

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 α IIb β 3 integrin expressed at high density on the platelet surface and also in intracellular pools. Activated α IIb β 3 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 α IIb β 3 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 $\alpha\text{IIb}\beta_3$, a member of the integrin superfamily of cellular receptors (reviewed by Coller and Shattil⁸). Electron microscopy initially showed GPIIb-IIIa to have a head and two legs; later crystallography strikingly revealed $\alpha\text{IIb}\beta_3$ 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 β_3 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 β_3 ; 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 $\alpha\text{IIb}\beta_3$, platelets contain trace amounts of $\alpha\text{v}\beta_3$ 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 $\alpha\text{IIb}\beta_3$ <5% or absent. Platelet Fg storage pool lacking or negligible. AR inheritance.	- The most common type of GT, given by defects in <i>ITGA2B</i> and <i>ITGB3</i> genes. With <i>ITGA2B</i> defects $\alpha\text{v}\beta_3$ may still be present and functional. Patients susceptible to form isoantibodies reactive with $\alpha\text{IIb}\beta_3$ and/or $\alpha\text{v}\beta_3$ after blood transfusion or pregnancy.
Type II Subgroup	
- Absence of platelet aggregation but clot retraction can be partial or normal. Residual $\alpha\text{IIb}\beta_3$ 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 <i>ITGA2B</i> and <i>ITGB3</i> . 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 $\alpha\text{IIb}\beta_3$ 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 <i>ITGA2B</i> but mostly by <i>ITGB3</i> variants. Extracellular mutations directly or indirectly abrogate Fg-binding sites. Intracellular mutations stop signals for $\alpha\text{IIb}\beta_3$ activation. Clot retraction and Fg storage are mutation dependent. Can be confused with defects in <i>FERMT3</i> and <i>RASGRP2</i> that prevent kindlin-3 (LAD-III disease) and CalDAG-GEFI signaling.
Upregulated $\alpha\text{IIb}\beta_3$ and Macrothrombocytopenia (MTP)	
- Much reduced platelet aggregation with clot retraction and Fg storage again variable. Residual $\alpha\text{IIb}\beta_3$ 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 $\alpha\text{IIb}\beta_3$ interfering with megakaryocyte maturation and platelet biogenesis with enlarged platelets in variable numbers. Bleeding mostly due to defective $\alpha\text{IIb}\beta_3$ function. Single allele mutations on <i>ITGA2B</i> but mostly <i>ITGB3</i> . 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 $\alpha\text{IIb}\beta_3$.

Abbreviations: AR, autosomal recessive; AD, autosomal dominant; LAD-III, leukocyte adhesion deficiency syndrome type III.

