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Biological, clinical features and modelling of heterozygous variants of glycoprotein Ib platelet subunit alpha (GP1BA) and glycoprotein Ib platelet subunit beta (GP1BB) genes responsible for constitutional thrombocytopenia

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5 2 GP1BB genes responsible for constitutional thrombocytopenia
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3 30 **Abstract:** Constitutional thrombocytopenias are rare disorders, often difficult to discriminate
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5 31 from acquired thrombocytopenias. More than 80 genes have been described as being at the
6
7 32 origin of these diseases. Among them, several variants of the *GP1BA* and *GP1BB* genes, coding
8
9 33 for the GpIb-IX-V glycoprotein complex, have been reported in the literature. The study
10
11 34 reported here aimed at describing newly identified monoallelic anomalies affecting the *GP1BA*
12
13 35 and *GP1BB* genes on a clinical, biological and molecular level. In a cohort of 9 patients with
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15 36 macrothrombocytopenia, eight heterozygous variants of the *GP1BA* or *GP1BB* genes were
16
17 37 identified. Five of them had never been described in the heterozygous state. Computer
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19 38 modelling disclosed structure/function relationships of these five variants.
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40 Introduction

41 Constitutional thrombocytopenias (CT) are rare diseases difficult to distinguish from acquired
42 thrombocytopenias, in particular immune thrombocytopenia (ITP). A CT diagnosis must be
43 evoked in the presence of a number of characteristics: i)thrombocytopenia known since
44 childhood, ii)family history, iii)poor response to ITP treatment and iv)analysis of platelets and
45 megakaryocytes morphology and platelet functions. CT confirmation then relies on molecular
46 analysis. Advances in sequencing techniques have made possible to better characterize the
47 genetic abnormalities at the origin of these pathologies, as they often affect genes involved in
48 megakaryopoiesis and platelet formation.

49 More than 80 genes have thus been described as being potentially responsible for CT. Among
50 them, some are coding for molecules of the membrane glycoprotein complex Ib-IX-V (GPIb-
51 IX-V). However, publications concerning these mutations are rare and, to date, less than 30
52 such variants have been reported in the literature.

53 The GPIb-IX-V glycoprotein complex, the platelet receptor for Willebrand factor (VWF), is
54 essential for primary hemostasis since it allows platelets to bind subendothelial collagen
55 molecules, which become accessible in case of vascular injury. It is composed of 4 subunits,
56 respectively GPIb α , Ib β , IX and V, encoded respectively by the *GP1BA*, *GP1BB*, *GP9* and *GP5*
57 genes. Homozygous or double heterozygous deleterious sequence variants of *GP1BA*, *GP1BB*
58 and *GP9* are at the origin of Bernard-Soulier syndrome (BSS), characterized by
59 thrombocytopenia with increased platelet size and the presence of giant platelets, as well as
60 severe platelet function disorders related to quantitative or qualitative deficiency of the GPIb-
61 V-IX complex (1-3). However, some variants are responsible for an attenuated disease
62 phenotype when present in the heterozygous state. The latter are called monoallelic BSS with
63 an autosomal dominant transmission (4).

64 To date, 13 *GP1BA* variants (4-16) and 14 *GP1BB* variants (15-22), considered to be responsible
65 for monoallelic BSS, have been described in the literature (Table 1). No variant responsible for
66 monoallelic BSS has been identified so far in the *GP9* gene.

67 From a molecular point of view, all described heterozygous *GP1BA* variants show clustered
68 substitutions within the leucine-rich repeat (LRR) domains of GPIb α , located in the ligand-

1
2
3 69 binding site (LBD) (residue His1-Thr266) which contains the VWF-binding site (5-7). There also
4
5 70 are variants of GPIb β similarly involving the LRR domain of the protein.
6

7
8 71 Clinically, patients present with moderate mucocutaneous bleeding, such as epistaxis,
9
10 72 ecchymosis, menorrhagia and sometimes prolonged post-surgical bleeding (4-23). Platelet
11
12 73 counts show moderate thrombocytopenia with an increase in mean platelet volume.
13
14 74 Morphologic analysis discloses giant platelets in variable proportions. Surface expression of
15
16 75 the GPIb-IX-V complex, assessed by flow cytometry, shows decreased or normal levels,
17
18 76 depending on the type of antibody used and the degree of damage to the complex (11).
19
20 77 Platelet functional studies often show decreased aggregation in the presence of ristocetin and,
21
22 78 in most cases, normal aggregation in the presence of ADP and collagen (6,8,9,12,14,20-22).

23
24 79 From a pathophysiological point of view, the origin of MTP is still not completely elucidated.
25
26 80 The GPIb-IX-V complex is closely linked to the cytoskeleton, required to sustain platelet shape
27
28 81 (2) and also involved in megakaryopoiesis since proplatelet formation is mediated by actin
29
30 82 filaments remodelling (24). Moreover, published data indicate that macrothrombocytopenia
31
32 83 (MTP) may be due to abnormal interactions between the intracytoplasmic portion of GPIb α
33
34 84 and actin-binding proteins. Indeed, the latter monitor MK maturation and production of the
35
36 85 demarcation membrane system, thus controlling platelet size (25,26). This suggests that a
37
38 86 GPIb-IX-V complex deficiency would have a direct impact on platelet production and would be
39
40 87 the cause of MTP.

41
42 88 At the molecular level, variants that cause monoallelic BSS appear to have a dominant
43
44 89 negative effect (11,21). Indeed, in the majority of heterozygous carriers, including relatives of
45
46 90 patients with classical BSS, a normal copy of the GP1BA or GP1BB genes is found without
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48 91 thrombocytopenia nor giant platelets (24). Thus, only specific variants cause MTP in
49
50 92 monoallelic BSS, inducing a significant deficit - qualitative or quantitative - of the GPIb-IX-V
51
52 93 complex, despite the presence of a normal GP1BA or GP1BB allele (27). The putative effect of
53
54 94 these dominant-negative variants is the presence of an abnormal GPIb subunit that
55
56 95 compromises the delivery of the GPIb-IX-V complex to the surface, resulting in a quantitative
57
58 96 and qualitative GPIb-IX-V deficit (28). Indeed, most variants affect residues that are embedded
59
60 97 in the protein structure and disrupt proper folding of the LRR region, thus impairing the
98
99 98 formation of the GPIb-V-IX complex (4,5,7-9,11,15,20,21,28-30)

99

100 The aim of the study reported here was therefore to describe clinically, biologically and
101 molecularly new monoallelic variants affecting the *GP1BA* and *GP1BB* genes. Moreover, the
102 structure/function relationship of each variant was analysed in depth by molecular modelling
103 of the mutated protein.

