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A new heterozygous mutation in GPIBA gene responsible for macrothrombocytopenia

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Congenital macrothrombocytopenias are a heterogeneous group of rare inherited disorders characterized by decreased platelets count with enlarged platelet size. However, patients with isolated macrothrombocytopenia are often misdiagnosed with idiopathic thrombocytopenic purpura (ITP) and wrongly treated with immunoglobulin injection, steroid administration and splenectomy.

Bernard-Soulier Syndrome (BSS) is a bleeding disorder caused by defects in the platelet GPIb/IX/V complex, a receptor for von Willebrand factor (vWF) and thrombin. Patients show a macrothrombocytopenia and their platelets do not agglutinate in response to ristocetin, while maintaining a normal aggregation in response to a variety of aggregating agents. GPIb/IX/V complex consists of two GPIb α and four GPIb β subunits stabilized by disulfide bonds (Luo *et al.*, 2007). This heterodimer is non-covalently associated with two GP1X and one GPV subunits. The N-terminal residues of GPIb α form seven leucine-rich repeats (LRRs) and include the binding sites for vWF and thrombin. The BSS disease is due to homozygous mutations in *GPIBA*, *GPIBB* or *GP9* genes encoding GPIb/IX/V complex (Savoia *et al.*, 2014). However, some families with hereditary macrothrombocytopenia and mild or no bleeding diathesis were described with heterozygous mutations in both *GPIBA* (Savoia *et al.*, 2001; Vettore *et al.*, 2008) or *GPIBB* gene (Savoia *et al.*, 2014; Kunishima *et al.*, 2001). These patients show mild thrombocytopenia with slightly increased mean platelet volume (MPV), a variable percentage of giant platelets, a modest decrease of cell-surface expression of the GPIb/IX/V complex, sometimes leading to a reduced aggregation in response to ristocetin. However, due to the absence or paucity of biological signs, the use of gene sequencing is mandatory to pinpoint the GPIb complex involvement. The International Consortium for the study of BSS described 60 gene variations in *GP1BA* (28%), 59 in *GPIBB* (28%) and 92 in *GP9* (44%) (Savoia *et al.*, 2014). Most of variations (85%) were homozygous and most cases were products of consanguineous marriages. The most common reported variation is a *GP1BA* missense mutation, leading to an amino acid substitution (Ala172Val), known as the Bolzano variant located in the sixth LRR (Savoia *et al.*, 2001). Here, we describe a French family with a new mutation in *GPIBA* gene located in the fifth LRR.

The proband shows a mild thrombocytopenia ($89 \times 10^9/L$) with large platelets (MPV: 13.1 fL; Advia[®], Siemens) without bleeding diathesis. Platelets aggregated normally with ADP and collagen but agglutination response to 1.25 mg/mL ristocetin as the GPIb/IX/V complex

platelet surface expression were modestly decreased (Table 1).

The member 2 (his sister) exhibits more pronounced bleeding symptoms with menorrhagia, epistaxis, postoperative bleeding that did not require transfusion (Table 1). Platelet volume in the two patients was slightly elevated (11.5-13.1 fL) with 17% of large platelets, but without giant platelets on the peripheral blood smear (Table 1 and Figure 1A). Pedigree analysis reveals an autosomal dominant inheritance pattern since several members of different generations exhibit macrothrombocytopenia (Supplementary data).

The morphology of the propositus bone marrow cells revealed small megakaryocytes with reduced and vacuolated cytoplasm (Figure 1B). *In vitro* study of MKs derived from peripheral blood CD34⁺ cells in presence of TPO and SCF revealed a reduction in the percentage of mature CD41⁺CD42⁺ in the propositus as compared to control whereas the ploidy level was not affected (Figure 1C).

Sanger sequencing of the patient's *GPIBA*, *GPIBB*, and *GPIX* gene revealed a novel single nucleotide mutation located in *GPIBA* (Figure 1D). The substituted residue was highly conserved in *GPIBA* orthologs from ten distantly related species. This variation was called deleterious by diverse prediction algorithms (Polyphen-2 score 1, SIFT score 0) and was not reported in the ExAC and 1000G databases (Supplementary data).

The first reported thrombocytopenic patients with large platelets, slightly reduced aggregation to ristocetin and GPIb/IX/V platelet surface expression carried monoallelic Ala172Val substitution in GPIb α (Savoia *et al.*, 2001) known as the Bolzano variant. Other monoallelic mutations in the *GPIBA* gene have since been identified (Savoia *et al.*, 2001; Vettore *et al.*, 2008) and some were also discovered in the *GPIBB* gene associated with macrocytosis though not always with thrombocytopenia (Savoia *et al.*, 2014; Kunishima *et al.*, 2001). Macrothrombocytopenia due to haploinsufficiency of the *GPIBB* gene is also a finding frequently observed in patients with the diGeorge syndrome (*del22q11*) (Liang *et al.*, 2007) but these patients are mainly diagnosed according to extra-hematological symptoms. In our laboratory, we sequenced *GPIBA*, *GPIBB* and *GPIX* genes in all patients with mild macrothrombocytopenia and we found only one family carrying the substitution of asparagine by a serine at position 150 that was not yet listed by the International Consortium for the study of BSS. Thus, in contrast to the Bolzano variant, our results do not support a mutational founder effect. Like the Bolzano variant, we have observed a variable phenotype among the mutation carriers despite similar platelet phenotype. A combination of genetic, environmental and lifestyle factors may explain the discrepancies such as gender. Indeed, the individual with the highest bleeding score predominantly suffered from gynecological and obstetrical bleeding.