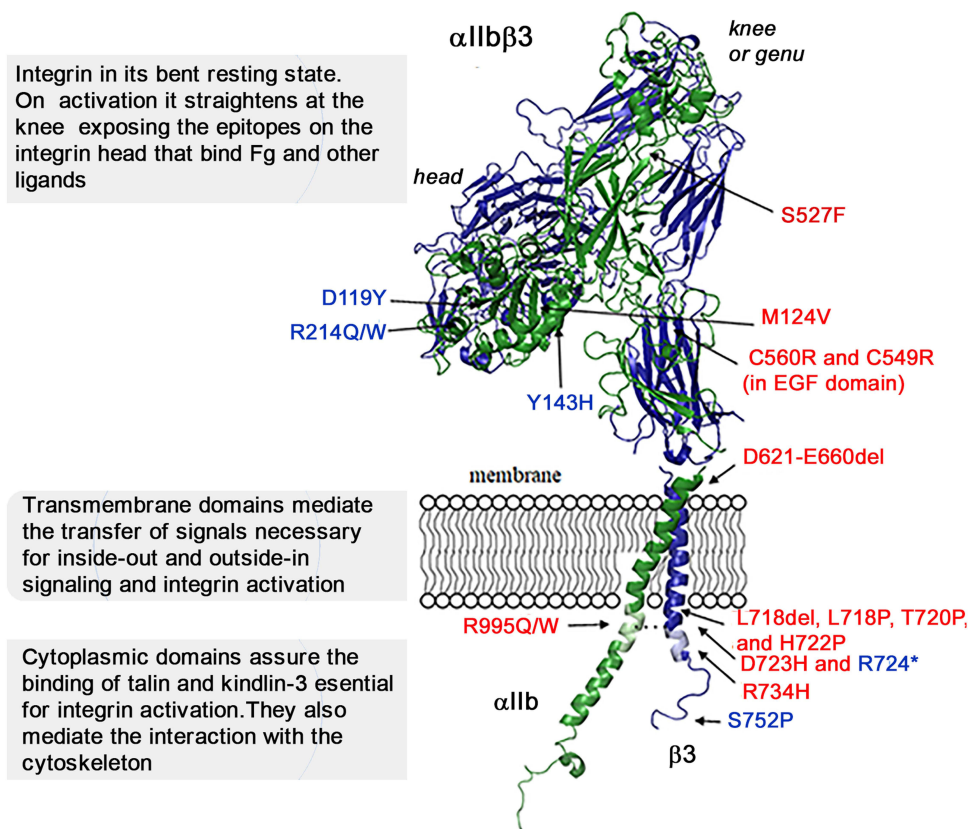


Figure 1 Structural representation of α IIb β 3 in its bent conformation showing the mutations that give rise to selected variant forms of GT or to related phenotypes. This model is based on the crystal structure of α IIb β 3; it was constructed using the PyMol Molecular Graphics System, version 1.3 Schrödinger, LLC and 3fcs and 2knc pdb files as described.¹⁶ The α IIb subunit is in green and β 3 is in blue. Precision crystallography and modeling showed that α IIb has 4 major extracellular domains (β -propeller, thigh, calf-1 and calf-2) whereas β 3 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 β 3 extracellular head prevent binding of Fg or other adhesive proteins to the opened integrin headpiece following platelet activation, while those in the β 3 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 α IIb β 3 and often associated with MTP accompanied by a variable loss of α IIb β 3 function. All mutations are detailed and referenced in the text.

when *ITGA2B* mutations are the cause.¹⁸ While α IIb β 3 is more or less specific for MKs and platelets, α v β 3 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 α IIb β 3, 2) whether this residual α IIb β 3 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 α 2 β 1 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 α IIB β 3 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 α IIB β 3 complexes; a less likely occurrence at a low surface density of α IIB β 3 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 α IIB β 3 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 α IIB β 3 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 β 3 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 α IIB. 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 al²⁶ 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 α IIB β 3, 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 α IIB β 3 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 α IIB.^{27,28} A twofold difference in residual platelet α IIB β 3 content between the probands suggests an influence of other undefined factors. The maturation or trafficking of pro- α IIB β 3 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 α IIB β 3 expression in some cases.²⁹ While complex-dependent MoAbs bound minimally, WB revealed 30–35% of each subunit with α IIB showing signs of proteolysis. Platelet α v β 3 was somewhat increased. Expressed in CHO cells the surface expression of the mutated α IIB β 3 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 α IIB β -propeller.

Ambo et al³⁰ reported two Japanese women with platelets having residual α IIB β 3 who were homozygous for p.Q747P affecting the α IIB 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% α IIB β 3 expression.³¹ In CHO cells α IIBp747 β 3 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 α Ib β 3, 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 α IbR674 β 3 in CHO cells confirmed the platelet phenotype with intracellular retention of pro- α IbR674 by the chaperone BiP explaining a reduced transport to the Golgi apparatus and then to the surface membrane.³³

Basani et al³⁴ 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 α Ib β 3 unable to bind PAC-1 and with a much-reduced Fg storage pool. Expression of α Ib β 3 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 α Ib β 3 density. For the Chinese patient, p.P145L was given by a different nucleotide change. Mutation scanning of α IbP145 in transfected COS-1 cells showed that only p.P145K abrogated α Ib synthesis, while other mutants including p.P145L allowed pro- α Ib β 3 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 al³⁵ Mitchell et al³⁶ reported a boy with parents from Puerto Rico and Canada and with platelets containing 3–6% α Ib β 3 and an Italian/Sicilian family where MoAb binding showed 7–8% α Ib β 3 for four family members. As few clinical and biological details were given these cases are not included in [Suppl Table I](#). For both families, α v β 3 was unaffected and WB confirmed residual mature α Ib and β 3 in their platelets. The American proband was compound heterozygous for p.V298F near the second Ca²⁺-binding domain in blade 5 of the α Ib β -propeller, and p.Y380* that prevents α Ib expression. The Sicilian family combined p.C674R in the α Ib calf-1 domain and a p.I374T missense mutation within the third Ca²⁺-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 α Ib β 3 in 293T cells pulse-chase experiments

