manouche gypsy mutation (on *ITGA2B*) with αvβ3 in platelets and other cells expressing β3 homozygous for the rare HPA-1b allele. Thus HPA typing is recommended in GT and especially for affected women.

How Other Gene Defects Can Modify the GT Phenotype

High-throughput gene screening allows the simultaneous evaluation of a wide variety of gene variants that can influence the GT phenotype. In addition to the examples already illustrated in this review and in the literature for other IPDs,[96] it allowed us to find a heterozygous p.G146R mutation in *TUBB1* encoding β-tubulin in a patient who combined MTP and type I GT caused by compound heterozygosity of p.P189S and p.C210S in *ITGB3*.[107] It is becoming more and more clear that bleeding in GT depends not only on the mutations that define the pathology but involves variants of a wide variety of genes involving coagulation factors and the vasculature as previously discussed for other IPDs.[77,78] For example, Deshpande et al[108] identified a combination of GT and

mild FVII deficiency in a 13-yr-old Indian boy with GT and a moderately severe bleeding syndrome. His platelets lacked αIIbβ3 due to compound heterozygosity for p. Q132K and p.K650T in *ITGB3*. Three polymorphisms (two in the promoter region) of *F7* were predicted to cause the FVII deficiency. In a pioneering study, Owaidah et al[109] performed targeted sequencing of 72 family members of GT patients in Saudi Arabia and as well as identifying 17 mutations within *ITGA2B* and *ITGB3* they found possible disease-influencing variants in *ITGA2, VWF* and *F8*. Notwithstanding, the genes analyzed should also include variants potentially protectrice against severe bleeding such as Factor V Leiden but for which the jury is out at the moment with regard to their influence in GT.[19,95]

Human Stem Cell Transplants (HSCT) and Gene Therapy

Restoring hemostasis in GT patients with life-threatening bleeding by HSCT has proved remarkably successful. Pioneering studies were performed in Paris, first on a brother and later on his sister with severe type I GT and antibodies to αIIbβ3 making them refractory to transfusions.[110] Both received bone marrow transplants (BMT) from an asymptomatic sibling but under evolving conditions. Platelet function and αIIbβ3 expression was restored and remained stable for years. Significantly antibodies to αIIbβ3 were no longer present. Flood et al[111] performed BMT on three type I patients with severe bleeding. Two donors were matched family members but the third was unrelated. A conditioning regimen consisting of busulfan, cyclophosphamide and fludarabine met with isolated episodes of graft versus host disease (GVHD); thrombocytopenia due to antibodies to αIIbβ3 was successfully treated by immunosuppressive therapy and intravenous gammaglobulin (IVIgG). Complete and long-term donor engraftment was observed. Poon et al[100] reviewed data from an international registry for BMTs in GT composed of 43 patients. Despite variable conditioning regimes and donor graft sources, there was an 81% survival at a median 47 months. Clearly, HSCT can restore the quality of life in GT; notwithstanding, it remains a last resort due to the possible complications and especially GVHD. The future for treating GT patients with severe bleeding remains gene therapy, yet remarkably this remains at an experimental stage. David Wilcox et al in Milwaukee have been pioneers. First, they transduced BM cells using a lentivirus vector containing a cDNA cassette encoding human β3 and transplanted them into irradiated β3-/- mice.[112] Human β3 complexed with murine αIIb but platelets in the mice only expressed around 12% of the normal levels of functional αIIbβ3. Notwithstanding their platelet Fg normalized and their tail bleeding times improved. Antibodies to αIIbβ3 were observed in one mouse, but perfusion of IVIgG slowed platelet clearance. Studies followed on dogs with an *ITGA2B* mutation and type I GT.[113] Bleeding was corrected, but the expression of chimeric αIIbβ3 in the platelets again remained low with only about 10% platelets expressing around 5000 copies of αIIbβ3. One of four dogs formed antibodies to αIIbβ3 that were eliminated by IVIgG. However, questions are raised as platelets of patients with type II GT and uniformly expressing 10% of functional αIIbβ3 can have a serious bleeding syndrome. Clearly improved protocols are required. Two alternative procedures are promising. The first is the use of patient-derived induced pluripotent stem cells (iPSCs). Sullivan et al[114] designed a specific construct to reprogram monocytes from two patients with type I GT to produce iPSCs with restored synthesis of αIIbβ3. Their procedure led to corrected αIIbβ3 expression and function in MKs. Hu et al[115] generated iPSCs from skin fibroblasts from a boy with type I GT and compound heterozygosity in *ITGA2B*. Differentiation of the iPSCs into MKs and restoration of the defective gene led to platelets expressing αIIbβ3; nonetheless, the difficulties encountered led the authors to suggest that blood cells were a better source for iPSCs. Notwithstanding, CRISPR/Cas technology has opened new concepts for directly correcting gene defects in rare diseases and GT will be an obvious choice. As proof of concept, Zhang et al[116] edited the genome of heterologous cells and then iPScs to change HPAs. More precisely, iPSCs expressing HPA-1a (β3Leu33) were converted to HPA-1b (β3Pro33) and then differentiated into progenitor cells. The advantage of CRISPR/Cas procedures is that the correction is made on the patient's own cells; therefore, the question of immune tolerance is of lesser importance. This field offers much scope for future years.

Conclusions

Looking back over a long career that nears 50 years has enabled me to review the progress made and to offer thoughts for the future. Clearly, an improved point-of-care test to better evaluate bleeding risk under the flow conditions of the microcirculation is needed as is a spot

test to allow the rapid detection of antibodies to αIIbβ3 prior to surgery or delivery. While the classification of GT patients into type I, type II and variant subgroups has served a useful purpose, genotyping has confirmed that GT as a whole is not divided into subgroups but consists of a range of genetic defects leading to an absence of αIIbβ3 or residual amounts that vary in quantity and function with no clear boundaries between them. An international consensus is required to determine if AD GT-like MTP should be given separate status in a similar way to platelet-type VWD. The place of genotyping and the use of high-throughput sequencing procedures will gain in importance and their early use will allow for more rapid and targeted biological testing. Finally and most importantly, while gene mutations define the disease, bleeding severity depends on a range of other factors, both epidemiological and genetic and defining these must be the priority in coming years as personalized medicine and gene therapy become options for patient care. Notwithstanding, differential diagnosis and biological studies will remain an essential part of accurate patient phenotyping and patient management, especially in third-world countries.[117] The place of new technologies both in platelet function testing and in genotyping will need continued and constant evaluation.

Note

Human Genome Variation Society (HGVS) nomenclature is used throughout for cDNA and protein numbering. For nucleotide numbering, the A nucleotide of the ATG start codon was designated +1 (cDNA *ITGA2B* and *ITGB3* GenBank accession numbers NM_000419.3 and NM_000212.2, respectively). For amino acid numbering +1 corresponds to the initiating Met with signal peptide included. However, as the numbering for the mature protein was used for the crystal structure of αIIbβ3 [see Xiao et al[10] and Nurden et al[15]], mature protein numbering is used here for missense and other mutations affecting protein structure. This involves subtracting 31 amino acids from the HGVS numbering of αIIb and 26 amino acids for β3.

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Disclosure

The author has no disclosures to declare.

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