104

105 **Materials and methods**

106 This non-interventional, retrospective and prospective bicentric cohort study was submitted
107 and approved by the GNEDS (Groupe Nantais d'Ethique dans le Domaine de la Santé). All
108 patients were informed of the study and none refused to participate.

109 The cohort consists of 9 patients with MTP, recruited from the Clinical Haemostasis centres of
110 Nantes and Rennes University Hospital. For all of them, a heterozygous variant of the *GP1BA*
111 or *GP1BB* genes had been identified in the context of thrombocytopenia exploration.

112 Platelet counts were assessed by impedancemetry and fluorocytometry, together with mean
113 platelet volume (MPV) and immature platelet fraction (IPF) assessment, using an XN9000
114 instrument (Sysmex, Kobe, Japan). Platelet aggregation assays were performed on citrated
115 platelet-rich plasma with the following agonists: adenosine diphosphate (ADP) (5 μ M),
116 epinephrine (5 μ M), arachidonic acid (1 mM), collagen (1.25 μ g/mL) and ristocetin (1.5 mg/mL,
117 1.2 mg/mL and 0.7 mg/mL). GPIb-IX-V expression was measured by flow cytometry using a
118 quantification kit (PLT GP/receptors, Biocytex, Marseille France) on a Navios (Beckman
119 Coulter, Miami, FL) instrument.

120 The search for sequence variants was performed by high throughput sequencing on a MiSeq
121 sequencer (Illumina, San Diego, CA) targeting coding sequences and the intron-exon junctions
122 of the *GP1BA*, *GP1BB* and *GP9* genes with an average depth of 300 reads. Mutated and normal
123 reads were assessed with an automated algorithm (SOPHiA Genetics, Lausanne, Switzerland)
124 detecting the homozygous or heterozygous status of each of the variants. The presence of
125 each variant and its status were also systematically confirmed by Sanger sequencing. Only the
126 probands were available, and it was not possible to perform familial triad analyses as reported
127 by Sivapalaratnam et al.(20).

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2
3 128 The possible pathogenicity of the variants was assessed using recommendations from the
4
5 129 American College of Medical Genetics and Genomics and Clinvar (Supplemental table 1).

6
7 130 Structural visualization of proteins of interest was performed from genomic characteristics, in
8
9 131 order to visualize the localization and potential structural impact of variants on the GPIb-IX-V
10
11 132 complex. Protein sequences were retrieved from UniProt (<https://www.uniprot.org/>):
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13 133 UniProtKB - P07359 (GP1BA_HUMAN), UniProtKB - P13224 (GP1BB_HUMAN) and residues
14
15 134 numbering used on the figures refers to these sequences. Protein structures were obtained
16
17 135 from the Protein Data Bank (PDB, <https://www.rcsb.org/>). For GPIb α , the following PDB codes
18
19 136 were used: 1GWB (GPIb α wild type, residues 18 to 283), 1SQ0 (GPIb α carrying N37D and
20
21 137 N175D mutations, in complex with VWF) and 1P8V (GPIb α carrying the N37D mutation, in
22
23 138 complex with α -trombin). For GPIb β , the PDB code 3RFE, corresponding to wild-type GPIb β
24
25 139 subunit (residues 27 to 143) was used.

26
27 140 Finally, the structural amino-acid residues of interest were identified and visualized with the
28
29 141 BIOVIA Discovery Studio Visualizer (Dassault Systèmes, San Diego, CA).

30 31 142 **Results**

32
33 143 Eight pathogenic variants were found for the 9 patients included in the study, respectively 3
34
35 144 in the *GP1BA* gene and 5 in the *GP1BB* gene (according to the ACMG criteria).

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37 145 Among these, 3 have not been previously described in MTP (respectively *GP1BA* [p.Leu155Pro],
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39 146 *GP1BB* [p.Leu168dup] and gene deletion) but 2 (*GP1BB* [p.Cys141*] and [p.Leu137Pro] had
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41 147 already been found in the homozygous state in a BSS context. Moreover, *GP1BA* [p.Leu73Phe]
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43 148 and [p.Asn150Ser] as well as *GP1BB* [p.Leu16Pro] had already been described in a
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45 149 heterozygous state.

46 47 150 **GP1BA gene**

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49
50 151 Patients with *GP1BA* variants (Table 2) displayed a very discreet haemorrhagic
51
52 152 symptomatology, mainly affecting mucosal sites when present. Platelet counts showed
53
54 153 moderate thrombocytopenia (49 to 79x10⁹/L), variable MPV (11.2 to 14.2 fL), and, in the 2
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56 154 patients tested, an increase in IPF. GPIb α expression was low in one patient. Abnormalities in
57
58 155 platelet function were observed in the presence of ADP, collagen and arachidonic acid. The
59
60 156 platelet count was too low to perform aggregometry in one case.

