

**α IIb β 3 integrin : New allelic variants in Glanzmann Thrombasthenia,
effects on mRNA splicing, expression and structure-function**

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Short title :

New mutations in Glanzmann patients and carriers

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6 **Abstract**
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10 Glanzmann thrombasthenia (GT) is an autosomal recessive inherited bleeding disorder
11 characterized by an impaired platelet aggregation due to defects in integrin α IIB β 3 (ITGA2B,
12 ITGB3), a fibrinogen receptor. Mutations from 24 GT patients and 2 carriers of various
13 origins, Caucasian, North-African and Asian were characterized. Promoter and exon
14 sequences of α IIB and β 3 genes were amplified and directly sequenced. Among 29 identified
15 mutations, 17 new allelic variants resulting from nonsense, missense and deletion / insertion
16 mutations were described. RNA alterations were evaluated by using web-servers. The α IIB
17 p.S926L, p.V903F, and β 3 p.C38Y, p.M118R, p.G221D substitutions prevented complex
18 expression at the surface of Cos-7 cells by altering the α IIB or the β 3 subunit structure. As
19 shown by free energy analyses applied on the resolved structure of α IIB β 3 and structural
20 modeling of the mutant, the p.K253M substitution of β 3 helped to define a key role of the
21 K253 in the interaction of the α IIB β -propeller and the β 3 β -I domains. Finally, the α IIB
22 p.Q595H substitution allowed cell surface expression of the complex but its corresponding
23 c.2800G>T mutation is predicted to alter normal RNA splicing. Characterization of GT
24 mutations is of interest for prenatal diagnosis, genetic counseling and provides useful tools for
25 structure-function relationship studies of α IIB β 3.
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54 **Keywords**

55 α IIB β 3, ITGA2B, ITGB3, Glanzmann, protein structure modeling
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INTRODUCTION

Glanzmann thrombasthenia (GT) is an autosomal recessive bleeding disorder characterized by a prolonged bleeding time and an impaired platelet aggregation in response to physiological agonists such as ADP, thrombin, collagen and epinephrine [Caen, 1972]. GT results from quantitative and / or qualitative defects of the fibrinogen (Fg) platelet receptor, the glycoprotein α IIb β 3 complex (OMIM accession number #273800). Classically GT has been divided into 3 subtypes defined by the α IIb β 3 content of patient's platelets : Type I, no or only trace amounts of α IIb β 3; Type II and variant, respectively with low and near normal platelet surface content of functionally impaired α IIb β 3 [Nurden, 2005]. To date, more than 100 different mutations involved in GT phenotype have been described in the α IIb gene and 60 in the β 3 gene (<http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu>). The nature of GT mutations is highly variable excepting some that are commonly identified in consanguineous populations such as the α IIb c.409-3_418del (IVS3-3del13) and the β 3 c.2031_2041del (2031del11) respectively detected in Arab and Iraqi-Jewish GT populations [Newman et al., 1991], the α IIb c.1544+1G>A (IVS15+1/G>A) mutation in French Gypsies [Schlegel et al., 1995], or the β 3 c.1053_1058del (1053CCCAGG) deletion in North-Africans [Morel-Kopp et al., 1997].

Characterization of GT mutations in patients is critical for prenatal diagnosis and genetic counseling. We report here the characterization of 29 mutations identified in 24 thrombasthenic patients and 2 asymptomatic carriers. Using site directed mutagenesis, cell expression models, *in silico* tools and the resolved structure of the α IIb β 3 complex, we have analyzed the pathogenic role of 17 newly described allelic variants. Structural modeling studies of an allelic variant of GT has revealed the importance of the Lys253 in the interaction of the α IIb β -propeller and the β 3 β -I domains.