showed much of the mutated pro- α Ib to be retained in the ER. Jayo et al³⁷ 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 α Ib β 3 but α v β 3 was normally present. Residual Fg binding indicated a qualitative defect. Sequencing of reverse-transcribed α Ib mRNA revealed compound heterozygosity: a c.C2829T transition giving a p.P912L substitution within the α Ib light chain and a c.C1750T transition in exon 17 of *ITGA2B* that gave p.R553* predicting truncated α Ib 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 α Ib β 3L912 reaches the surface but with a fivefold reduction in the rate of expression. Enigmatically, exontrap analysis of the mutant α Ib 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 α IbL912 β 3 only concerned the full-length transcript.

More recent studies using in silico analysis and molecular dynamics simulations of variants affecting the α Ib 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 al³⁹ also reviewed structural modifications in and around the α Ib genu, a region that is the fulcrum of the bent “resting” state of α Ib β 3. 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 α Ib β 3 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 β 3 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 α Ib β 3 expression was reduced to 6–14% and α v β 3 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 α Ib β 3 yet the cells retained an ability to attach to surface-bound Fg. Novel integrin β 3 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 α Ib β 3 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 α Ib β 3 expression for p.H280P and p.G579S but surprisingly a near normal expression for p.C560F. The expression of α v β 3 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 α Ib β 3 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 α Ib and β 3 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 α Ib and β 3. 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 α Ib β 3 in her father's platelets confirmed the non-expression of the truncated β 3. 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 β 3 conformation. In transfected cells, α Ib β 3P262 was unstable and only small amounts reached the surface. Pulse-chase experiments confirmed that α Ib β 3P262 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 al⁴³ described a young boy with bleeding since birth. His platelets bound

only trace amounts of MoAbs to α Ib β 3, but in WB α Ib and β 3 were 10% to 30% of normal, unusually his β 3 migrated as a high molecular weight band, thought to be β 3 dimer as its migration normalized after disulfide reduction. Platelet Fg was <20% of normal. He was homozygous for a p.C506Y mutation in β 3; this created an unpaired cysteine in the EGF-2 region. Different models proposed that C506 could link with C495 or C501 in β 3.

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 α Ib β 3 antagonist. Her platelets expressed α Ib β 3 at 24% of normal levels by FC but failed to bind Fg or PAC-1 after activation with ADP. Thus, once more, the residual α Ib β 3 had both quantitative and qualitative defects. Platelet Fg was virtually absent. Sequencing of cDNA revealed a homozygous p.T176I mutation in *ITGA2B*. Interestingly, α IbI176 locates to the blade 3 1:2 loop of the α Ib β -propeller suggesting that it induces conformation changes through allosteric effects rather than direct interference. Expression of the mutated α Ib β 3 in COS-7 or CHO cells was confirmed as being much reduced.

Morel-Kopp et al⁴⁵ reported a homozygous p.L196P β 3 variant in a French woman with type II GT and mild bleeding. Her platelets expressed only 4100 copies of α Ib β 3 yet her intra-platelet pool of Fg was as high as 50%. A low expression of α Ib β 3P196 was confirmed in transfected CHO cells with increased cytoplasmic pro- α Ib suggesting altered pro- α Ib β 3 processing. Intriguingly, cells expressing α v β 3P196 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 α Ib β 3P196. Nevertheless, α Ib β 3P196 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% α Ib β 3 but WB again revealed a substantial pool of Fg a finding suggestive of α Ib β 3 recycling. His mother, a sister and son, all heterozygous for β 3 p.L196P had platelets with intermediate levels of α Ib β 3. 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 α IIB β 3 expression.

Splice Site and Other Mutations in Type II GT

Mutations in the calf-1 and calf-2 domains of α IIB are not directly involved in ligand binding, but small discontinuous contacts link them to the EGF-3, EGF-4 and β -tail domains of β 3.^{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- α IIB complexed with β 3 in transfected BHK cells, but little mature α IIB resulted. Golgi mediated complex mannose glycosylation was not seen and immunolocalization confirmed that mutated α IIB β 3 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 al¹⁷) 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 α IIB β 3 in his platelets. Although his platelets failed to aggregate, clot retraction was partial and platelet Fg was abundant and suggestive of α IIB β 3 recycling. Despite his residual α IIB β 3, 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% α IIB β 3 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 α IIB β 3 in her platelets.