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3 157 The location in the GP1b α subunit of the 3 mutated residues is depicted on Figure 1.
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5
6 158 *p.(Leu73Phe)*
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8 159 This variant had already been described by Miller *et al.* in 1992 in a patient with MTP (7). The
9
10 160 leucine residue at position 73 is located within the second leucine rich repeat (LRR) domain of
11
12 161 the GPIb α subunit. Its substitution with a phenylalanine, which is a larger amino acid, may
13
14 162 create steric hindrance and disrupt the structure. Furthermore, adjacent residues are involved
15
16 163 in VWF binding, making it likely that the *p.(Leu73Phe)* variant could affect this molecular
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18 164 interaction (Figure 2).
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20 165 *p.(Asn150Ser)*
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22 166 Sequencing disclosed a c.449A>G substitution predicting a *p.(Asn150Ser)* missense. This was
23
24 167 previously described by Ghalloussi *et al.* in 2017 (9). This team suggested that this variant
25
26 168 would affect megakaryopoiesis by decreasing the number of mature megakaryocytes (9).
27
28 169 Structurally, residue 150 is positioned at the end of the 5th LRR domain (Figure 1). Asn150 is
29
30 170 buried but its lateral chain participates in the maintenance of the structure via hydrogen
31
32 171 bonds. Its substitution to a serine, which is a smaller amino acid, may have an impact on the
33
34 172 conformation of GPIb α (Figure 3).
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36 173 *p.(Leu155Pro)*
37

38 174 From a structural point of view, residue 155 is positioned at the beginning of the 6th LRR
39
40 175 domain (Figure 1). Because the leucine at position 155 is buried, its replacement to a proline
41
42 176 may strongly disrupt the structure of the hydrophobic core (Figure 4). Moreover, the 2
43
44 177 residues upstream of Leu155 are also proline residues (Pro156, Pro157), and the
45
46 178 *p.(Leu155Pro)* variant will result in 3 consecutive prolines, likely to form a polyproline helix
47
48 179 that could induce the formation of a twist and would thus distort the initial structure of GPIb α .
49
50 180 This could impair interactions with α -thrombin (Figure 4).
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52 181

53
54 182 *GP1BB* gene
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56
57 183 The haemorrhagic symptomatology of patients with *GP1BB* gene variants (Table 3) is variable,
58
59 184 depending on the variant, and mostly affects mucosal sites when present. Platelet counts
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3 185 showed very moderate thrombocytopenia (from 94 to $147 \times 10^9/L$), a variable but most often
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5 186 clearly increased MPV (from 11 to 14.3 fL) and increased IPF in all the patients tested. Low
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7 187 GPIb α expression was disclosed in 3/4 patients. Abnormalities of platelet functions were
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9 188 observed in the presence of low doses of ristocetin in 4 of the 5 patients tested.

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11 189 From a molecular point of view, because of the location of the variants, prediction of their
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13 190 impact was possible for 2 out of 5 (Figure 5).

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15 191 *p.(Leu137Pro)*

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18 192 The heterozygous c.410T>C variant results in the p.(Leu137Pro) substitution, already
19
20 193 described in the homozygous state in BSS (31). This residue is buried and located at the C-
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22 194 terminal domain of the GPIb β subunit, more precisely in the $\alpha 2$ helix. Its replacement by a
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24 195 proline is likely to strongly disrupt the helix structure, which would impact subunits assembly
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26 196 of the GPIb-IX-V complex, and would explain the quantitative deficit observed in this patient
27
28 197 (Figure 5).

29
30 198 *p.(Cys141*)*

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32 199 A heterozygous c.423C>A substitution was disclosed in the *GP1BB* gene, resulting in a stop
33
34 200 codon that predicts the nonsense variant p.(Cys141*). Cysteine 141 is positioned at the end
35
36 201 of the C-terminal domain of GPIb β , and is involved in a disulphide bridge with cysteine 95
37
38 202 (Figure 5). The p.(Cys141*) variant would lead to a soluble form of GPIb β with the added loss
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40 203 of the disulphide bridge with Cysteine 95, that could impact the C-terminal domain
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42 204 conformation. This soluble form could either interact with other members of the GPIb-IX-V
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44 205 complex or compete with membrane-bound GPIb β if both forms are present. This variant has
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46 206 been reported in the homozygous state in BSS (22) (Figure 5).

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48 207 *p.(Leu16Pro)*

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50 208 This variant, previously described (20,21), was found in 2 siblings. In these two patients,
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52 209 sequencing disclosed a heterozygous c.47T>C variant on the *GP1BB* gene, predicting a
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54 210 p.(Leu16Pro) missense. This residue is located at the level of the signal peptide, therefore not
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56 211 visible on the available structures of GPIb β . This mutation might lead to abnormal addressing
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58 212 or cleavage of the mature protein, thus leading to a quantitative GPIb β deficiency.

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60 213 *p.(Leu168dup)*

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3 214 Sequencing identified a heterozygous variant c.502_504dup that predicts a duplication of the
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5 215 Leu168 residue. The latter is located at the transmembrane domain of the GPIIb β subunit and
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7 216 not present in GPIIb β available structures. The variant is likely to disrupt the helix structure of
8
9 217 this domain and its association with the other subunits of the complex.

10
11 218 *Exon deletion*

12
13 219 Sequencing found a deletion of *GP1BB* gene exon 2 in the heterozygous state in one patient.
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15 220 This translated in decreased expression of the GPIIb-GPIV-GPIX complex as shown by GPIIb α
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17 221 assessment in flow cytometry (table 2).
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21
22 223 **Discussion**

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24 224 This molecular study of patients with constitutional thrombocytopenia allowed for the
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26 225 discovery of five variants that had never been described before in MTP. One is located on the
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28 226 *GP1BA* gene and four on the *GP1BB* gene. Of note, p.(Leu137Pro) and p.(Cys141*), on the
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30 227 *GP1BB* gene, had previously been described as homozygous pathogenic variants in BSS cases
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32 228 (31,32). Here, although only the probands (and not their parents or siblings) were tested,
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34 229 heterozygosity was confirmed by NGS and Sanger, demonstrating the presence of normal and
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36 230 variant sequences.

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38 231 An attempt to predict and explain the impact of these variants based on the location of the
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40 232 residues involved on the available structures of the extracellular domain of GPIIb α and GPIIb β
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42 233 was possible for 5 of the 8 variants observed. Of note, these data are somehow speculative
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44 234 since it was not intended in this study to perform functional studies of the variant proteins.
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46 235 Indeed, two are located either at the level of the signal peptide or in the transmembrane
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48 236 region and one results in a total loss of the coding region. The data obtained confirm and
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50 237 complete available literature data.