MATERIAL AND METHODS

Patients

Blood samples from patients were sent to our laboratory for confirmation of GT diagnosis and genetic analysis. Propositi and their detailed platelet phenotype are listed in Supp Table S1. Propositi 4 and 15 are GT carriers. Propositus 4 who had presented mild bleeding and healing difficulties during tympanoplasty was addressed to the laboratory for GT investigation after flow cytometry analysis revealed a 50 % reduction of α IIB β 3 platelet surface expression. Propositus 15 was unexpectedly found to be a GT type I carrier during neonatal alloimmune thrombocytopenia diagnosis following in utero fetal death. Her platelets were phenotyped HPA-3a negative but genetically HPA-3a/b as determined by PCR-Sequence Specific Primer testing (data not shown).

As shown in Supp Table SI, 19 propositi of 22 of confirmed platelet phenotype_ (86 %) were found to be of type I (< 5 % of α IIB β 3 and < 25 % of Fg normal platelet contents). Three propositi were found to be of type II as their platelets presented low but significant α IIB β 3 content (\geq 10 %) and a relatively high level of intra-platelet Fg (\geq 50 %). None of these patients had been previously studied for molecular gene defects. Patients described herein gave their informed consent for genetic analysis.

Monoclonal antibodies

Monoclonal antibodies (Moab) SZ1 (anti-GPIX), SZ22 (anti- α IIB), SZ21 (anti- β 3), P2 (anti- α IIB β 3) and E3 (anti-Fg) were purchased from Beckmann Coulter-France (Villepinte, France). Moabs AP2 (anti- α IIB β 3) and AP3 (anti- β 3) were obtained from Lucron Bioproducts

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3 (Lille, France). Moab Y2-51 (anti- β 3) was purchased from DAKO (Trappes, France). Moab
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5 XIIF9 to β 3 was a generous gift from G.Vezon (CRTS, Bordeaux, France). In some flow
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7 cytometry experiments, Moabs SZ22, SZ21 and P2 were directly conjugated to FITC.
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10 11 12 13 **Blood samples**

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15 Blood samples from patients were collected by venipuncture using 5mM ethylene-diamine-
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17 tetra-acetic acid as an anticoagulant. Platelet were separated by differential centrifugation and
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19 used or stored in 70% ethanol at -80°C until RNA was extracted. In some cases, 500 μl of
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21 whole blood were mixed with 1.3 ml of RNA Later (Applied Biosystem, Courtaboeuf,
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23 France) and were kept frozen at -80°C for subsequent RNA extraction.
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33 34 35 36 **Flow cytometry**

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38 For platelet glycoprotein and Fg content analysis, 50 μl of EDTA anticoagulated whole
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40 blood was diluted in 500 μl of phosphate saline buffer (PBS) pH 7.2 containing 1 % (wt/vol)
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42 BSA-fraction V (Euromedex, Souffelweyersheim, France) and 3 mM EDTA (PBE buffer).
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44 Cell were fixed by adding 1.5 ml of PBS - 3 mM EDTA, pH 7.2, containing 1.33 % (vol/vol)
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46 of a methanol-stabilized formaldehyde solution (Fisher Scientific, Illkirch, France) for 15 min
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48 at room temperature (RT). The reaction was stopped by adding 2 ml of PBE before cells were
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50 pelleted by centrifugation at 1500 g for 10 min. Pelleted cells were washed in 2 ml PBE
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52 before to be resuspended in 1 ml PBE containing 0.1 % (wt/vol) sodium azide and store at 4°C
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54 until use. 40 μl of the fixed cells suspension were incubated with 3 $\mu\text{g}/\text{ml}$ Moab in PBE (100
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56 μl final volume), for 1 h at RT. After two washes in 2 ml PBE, the bound Moabs were
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58 detected by using a polyclonal goat anti-mouse IgG conjugated to FITC (Jackson
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60 Immunoresearch Europe, Newmarket, UK) diluted in PBE (1 μl for 10 ml) for 30 min at RT.
Following one wash in 2 ml PBE, mean fluorescence intensity of cells was analyzed on an