Mutations Reducing α IIB β 3 Expression and Overlapping That of Variant Forms

The classification of type I; type II and variant GT on α IIB β 3 numbers alone is purely arbitrary for borderline cases. For example, Jackson et al⁵¹ described a girl with

platelets unable to bind Fg or aggregate when stimulated but with a normal clot retraction. Her platelets expressed 27% α IIB, 16% β 3 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 α IIB β 3 was unstable and failed to express LIBS-binding sites when challenged. She had a homozygous p.S162L substitution in β 3 with a destabilizing effect on α IIB β 3. 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 β 3. Expression of mutated α IIB β 3L162 in COS-7 cells led to pro- α IIB β 3 synthesis but delayed maturation with only small amounts of mature α IIB β 3 and a rapid proteolysis of both subunits. This patient nicely emphasizes how the α IIB β 3 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% α IIB and β 3 as assessed using MoAbs to the subunits but bound only 13% of a complex-dependent MoAb. Her α IIB β 3 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 α IIB β -propeller and a null allele; the latter largely accounting for the low α IIB β 3 density. Transfected HEK cells normally expressed α IIBH143 β 3 but failed to bind PAC-1 or Fg in the presence of an activating MoAb. Identical results obtained for α IIBA143 β 3 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 α IIB (Figure 1). Notwithstanding, cells transfected with α IIBH143 β 3 underwent a partial clot retraction, whereas the KO variant abrogated it.

Variant Forms of GT and α IIB β 3 Functioning

Activation of α IIB β 3 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 α IIB β 3 initiate “outside-in” signaling and responses such as platelet spreading on Fg and clot retraction where α IIB β 3 transmits forces generated by actin and myosin. Important is

phosphorylation of the $\beta 3$ -cytoplasmic tail and c-Src and RhoA activities with retraction regulated by a molecular switch involving $\beta 3$ dephosphorylation and cleavage at Y759.⁵³ Variants that mostly possess 50% or more α IIB $\beta 3$ 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 α IIB $\beta 3$ 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 $\beta 3$.⁵⁴ Also of major importance were homozygous p.R214Q or W substitutions within the ADMIDAS (adjacent to MIDAS) domain of $\beta 3$.^{55,56} Here, α IIB $\beta 3$ 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 α IIB $\beta 3$ with dithiothreitol restored the Fg-binding capacity. These extracellular variant forms occurred in patients with clinically severe GT. While mostly the mutations affect $\beta 3$, the Japanese KO variant (also associated with severe bleeding) is an exception.⁵⁷ Here, platelets fully expressed α IIB $\beta 3$ that is unable to bind Fg or PAC-1 when stimulated and clot retraction is subnormal. In addition, α IIB $\beta 3$ was refractory to the activating MoAb PT25. A homozygous p.R,T160-161 insert on the upper face of the α IIB 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 α IIB $\beta 3$.⁵⁸ Uniquely, he experienced severe limb deep vein thrombosis (DVT) after a long air flight.^{3,19} His α IIB $\beta 3$ was predominantly in an internal platelet pool. Possessing a heterozygous $\beta 3$ 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 α IIB $\beta 3$ of internal membranes

after platelets were challenged with thrombin.⁵⁹ An RGDS-affinity column retained detergent solubilized α IIB $\beta 3$ from his platelets confirming an intrinsic Fg-binding capacity.⁵⁸ As his daughter's platelets aggregated normally while retaining 50% α IIB $\beta 3$ 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 α IIB $\beta 3$ activation.^{8,15} A similar phenotype in a black American girl with severe bleeding and platelets expressing intermediate levels of non-functional α IIB $\beta 3$ 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 $\beta 3$ cytoplasmic domain.⁶⁰ This patient lacked not only the kindlin-3 binding site but also a talin-binding domain essential for α IIB $\beta 3$ signaling.⁶¹ CHO cells expressing α IIB $\beta 3$ R724* failed to spread on Fg associated with a loss of FAK phosphorylation.