51
52 238 The p.(Leu16Pro) substitution, found at the level of the signal peptide, is likely to cause
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54 239 abnormalities in GPIIb β addressing and cleavage, resulting in a quantitative deficiency.
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56 240 Deletion of the *GP1BB* gene causes a complete absence of GPIIb β expression.

57
58 241 Concerning the clinico-biological phenotype of the patients, several results are interesting to
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60 242 note. Their hemorrhagic symptomatology is discrete or non-existent and mainly affects

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3 243 mucosal sites. This is compatible with the previous reports (3-23). Moreover, as expected, von
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5 244 Willebrand levels of the patients were normal, without discrepancy between activity and
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7 245 antigen assays.

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9 246 MTP is almost always observed in these patients, whether the variant involves the *GP1BA* or
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11 247 *GP1BB* gene.

12
13 248 Several authors have indeed pointed out that the GPIb α variants associated to MTP were
14
15 249 especially located at the LRR domains (5-7,19,23). Indeed, here p.(Leu73Phe), p.(Asn150Ser)
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17 250 and p.(Leu155Pro) mutations are positioned within the LRR2, LRR5 and LRR6 domains
18
19 251 respectively. All three could generate conformational changes in the GPIb α subunit, with
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21 252 increasing functional impact when the modification is located near the VWF binding site.

22
23 253 Concerning GPIb β , it has previously been hypothesized (19,22) that MTP-associated variants
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25 254 result in a decreased expression of the molecule, which impacts all subunits of the GPIb-IX-V
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27 255 complex. This hypothesis is supported by our observations. Both p.(Leu137Pro) and
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29 256 p.(Leu168dup) alterations could result in structural changes of GPIb β which might interfere
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31 257 with a correct assembly of the GPIb-IX-V complex. The p.(Cys141*) variant results in a
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33 258 truncated protein which could be responsible for the production of a soluble form of GPIb β .

34
35 259 The origin of MTP remains debated. It has been proposed that MTP would not be due to the
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37 260 absence of an allele but to the presence of an abnormal GPIb-V-IX complex leading to a
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39 261 dominant negative effect (20). Nevertheless, the majority of first-degree relatives of patients
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41 262 with BSS, who therefore have a *GP1BB* allele with a deleterious sequence variation and a
42
43 263 healthy allele, do not have MTP. In addition, only 25% of the patients with a 22q11 deletion
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45 264 syndrome, who have a complete deletion of a *GP1BB* allele, present MTP (28). In our series,
46
47 265 similarly to these cases of 22q11 deletion, the patient presenting a complete deletion of
48
49 266 *GP1BB* was diagnosed with MTP. The mechanism by which only some mutations of *GP1BA* or
50
51 267 *GP1BB* with hemizyosity for these genes present with MTP still remains unclear although
52
53 268 interactions between GPIb-V-IX complex subunits are probably essential (16, 32-34). One
54
55 269 hypothesis would be that the concentration of the GPIb-V-IX complex on the platelet surface
56
57 270 is involved in the terminal phases of megakaryopoiesis. This could be confirmed by studying
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59 271 the concentration of the GPIb-V-IX complex in patients with a monoallelic abnormality of the
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272 *GP1BA* or *GP1BB* gene without MTP. Alternately, in vitro models of megakaryopoiesis from

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3 273 stem cells issued from such patients could shed some light on this phenomenon. Another
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5 274 hypothesis could be that cryptic anomalies of the second allele are present in patients with
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7 275 MTP, that might be evidenced in whole genome analyses.
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9 276 The expression of GPIb α detected by flow cytometry, which reflects the whole GPIb-V-IX
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11 277 complex, was moderately decreased in most of cases, whatever the variant gene. This
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13 278 suggests that the proteins resulting from abnormal alleles do not allow the complex to be
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15 279 properly expressed at the membrane surface.
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17 280 From a functional point of view, the study of platelet aggregation unexpectedly revealed
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19 281 anomalies with ADP, arachidonic acid or collagen in association with GPIb α mutations. There
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21 282 is no clear explanation for this but, paralleling observations in homozygous BSS, this could be
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23 283 the consequence of an abnormal expression of the CD9 tetraspanin. CD9 and GPIIbIIIa are
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25 284 stored in the same intracytoplasmic platelet structures and migrate to similar membrane
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27 285 areas, which is consistent with a possible role of CD9 in platelet aggregation, modulating
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29 286 GPIIbIIIa and consistent with the abnormalities of platelet aggregation in the presence of other
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31 287 activators than ristocetin (14, 35, 36). More intriguingly, ristocetin aggregation was normal for
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33 288 these patients, although the VWF receptor, located in the LRR part of GPIb α , is encoded by
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35 289 the *GP1BA* gene. It could be that variants in the *GP1BA* gene do not directly impact the binding
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37 290 site of the VWF A1 domain (residues 60-128) nor the thumb region of the ligand binding
38
39 291 domain, which is important for the interaction between GPIb and VWF (37,38). Moreover,
40
41 292 again surprisingly, 4/5 patients with GPIb β variants tested displayed abnormalities affecting
42
43 293 platelet aggregation with intermediate concentrations of ristocetin. This suggests that the
44
45 294 GPIb β protein is essential to ensure the proper conformation of GPIb allowing for VWF binding
46
47 295 (39-41). In addition, several studies indicate that the binding of GPIb β to GPIb α protects the
48
49 296 latter from lysosomal degradation in (42,43). It is therefore possible that the binding defect
50
51 297 between the two subunits leads to excessive degradation of GPIb α , resulting in an abnormal
52
53 298 functional response to Willebrand factor

54
55 299 This work also demonstrates once again the interest of assessing the IPF, which corresponds
56
57 300 to the fraction of circulating immature platelets, in order to differentiate constitutional from
58
59 301 acquired thrombocytopenias. Indeed, in this cohort, the IPF value was significantly increased
60
302 in all patients whatever the *GP1BA* or *GP1BB* variant. This is in agreement with previous

1
2
3 303 studies that have attempted to define IPF thresholds to distinguish central from peripheral
4
5 304 thrombocytopenias (44,45). It has been shown that, in MTP, the IPF is increased (3 to 5 times
6
7 305 higher than in ITP) (46,47). Nevertheless, there are no validated normal values for IPF levels
8
9 306 to date, although several studies have measured IPF in cohorts of healthy adults, yielding
10
11 307 ranges of 0.2 to 7.7% (44-47). Finally, a recent study found that an IPF level greater than
12
13 308 $11 \times 10^9/L$ is a strong argument in favour of constitutional thrombocytopenia (47), which is
14
15 309 confirmed here.