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3 XL-MCL Flow cytometer (Beckman Coulter, Villepinte, France). To detect intraplatelet Fg,
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5 each incubation steps were done in presence of 1% (wt/vol) saponine (Fischer Scientific).
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8 Amounts of proteins are expressed as a percentage of the mean amounts obtained with normal
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10 platelets from 10 healthy donors.
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16 To analyze α IIb β 3 wild type or mutant complexes expressed at the surface of transfected
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18 Cos-7 cells, cells were collected by Trypsin-EDTA treatment and washed 2 times in PBS-1%
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20 (wt/vol) BSA. 5×10^5 cells were incubated in 100 μ l PBS containing 3 μ g/ml Moabs (SZ22,
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22 SZ21 or P2) conjugated to FITC for 1 h at RT. Cells were washed twice in 2 ml PBS-BSA
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24 before to be analyzed by flow cytometry. Dead cells were discarded from analysis by using
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26 the 7-AAD viability dye (Beckmann-Coulter). Results are expressed as a percentage of
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28 positive cells for the concerned antigen.
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DNA/RNA extractions

Genomic DNA was isolated from peripheral blood according to a published procedure [Jallu et al., 2002] or by using the MagNA Pure Compact Instrument and the Nucleic Acid Isolation Kit I (Roche Diagnostics, Meylan, France). In some cases, DNA samples were obtained by using QIAamp DNA Blood Mini kit (Qiagen, Courtaboeuf, France). RNA extractions were done on platelets by using the Rneasy Protect MiniKit (Qiagen). DNAs and RNAs were extracted according to the manufacturers' instructions.

Sequencing analysis

Sequencing was performed as previously described [Quintanar et al., 1998] or by GenoScreen (Lille, France). Primers defined by Kato and coworkers [Kato et al., 1992] and Jin and co-workers [Jin et al., 1993] were respectively used to amplify coding sequences of

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3 α IIb and β 3 genes from genomic DNA. Primers to amplify promoter sequences of α IIb and
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5 β 3 have been respectively defined according to the genes' reference sequences NG_008331.1
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7 and NG_008332. α IIb promoter sequences were studied by using the following primer pairs
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9 ("a" forward, "b" reverse) :

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12 IIbP1a CGCCAGAATTCTCAGTCACGAAGCTTGGCTCAAGACGGAG

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14 IIbP1b CCAGCCAGATATCAGCTATGTACTACCACCGTGCTAGTCC

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16 IIbP2a CGCCAGAATTCTCAGTCACGGGTAAAGATTCAAGAGACAT

17
18 IIbP2b CCAGCCAGATATCAGCTATGTCAGATTCCTCCACAGGAAG

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20 IIbP3a CGCCAGAATTCTCAGTCACGGAAGGGAAGGAGGAGGAGCT

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22 IIbP3b CCAGCCAGATATCAGCTATGCTTCCCTTACGGCTCACCTC

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25 Underlined sequences are non genomic sequence for sequencing PCR products. Promoter
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27 and exon 1 sequences of β 3 gene were amplified with the primer pair

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29 IIIaE1a ATGTGGTCTTGCCCTCAACAGGTAG

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31 IIIaE1b CACGCTCTCACCCAGGAAGTTACAG by using the Advantage GC genomic
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33 PCR kit (Clontech, Saint-Germain-en-Laye, France) and then sequencing was done with the
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35 forward primer 3aseq-P1a CCGGAAAACCAAATAAGGC and the reverse primer 3aseq-
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37 P1b GACCAGTCCTCCGCGTTTGC.