Extracellular Activating Mutations in α IIB $\beta 3$

Activating mutations within extracellular domains mostly concern cysteine residues, and $\beta 3$ is rich in conserved disulfides of which 31 localize to the EGF domains. Pioneering was a homozygous p.C560R substitution in the $\beta 3$ EGF-3 domain of a Frenchman whose platelets expressed 20% α IIB $\beta 3$ (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 α IIB $\beta 3$ R560 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 al⁶³ identified a founder p.C549R variant in $\beta 3$ 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 α IIB β 3 spontaneously and maximally bound PAC-1, while WB showed a higher than normal presence of pro- α IIB much of which was retained in the ER. The affected C549 forms a highly conserved disulfide with C558 in EGF-3. Kamata et al⁶⁴ investigated the role of β 3 disulfides by systematically substituting cysteine with serine followed by the expression of the recombinant α IIB β 3 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 al⁶⁵ and involved a heterozygous p.S527F substitution in the I-EGF-3 domain of β 3. The proband was a young Arab man with mild bleeding and much decreased platelet aggregation with ADP despite normal α IIB β 3 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 α IIB β 3F527 although their binding increased when RGDS was present. β 3F527 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 α IIB and β 3 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 β 3 MIDAS domain. Homozygous p.D119Y gives rise to the classic CAM variant (see the preceding section).⁵⁴ CHO cells transfected with α IIB and β 3V124 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 α IIB β 3V124 was constitutively active. The failure of the patient's platelets to spontaneously aggregate was probably due to the dominance of the loss-of-function α IIB β 3Y119. About 50% of the recombinant α IIB β 3V124 was present as pro- α IIB β 3 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 α IIB β 3 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 ($100\text{--}160 \times 10^9/\text{L}$), some enlarged platelets and defective platelet aggregation.^{67,68} Although surface α IIB β 3 was 10–20% of normal, his platelets mediated clot retraction and stored Fg; a normal α IIB β 3 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 α IIB cytoplasmic tail. Expression of α IIBQ995 β 3 in Cos-7 and CHO cells confirmed a reduced transport to the cell surface. Although the mutated α IIB β 3 did not bind Fg spontaneously, there was weak binding of PAC-1. Significantly, α IIBR995 forms an intracellular salt bridge with β 3D723 and weakening or breaking this link upgrades the activation status of α IIB β 3 (Figure 1).⁶⁹ Family studies showed that R995Q came from his father, but discrepancies in their α IIB β 3 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 α IIB β 3 expression was 50% to 70% of normal. Spontaneous PAC-1 binding to platelets or transfected 293T cells confirmed that α IIB β 3 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 β 3 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 α Ib and the third p.D723H in β 3. 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 α Ib β 3 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 al⁷³ 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 α Ib β 3.

Mutations in the single-pass transmembrane domains of α Ib β 3 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 α Ib β 3 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 β 3 was noted in their platelets and a dominant negative effect was hypothesized. A heterozygous p.L718P mutation in β 3 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 α Ib β 3 was selectively reduced. Her platelets spread poorly on Fg with defective lamellipodia. CHO cells expressing recombinant α Ib β 3P718 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 β 3 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 al⁷³ have added p.G976V in α Ib and p.T720P, p.H722P and p.R734H (p.

R760H) in β 3 to the list causing MTP with GT-like functional defects (Figure 1).

These patients raise important questions. Quite clearly, the conformational status of α Ib β 3 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 al⁷⁹ 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 β 3 p.R67Q variant had normal α Ib β 3 expression and clot retraction. Missense mutations affecting α Ib primarily concerned the β -propeller or thigh domains while those in β 3 were distributed within the PSI, β -I and hybrid domains. Nelson et al⁸⁰ followed with a further 15 patients from the same communities. Significantly, 14 showed no α Ib in WB and 10 had no β 3, while for 4 others, β 3 was severely reduced. Platelet Fg was undetectable for 13 patients, severely reduced in 1; only 1 patient had normal platelet surface α Ib β 3 expression and normal Fg. Disease-causing mutations were identified for 11 patients with type I GT predominating.

Jallu et al⁸¹ 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 α Ib β 3 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 α IIB β 3. Two type II patients had platelets with substantial pools of Fg (51% and 83%, respectively). Sandrock-Lang et al⁸² 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 α IIB 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 re-occurred in two nonrelated French families with type II GT and compound heterozygosity; expression studies confirmed an altered maturation of pro- α IIBF903 β 3. 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 β 3 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-