16
17 310 Several questions however remain pending regarding these observations. First, patients with
18
19 311 these forms of MTP have very variable clinical features, both in the literature and in this
20
21 312 cohort. The spectrum of clinical manifestations ranges from the absence of haemorrhagic
22
23 313 signs (4 patients) to severe hematoma and minor haemorrhagic signs. It was also noted that,
24
25 314 in some cases, patients carrying the same variant (within the same family) presented
26
27 315 hemorrhagic signs of different severity. It is therefore likely that any given mutation is one
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29 316 element, among others, involved in the bleeding phenotype.

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31 317 In an attempt to explain the heterogeneity of the clinical-biological phenotype between the
32
33 318 different patients of this series, we can refer to the recent studies of polygenic scores for FBC
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35 319 traits (46) which seem to be especially relevant in the context of MTP. This model predicts that
36
37 320 associated gene variants can explain a large part of the heritability of traits through the
38
39 321 transregulation of major genes. For example, a network involving two genes encoding the
40
41 322 GPIb-V-IX receptor, GP1BB and GP9, and 8 other highly coexpressed genes can be mentioned.
42
43 323 These genes are all trans-regulated by the ARHGEF3 gene, which is one of the main genes
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45 324 regulating megakaryopoiesis and could therefore influence the severity of thrombocytopenia
46
47 325 as well as platelet size (48, 49).

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49 326 The pathophysiological mechanisms of the impact of these mutations on megakaryopoiesis
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51 327 remain to be elucidated. For this, it would be necessary to analyze larger series of patients
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53 328 and to carry out directed mutagenesis studies. It would also be interesting to compare the
54
55 329 proteomics of these variants with that of normal individuals, as performed by immunoblot for
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57 330 *GP1BA* p.Cys33Tyr by Skalníková et al. (13).

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59 331 Finally, the results of this study suggest that variants affecting the *GP1BA* and *GP1BB* genes
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332 are a fairly frequent cause of MTP. It is nonetheless probably underestimated, especially since

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3 333 patients display moderate thrombocytopenia with an often mild hemorrhagic phenotype. It
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5 334 may be proposed that a better etiological investigation of thrombocytopenias should be
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7 335 performed in order to avoid inappropriate diagnoses that could be at the origin of useless or
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9 336 even deleterious therapies.

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3 338 Authorship statement
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6 339 DF performed the research and analysed the data and wrote the paper, QA performed the
7
8 340 research and contributed essential tools, BS saw the patients and performed the research, BP
9
10 341 performed the research and contributed essential tools, BA saw the patients and performed
11
12 342 the research, TM saw the patients and performed the research, SM saw the patients and
13
14 343 performed the research, TC saw the patients and performed the research, DN saw the patients
15
16 344 and performed the research, EM performed the research and contributed essential tools, GB
17
18 345 saw the patients and performed the research, BMC analysed the data and wrote the paper,
19
20 346 MF saw the patients, designed the research study, analysed the data and wrote the paper.

21 347 Conflicts of interest
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23 348 The authors report no conflicts of interest.
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26 349 Data availability.
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29 350 All data generated or analysed during this study are included in this article. Further enquiries
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31 351 can be directed to the corresponding author
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3 490 **Tables**

4
5 491 Table 1: Compilation of the literature about GP1BA and GP1BB heterozygous mutations in
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7 492 patients with thrombocytopenia including the mutations reported here.

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9 493
10 494 Table 2: Clinical and biological features of the three patients with a *GP1BA* gene mutation.
11
12 495 Normal values: platelet count: 150-450x10⁹/L; MPV (mean platelet volume): 7.4-11.7 fL; IPF
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14 496 (immature platelet fraction): 1.1-6.1%; GPIb α surface expression: 27000-49000 sites/platelet.
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16 497 AA: arachidonic acid; Col: collagen, ADP: adenosine diphosphate.

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18 498
19 499 Table 3: Clinical and biological features of the six patients with a *GP1BB* gene mutation.
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21 500 Normal values: platelet count: 150-450x10⁹/L; MPV (mean platelet volume): 7.4-11.7 fL; IPF
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23 501 (immature platelet fraction): 1.1-6.1%; GPIb α surface expression: 27000-49000 sites/platelet.
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25 502 ND : not done.

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30 504 **Figure legends**

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32 505 Figure 1: Ribbon drawing showing the crystal structure of the GPIb α N-terminal domain (PDB
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34 506 code 1GWB). The location of the residues related to the 3 mutations detected: p.(Leu73Phe)
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36 507 (L73), p. (Asn150Ser) (N150) and p.(Leu155Pro) (L155) are depicted in yellow. These 3 residues
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38 508 are buried and may participate in the maintenance of the structure of GPIb α especially via
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40 509 hydrogen bonds. Their mutation into phenylalanine, serine and proline respectively, may have
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42 510 a significant impact on the conformation of the protein and therefore on binding to its
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44 511 partners such as VWF or α -thrombin.