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39 All identified mutations were confirmed by PCR-Restriction Fragment Length
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41 Polymorphism (PCR-RFLP) analysis on a second PCR product. Restriction enzymes are
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43 listed in tables 1 and 2 with the corresponding mutations. The experimental details of these
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45 RFLP assays can be obtained upon request. When the mutation did not introduce restriction
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47 modification, sequencing was done on a second PCR-product. For nucleotide numbering the
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49 A nucleotide of the ATG start was designated +1 (cDNA ITGA2B and ITGB3 GenBank
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51 accession numbers NM_000419.3 and NM_000212.2 respectively). For amino acid
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53 numbering used in the text, the first amino acids of the mature α IIb and β 3 proteins were
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3 designated +1. Residue numbering according to the Human Genome Variation Society
4 (HGVS) nomenclature that designed as +1 the translation initiating methionine
5 (http://www.hgvs.org/mutnomen) is also shown in tables 1 and 2. Both numberings differ by
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10 31 and 26 residues that correspond to the signaling peptides of α IIB and β 3 respectively. All
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12 identified allelic variations have been deposited on the NCBI dbSNP database
13 (http://www.ncbi.nlm.nih.gov/SNP/) and their corresponding accession numbers are indicated
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18 in tables 1 and 2.

21 22 **Site directed mutagenesis and in-vitro expression**

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24 Site directed mutagenesis and *in vitro* expression in Cos-7 cells were done as previously
25 described [Jallu et al., 2002] or by using the QuikChange® II XL Site-Directed Mutagenesis
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27 Kit (Agilent Technologies, Massy, France) according to the manufacturer's instructions. In
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29 some mutagenesis experiments, plasmid pcDNA3.1-Zeo containing the α IIB cDNA was used
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31 instead of pcDNA3.1-Neo (Invitrogen, Cergy Pontoise, France).

32 33 **Minigene study**

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35 To study a mutation predicted to affect mRNA splicing, an α IIB gene fragment covering
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37 exons 17 to 20 and containing the mutation was synthesized and introduced in the plasmid
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39 Exontrap (Mobitech, Goettingen, Germany). The wild type form of the sequence was obtained
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41 by site directed mutagenesis. Synthesis, cloning and site directed mutagenesis were done by
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43 the Epoch Biolabs, Inc. Company (Sugar Land, TX, USA). Following transient transfection in
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45 Cos cells, total RNA was extracted and mRNA amplified for the exon 17 to 20 and directly
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47 sequenced.
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Western-blot analysis

Trypsin-EDTA collected Cos-7 cells were washed 2 times in PBS and 1 time in 50 mM Tris-HCL pH 7.6, 150 mM NaCl. Cells were resuspended (1.5×10^7 cells) in 100 μ l of the same Tris buffer containing 1% (wt/vol) Triton-X 100 and 2 mM para-Methyl Sulfonyl Fluoride (PMSF). They were lysed for 30 min at 4°C and the lysate centrifuged at 16.000 g for 30 min at 4°C. The supernatant was diluted with 4 volumes of a 150 mM NaCl solution containing 2.5 % (wt/vol) Sulfate Dodecyl Sodium (SDS), 31.25 mM n-ethylmaleimide and 2.5 mM PMSF. The SDS lysate was heated to 100°C for 5 min before 35 μ l were used for western-blot analysis as previously described [Jallu et al., 1994] with a minor modification : bound antibodies were detected by using anti-mouse IgG conjugated to peroxidase associated with a chemiluminescence detection technique.

Computational analyses

The effect of mutations on intron splicing has been predicted *in silico* by using the following tools : Genscan (<http://genes.mit.edu/GENSCAN.html> [Burge and Karlin, 1998]) and NNSPLICE 0.9 (http://www.fruitfly.org/seq_tools/splice.html [Reese et al., 1997]). Modifications of the splicing factors binding sites pattern resulting from the mutations were evaluated by using the ESEFinder tool [Cartegni et al., 2003; Smith et al., 2006].