α IIB β 3C37 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 β 3 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% α IIB β 3 but no platelet aggregation and who was also homozygous for a *FERMT3* mutation.⁸³ *FERMT3* encodes kindlin-3 and its absence rendered the residual α IIB β 3 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 α IIB β 3 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 al⁸¹ 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 α IIB β 3 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 α IIB β 3. Podolnikova et al⁸⁵ showed how a cluster of newly exposed amino acids in the Fg γ 370-381 sequence mediate the interaction of immobilised Fg or fibrin with α IIB β 3. Much later, the same group revealed how this cluster had several potential contact sites within the α IIB β -propeller.⁸⁶ These differ from the activation-dependent sites on α IIB β 3 that mediate binding of the γ 404GAKQAGDV411 peptide or RGDS sequences of Fg and which respectively localize to the extremity of the α IIB β -propeller and to the β 3 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 α IIB β 3 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 al⁹² 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. Lorocho et al⁹³ 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% α IIB and 5% β 3 in platelets of the two patients studied. Signaling proteins such as kindlin-3, CaLDAG-GEFI, and Src were all normally present as were α v, GPIb-IX and GPVI. Downregulated in platelets were Fg, FXIIIB, carboxypeptidase-B2 (a fibrinolysis inhibitor) and plasminogen despite normal plasma levels. Fc γ RIIA and laminin- α 4 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. Fc γ RIIA increase was linked to increased dense granule secretion in response to immunoglobulin complexes. Recently, Blair et al⁹⁴ 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 α IIB β 3 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 al⁹⁶). 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 al⁹⁷ analysed next-generation sequencing data (WES and WGS strategies) from five international databases and assessed the frequency of missense mutations affecting α IIB and β 3 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 al³ 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 α Ib β 3 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 al¹⁰¹ 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 al¹⁰⁴ described pregnancy in a woman with type I GT and isoantibodies following blood transfusions. As the husband's platelets fully expressed α Ib β 3, the fetus was an obligate heterozygote. Fetal death occurred at 31 weeks due to intracranial hemorrhage. Although absent from platelets when α Ib β 3 and α v β 3 are lacking, HPA-systems on β 3 are a factor on platelets and other cells that express α v β 3 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 al¹⁰⁵ 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 α v β 3 in endothelial cells. Evidence for anti-HPA-1 alloantibodies reactive with α v β 3 in GT was provided by Fiore et al¹⁰⁶ who detected them in polytransfused patients with the French Manouche gypsy mutation (on *ITGA2B*) with α v β 3 in platelets and other cells expressing β 3 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 al¹⁰⁸ 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 α Ib β 3 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 al¹⁰⁹ 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 protective 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 α Ib β 3 making them refractory to transfusions.¹¹⁰ Both received bone marrow transplants (BMT) from an asymptomatic sibling but under evolving conditions. Platelet function and α Ib β 3 expression was restored and remained stable for years. Significantly antibodies to α Ib β 3 were no longer present. Flood et al¹¹¹ 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 α Ib β 3 was successfully treated by immunosuppressive therapy and intravenous gammaglobulin (IVIgG). Complete and long-term donor engraftment was observed. Poon et al¹⁰⁰ reviewed data from an international registry for BMTs in GT composed of 43 patients. Despite variable conditioning regimens 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 β 3 and transplanted them into irradiated β 3^{-/-} mice.¹¹² Human β 3 complexed with murine α Ib but platelets in the mice only expressed around 12% of the normal levels of functional α Ib β 3. Notwithstanding their platelet Fg normalized and their tail bleeding times improved. Antibodies to α Ib β 3 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 α Ib β 3 in the platelets again remained low with only about 10% platelets expressing around 5000 copies of α Ib β 3. One of four dogs formed antibodies to α Ib β 3 that were eliminated by IVIgG. However, questions are raised as platelets of patients with type II GT and uniformly expressing 10% of functional α Ib β 3 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 al¹¹⁴ designed a specific construct to reprogram monocytes from two patients with type I GT to produce iPSCs with restored synthesis of α Ib β 3. Their procedure led to corrected α Ib β 3 expression and function in MKs. Hu et al¹¹⁵ 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 α Ib β 3; 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 al¹¹⁶ edited the genome of heterologous cells and then iPSCs to change HPAs. More precisely, iPSCs expressing HPA-1a (β 3Leu33) were converted to HPA-1b (β 3Pro33) 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 α IIb β 3 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 α IIb β 3 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 α IIb β 3 [see Xiao et al¹⁰ and Nurden et al¹⁵], 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 α IIb and 26 amino acids for β 3.

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Disclosure

The author has no disclosures to declare.

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