45 512
46 513 Figure 2: (A) Ribbon diagram of the structure of the GPIb α unit (grey and pink shading) in
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48 514 complex with the VWF A1 domain (green) (PDB code 1SQ0). The Leu73 residue (L73) is showed
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50 515 in yellow in the vicinity of the β -finger residues (E21, V25, A26, D34), LRR1 (H53 and S55) and
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52 516 LRR2 (D79) of GpIb α , involved in interactions with VWF (dotted lines). The substitution of this
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54 517 residue with a phenylalanine, which is a larger residue, may impact the structure of GPIb α and
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56 518 its binding to VWF. (B) Zoom around residue Leu73 showing the interactions between VWF
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58 519 and GPIb α (pink dotted lines: hydrophobic, green dotted lines: hydrogen bond, orange dotted
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60 520 lines: electrostatic).

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5 522 Figure 3: Ribbon drawing of the GPIb α unit (PDB code 1GWB) in the area of residue Asn150
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7 523 (N150, in yellow) showing its participation in the hydrogen bonding network (green dotted
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9 524 lines). This residue is located at the end of the 5th LRR domain. Its substitution with a serine
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11 525 which is a smaller residue may disrupt this network, weakening the native conformation.
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14 527 Figure 4: (A) Ribbon diagram of the GPIb α unit complex structure (in grey and pink shading)
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16 528 with α -thrombin (orange) (PDB code 1P8V), showing residue Leu155 (L155, yellow) at the
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18 529 beginning of LRR6 and the two neighbouring prolines (P156 and P157) close to α -thrombin.
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20 530 The substitution of Leu155 with a proline will result in 3 consecutive prolines which may
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22 531 strongly constrain this area involved in the binding to α -thrombin. (B) Zoom around residue
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24 532 Leu155.
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27 534 Figure 5: (A) Structure of the GPIb β unit (PDB code 3RFE) showing the location of residues
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29 535 Leu137 (L137) and Cys141 (C141) (yellow) in the C-terminal capping region. The C-terminal
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31 536 capping region of GPIb β is involved in the formation of the GPb-IX-V complex. Leu137 is
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33 537 located more precisely in the α 2 helix. Its substitution with a proline is likely to strongly disrupt
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35 538 the helix structure which would certainly impact subunits assembly of the GPb-IX-V complex.
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37 539 Cysteine 141 is positioned at the end of the C-terminal domain of GPIb β and is engaged in a
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39 540 disulphide bridge with cysteine 95. The p.(Cys141*) variant would lead to a soluble form of
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41 541 GPIb β without the disulphide bridge with cysteine 95. The appearance of this soluble form
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43 542 could disturb the correct formation of the GPb-IX-V complex. (B) Zoom around residues
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45 543 Leu137 and Cys141.
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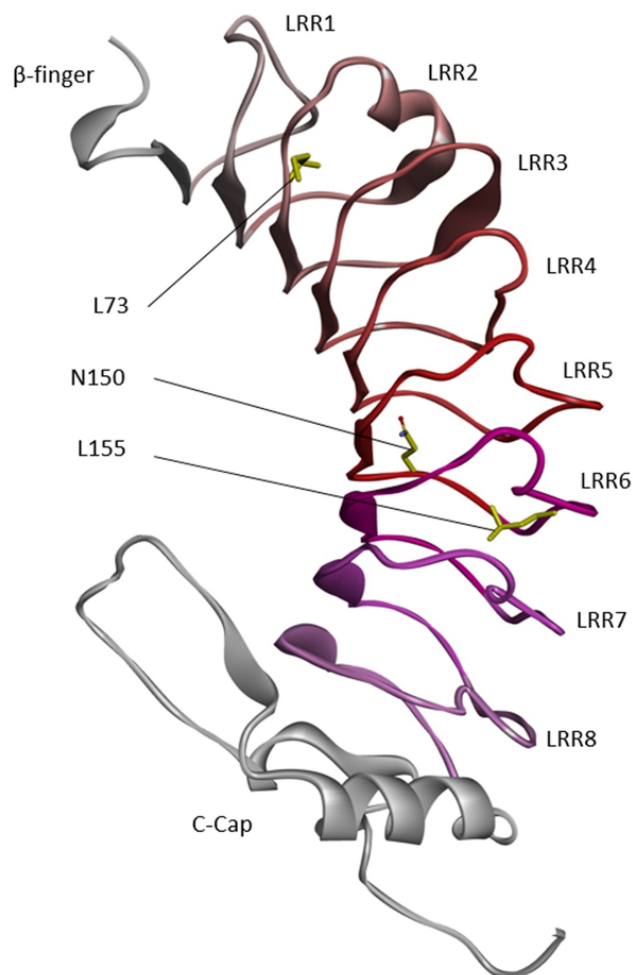


Figure 1: Ribbon drawing showing the crystal structure of the GPIIb N-terminal domain (PDB code 1GWB). The location of the residues related to the 3 mutations detected: p.(Leu73Phe) (L73), p. (Asn150Ser) (N150) and p.(Leu155Pro) (L155) are depicted in yellow. These 3 residues are buried and may participate in the maintenance of the structure of GPIIb especially via hydrogen bonds. Their mutation into phenylalanine, serine and proline respectively, may have a significant impact on the conformation of the protein and therefore on binding to its partners such as VWF or α -thrombin.

159x172mm (150 x 150 DPI)

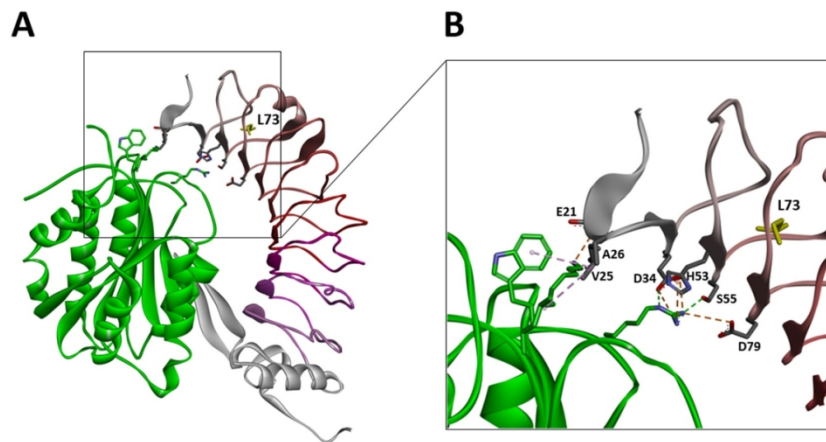


Figure 2: (A) Ribbon diagram of the structure of the GPIIb unit (grey and pink shading) in complex with the VWF A1 domain (green) (PDB code 1SQ0). The Leu73 residue (L73) is shown in yellow in the vicinity of the α -finger residues (E21, V25, A26, D34), LRR1 (H53 and S55) and LRR2 (D79) of GPIIb, involved in interactions with VWF (dotted lines). The substitution of this residue with a phenylalanine, which is a larger residue, may impact the structure of GPIIb and its binding to VWF. (B) Zoom around residue Leu73 showing the interactions between VWF and GPIIb (pink dotted lines: hydrophobic, green dotted lines: hydrogen bond, orange dotted lines: electrostatic).