To compare wild type and a mutant headpiece of α IIB β 3, modeling has been done using the structure (PDB code 2VDL) published by Springer et co-workers [Springer et al., 2008]. Residues 1-452 of α IIB and 109-352 of β 3 were taken into account. The interface between the protein chains has a correct resolution (2.75 Å) and the residues implicated at the interface have all good normalized B-factor values. A structural model of the mutant has been built. This model was checked using ProCheck software to analyze the quality of the modification [Laskowski et al., 1993] and the side-chain position was analyzed with SCWRL [Canutescu et

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3 al., 2003]. Finally, the FastContact web server (<http://structure.pitt.edu/servers/fastcontact/>)
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5 was used to estimate the direct electrostatic and desolvation interaction free energy between
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7 two proteins [Camacho and Zhang, 2005; Champ and Camacho, 2007]. The computational
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9 estimation of the binding free energy is based on a statistically determined desolvation contact
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11 potential and Coulomb electrostatics with a distance-dependent dielectric constant [Camacho
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13 and Zhang, 2005]. This free energy provides a reasonably good estimate of experimental
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15 binding affinities from complex crystal structures.
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20 21 22 RESULTS

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Clinical GT diagnosis was confirmed in our laboratory by immunological or biochemical studies (see Supp Table S1). Genomic DNA samples from patients were studied for molecular defects in promoter and exon sequences of genes coding for α IIB and β 3 by direct sequencing of the corresponding PCR products. Allelic variations identified in these genes are reported in tables 1 and 2. To avoid erroneous identification due to amplification errors, all sequence alterations have been confirmed on a second PCR product either by a PCR-RFLP technique when a restriction site modification was observed or by new sequencing analysis in other cases. Restriction enzymes that have been used are listed in tables 1 and 2 with their corresponding allelic variant. When available, DNAs from patient's relatives were also analyzed and used to confirm the allelic variations (data not shown).

51 52 53 54 55 56 57 58 59 60 **Sequence alterations detected in the α IIB gene**

For 18 propositi, a total of 20 mutations was identified in the gene coding for α IIB (see table 1). Five lead to nonsense codons : patients 1, 6, 7, 8 and 17. It should be noted that patient 17 was phenotype GT type II because the second mutation that had not been identified allowed residual expression of α IIB β 3 (see Supp Table S1). Three mutations directly affect a splice site : patients 9 and 12 with a single point mutation and 3 unrelated propositi (GT

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3 patients 3 and 5, and the carrier 4) who present the same 13 base pair deletion c.1442-
4 13_1442-1del. However for these latter propositi, an unknown common ancestry cannot be
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6 excluded as all are European Caucasians who live in the north-east of France. Two
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8 heterozygous single base insertions were identified in patient 2. These mutations are predicted
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10 to result in frame-shift anomalies creating a downstream stop codon and premature
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12 termination. A 10 base pair deletion identified in patient 14 also leads to a frame shift
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14 anomaly. Other gene anomalies identified affecting α IIb, were missense mutations. Three of
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16 them (patients 6, 10, 15 and 16) have not been described so far and 6 (patients 3, 11, 12, 13
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18 and 18) have already been reported (see references in Table 1). Among the 9 heterozygous
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20 compounds, 4 second obligatory mutations (patients 5, 7, 17 and 18) remain to be identified
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22 (NI abbreviation (not identified) in tables).
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33 **Sequence alterations detected in the β 3 gene**

34 Ten mutations were identified in the gene coding for the β 3 glycoprotein for 8 propositi
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36 (see Table 2). One single point mutation (patient 20) is responsible for a nonsense codon and
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38 a second (patient 19) disrupts an acceptor splice site. An homozygous 4 base pair deletion
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40 located downstream of the donor splice site (c.1125+3_1125+6del) of intron 8 has been
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42 identified as the unique mutation in patient 25. In patient 26, two silent homozygous
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44 mutations, c.1143C>A and c.1260G>A (20507C>A and 20624G>A with genomic
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46 numbering : accession number J05427) located in exon 9 are observed. These mutations have
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48 been reported to be responsible for type I GT [Jin et al., 1996]. Though silent, their
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50 association induces exon 9 skipping and the introduction of five intronic bases in the final
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52 transcript [Jin et al., 1996]. Analysis of the mRNA from patient 26 has confirmed this deletion
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54 / insertion mechanism (data not shown). Finally five single point missense mutations were
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3 identified (patients 21, 22, 23 and 24). In two heterozygous patients the second obligatory GT
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5 mutation remains to be determined.
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10 **Flow cytometry analysis of mutant complexes expressed in COS-7 cells**