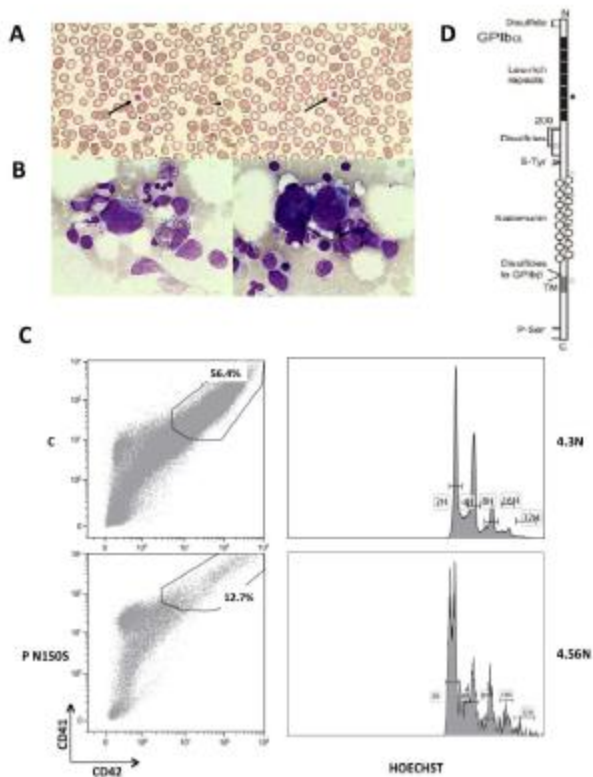
Balduini *et al.* (Balduini *et al.*, 2009) showed that megakaryocyte maturation was not affected in subjects with monoallelic Bolzano mutation while proplatelet formation was severely reduced *in vitro*. In this study, the maturation was evaluated according to the morphology of CD34⁺ derived megakaryocytes at day 12. We observed fewer megakaryocytes (CD41⁺CD42⁺ cells) at day 13 of culture without reduction of modal ploidy. Those results are consistent with those described in a murine model of BSS (Ware *et al.*, 2000).

We reported here a new monoallelic mutation in the *GPIBA* gene that slows-down megakaryocyte differentiation without reducing polyploidization.

Diagnosis of monoallelic BSS has to be suspected in case of thrombocytopenia, rather moderate with a dominant transmission pattern. The important element is the presence of macroplatelets (> 10%) but only few even no giant platelets (< 5%). These two criteria associated with discrete quantitative and qualitative alterations of the GPIb/IX/V complex may help to orient the diagnosis towards a defect in GPIb/IX/V complex.

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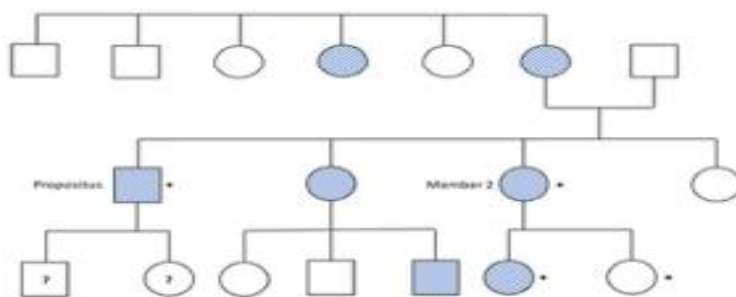


Blood smear (A) shows large platelets (arrow) and bone marrow smear (B) revealed the presence of some small megakaryocytes with reduced cytoplasm and sometimes vacuolated. MK differentiation was induced from control or patient peripheral blood CD34+ cells and analyzed at day 13 of culture (C).

Gates represent mature (CD41+CD42+). The ploidy level (N) was analyzed in the gate of CD41+CD42+ MKs. Mutation location in the protein (asterisk) (D) : amino acid substitutions is located in the fifth LRR. 199x199mm (300 x 300 DPI)

	Propositus	member 2
Age	29	37
Platelet count (10 ⁹ /L)	89	74
MPV (fl)	13.1	11.5
Bleeding score	0	1 to 2
RIPA by 1.25 mg/mL (Intensity, %)	77	62
Normal range : [85-94]		
RIPA by 1.25 mg/mL (Velocity, %)	41	33
Normal range : [85-212]		
GpIb expression (% of control)	69	100
% of large platelets/giant platelets	NP	17/0

Patients characterization : Age, platelet counts and platelet size (MPV) obtained by optical method, bleeding score, platelet aggregation in response to ristocetin (RIPA) 1.25 mg/mL and GpIb expression by flow cytometry (NP : not performed).



Family tree :
Members with macrothrombocytopenias are representing with striped square or circle.
Asterisks highlight patients which DNA was sequenced.
Platelet counts of members with question mark are unknown.

Cristallography studies : Cristallography studies predict that this mutation was probably damaging (by polyphen-2, SIFT and MutationTaster predictive algorithms). It results in the substitution of an asparagin by a serine at position 150. The physicochemical difference between Asn and Ser is very moderate (Grantham dist. : 46 [9-215]).