230x144mm (150 x 150 DPI)

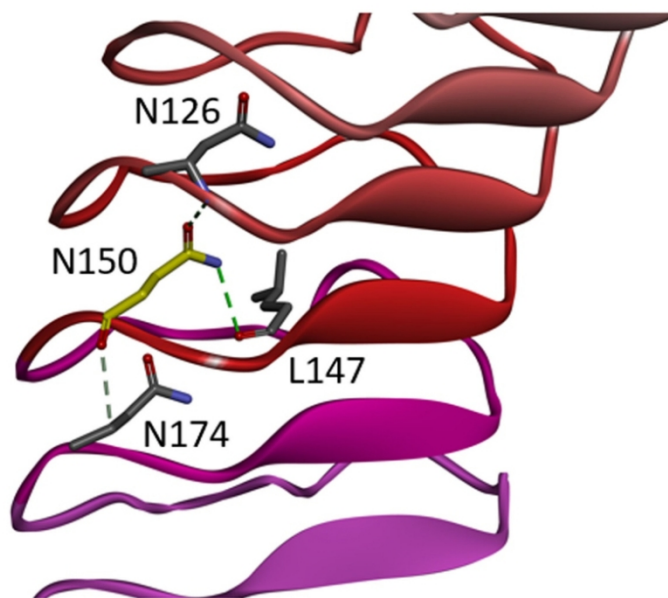


Figure 3: Ribbon drawing of the GPIIb/IIIa unit (PDB code 1GWB) in the area of residue Asn150 (N150, in yellow) showing its participation in the hydrogen bonding network (green dotted lines). This residue is located at the end of the 5th LRR domain. Its substitution with a serine which is a smaller residue may disrupt this network, weakening the native conformation.

197x168mm (150 x 150 DPI)

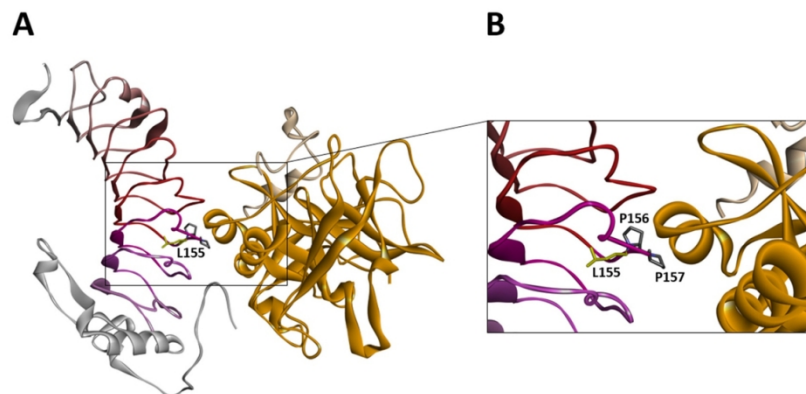


Figure 4: (A) Ribbon diagram of the GPIIb/IIIa unit complex structure (in grey and pink shading) with α -thrombin (orange) (PDB code 1P8V), showing residue Leu155 (L155, yellow) at the beginning of LRR6 and the two neighbouring prolines (P156 and P157) close to α -thrombin. The substitution of Leu155 with a proline will result in 3 consecutive prolines which may strongly constrain this area involved in the binding to α -thrombin. (B) Zoom around residue Leu155.

240x149mm (150 x 150 DPI)

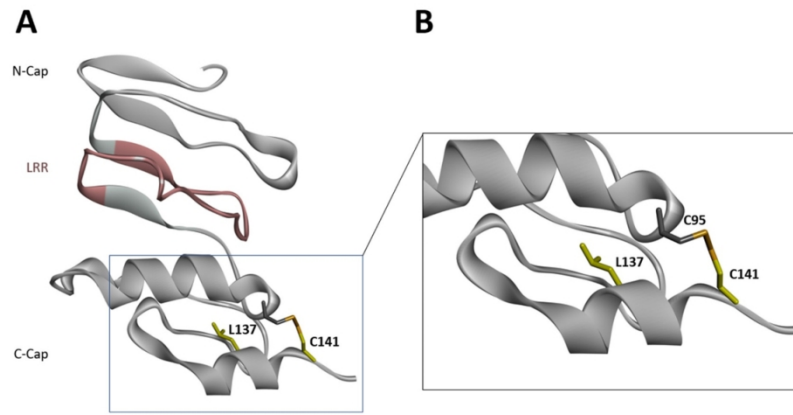


Figure 5: (A) Structure of the GPIIb/IIIa unit (PDB code 3RFE) showing the location of residues Leu137 (L137) and Cys141 (C141) (yellow) in the C-terminal capping region. The C-terminal capping region of GPIIb/IIIa is involved in the formation of the GPIIb/IIIa-IX-V complex. Leu137 is located more precisely in the α_2 helix. Its substitution with a proline is likely to strongly disrupt the helix structure which would certainly impact subunits assembly of the GPIIb/IIIa-IX-V complex. Cysteine 141 is positioned at the end of the C-terminal domain of GPIIb/IIIa and is engaged in a disulphide bridge with cysteine 95. The p.(Cys141*) variant would lead to a soluble form of GPIIb/IIIa without the disulphide bridge with cysteine 95. The appearance of this soluble form could disturb the correct formation of the GPIIb/IIIa-IX-V complex. (B) Zoom around residues Leu137 and Cys141.

242x148mm (150 x 150 DPI)

Table 1.

GPIIb Protein mutation	This study	Publications
p.(Cys20Gly)		Leinøe et al, 2021 (15)
p.(Val31Leu)		Ali et al, 2017 (10)
p.(Cys33Arg)		Ma, et al. 2021 (14)
p.(Cys33Tyr)		Skalnikova et al, 2022 (13), Leinøe et al, 2021 (15)
p.(Tyr54Asp)		Kunishima et al, 2006 (6)
p.(Leu57Phe)	✓	Miller et al., 1992 (7)
p.(Asn57 His)		Vettore et al, 2008 (5)
p.(Leu59 Arg)		Trizuljak et al, 2018 (11)
p.(Tyr70Asp)		Kunishima et al., 2006 (6)
p.(Leu83Phe)		Leinøe et al, 2021 (15)
p.(Arg127 Gln)		Bury et al., 2022 (16)
p.(Asn150Ser)	✓	Ghalloussi et al., 2018 (9)
p.(Leu155Pro)	✓	NR
p.(Ala172Val)		Savoia et al., 2001 (4), Noris et al., 2012 (8)

GPIIb Protein mutation	This study	Publications
p.(Met1*)		Sivapalaratnam et al., 2017 (20)
p.(Leu16Pro)	✓	Sivapalaratnam et al., 2017 (20), Babuty et al., 2019 (21)
p.(Pro27Ser)		Busonero et al. 2020 (18)
p.(Arg42Cys)		Kunishima et al., 2001 (19)
p.(Gly43Trp)		Sivapalaratnam et al., 2017 (20)
p.(Trp46*)		Sivapalaratnam et al., 2017 (20)
p.(Leu60Pro)		Ferrari et al., 2019 (23)
p.(Thr68Met)		Sivapalaratnam et al., 2017 (20)
p.(Pro79_Leu81del)		Sivapalaratnam et al., 2017 (20), Leinoe et al, 2021 (15)
p.(Pro79_Leu81dup)		Leinøe et al, 2021 (15)
p.(Arg110fs)		Dunstan-Harrison et al., 2022 (17)
p.(Tyr113Cys)		Sivapalaratnam et al., 2017 (20), Kurokawa et al., 2001 (22)
p.(Leu132Gln)		Savoia et al., 2001 (4)
p.(Leu137Pro)	✓	NR
p.(Cys141*)	✓	NR
p.(Ala150Argfs*43)		Sivapalaratnam et al., 2017 (20)
p.(Leu168dup)	✓	NR
Exon 2 deletion	✓	NR

NR : not previously reported in heterozygous state. Of note, all protein mutations are described according to the HGVS nomenclature

Table 1

<i>GP1BA</i> Mutations	Platelet count (x10 ⁹ /L)	MPV (fL)	IPF (%)	GPIb-V-IX expression (GPIb α sites/platelet)	Platelet functions (aggregometry)	Clinical features
c.217C>T p.Leu73Phe	49	11.2	67	17 341	Not assessed	Epistaxis
c.449A>G p.Asn150Ser	76	14.2	21.7	30 315	Decreased with AA, Col, ADP	Epistaxis and gum bleeding
c.464T>C p.Leu155Pro	79	12	ND	ND	Decreased with AA, Col, ADP	No bleeding

Table 2

<i>GP1BB</i> Mutations	Platelet count (x10 ⁹ /L)	MPV (fL)	IPF (%)	GP Ib-V-IX expression (GPIb α sites/platelet)	Platelet functions (aggregometry)	Clinical features
c.410T>C p.(Leu137Pro)	98	11.8	13	25955	Decreased with ristocetin low concentration	Gum bleeding
c.2423C>A p.(Cys141*)	99	12.9	15.7	ND	ND	No bleeding
c.47T>C p.(Leu16Pro)	105	14.1	27.8	37135	Decreased with ristocetin low concentration	Epstaxis, bruising, gum bleeding, hematomas and post-surgical bleeding
c.47T>C p.(Leu16Pro)	108	14.3	22.6	22202	Decreased with ristocetin low concentration	Epistaxis, gum bleeding
c.502_504dup p.(Leu168dup)	147	13.6	21.2	ND	Normal	Bruising, epistaxis, gum bleeding ant menorrhagia
Exon 2 deletion	94	14.3	35	11174	Slightly Decreased with ristocetin low concentration	No bleeding.

Biological, clinical features and modelization of heterozygous variants of GP1BA and GP1BB genes responsible for constitutional thrombocytopenia

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Supplemental table 1.

		ACMG scores			ClinVar
		Varsome	Franklin	InterVar	
GP1BA	p.(Leu73Phe)	4	3.9	4	4/5
	p.(Asn150Ser)	5	3.9	3	4/4
	p.(Leu155Pro)	3.5	3.1	3	fd
GP1BB	p.(Leu137Pro)	4	3.9	4	4
	p.(Cys141*)	4	5	4	4
	p.(Leu16Pro)	4	4.5	3	4/4
	p.(Leu168dup)	3.5	4.5	NC	fd

ACMG scores were calculated according to version 2 (with modulation of the different arguments) based on Richards et al., 2015 and other subsequent publications on the topic. These scores take into account many parameters at the variant locus including population data, literature, protein structure and function, bioinformatic predictions, segregation. ACMG scores are comprised between 1 and 5: 1: benign, 2: likely benign, 3: uncertain significance, 4: likely pathogenic, 5: pathogenic, NC: not calculated.

The three softwares used to perform these calculations can be accessed at

Varsome: <https://varsome.com/>

Franklin: <https://franklin.genoox.com/clinical-db/home>

InterVar: <https://wintervar.wglab.org/>

ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>): this database collates the ACMG scores calculated by the teams of experts over the world who have found the variant under consideration. fd: first description.