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12 Seven missense mutations not previously reported in the literature have been further
13 studied by expressing the mutant complexes in COS-7 cells to assess their role in the GT
14 phenotype. Cell surface expression of the mutant complexes was assessed by flow cytometry
15 using Moabs SZ22 to α IIB, SZ 21 to β 3 or P2 to the α IIB β 3 complex (see Figure 1). α IIB
16 p.S926L (patient 16) and p.V903F (patients 6 and 15) and β 3 p.C38Y (patient 21), p.M118R
17 (patient 22), p.G221D and p.K253M (patient 24) substitutions prevented normal complex
18 expression, mimicking the type I GT phenotype observed for the patients. For the α IIB
19 p.S926L (patient 16) and β 3 p.G221D and p.K253M (patient 24) mutants the percentage of
20 positive cells expressing β 3 was slightly higher than in other mutants tested (see Figure 1).
21 This low level expression could be correlated to the 10 % of β 3 expression at the surface of
22 the patients' platelets (see Supp Table S1).
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Surprisingly, the α IIB p.Q595H substitution identified in a GT type I patient did not affect
the expression of the mutant complex which is similar to that obtained with the wild type
form.

As control, untransfected cells did not significantly bind any of the Moabs tested and
irrelevant mouse IgG gave less than 1 % of positive cells whatever the transfectant tested.

In silico analyses of mRNA processing for missense allelic variations and for the intronic deletion c.1125+3_1125+6del

Some exonic single point mutations can alter normal processing of the mRNA and lead to
the synthesis of an aberrant mRNA, associated or not with its degradation. Genscan,

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NNSPLICE 0.9 and ESEFinder web servers were used to predict the effect of the missense mutations responsible for the substitutions that have been tested in flow cytometry. From this panel, the c.1878G>C responsible for the p.Q595H substitution was the unique variation predicted to modify normal splicing and to affect ESE binding sites. Splice site prediction by NNSPLICE 0.9 cannot identify the normal donor splice site of intron 18 in the mutant. Genscan analysis did not predict splice anomalies, but scores were lowered for both exons 18 (0.987 to 0.791) and 19 (0.995 to 0.849) implying a probability of correct splicing of 75 % instead of 99 % for the normal sequence. Furthermore ESEFinder shows mutation 1878G>C to increase the score of an SF2/ASF binding site from 2.67 to 3.28 and to create a new one with a score of 2.50 (threshold 1.95). Finally an SRp55 binding site that presents a high score (4.33 - threshold 2.67) encompassing the normal splice site is also created.

In the $\beta 3$ gene, the homozygous 4 bp deletion c.1125+3_1125+6del (patient 25) was the single intronic mutation identified which does not directly affect a splice site. However Genscan analysis predicts the use of a cryptic donor splice site located 70 bp downstream the normal one. If this transcript is not degraded, this would lead to an in frame introduction of 23 amino-acids between $\beta 3$ β -I and hybrid domains. ESEFinder shows no modifications but NNSPLICE 0.9 suggests the contiguous donor splice site would be ignored in the mutant.

Western-blot analysis of the p.C38Y $\beta 3$ mutation effect on the complex expression.

All the $\beta 3$ mutants tested strongly affect complex expression (Figure 1) but the p.C38Y substitution also strongly and specifically affects that of $\beta 3$ when tested with anti- $\beta 3$ Moab SZ21. To understand this specific effect, synthesis of the Y38 $\beta 3$ form of the complex in the transfected cells was analyzed by using a Western-blot technique with Moabs SZ22 to α Ib and SZ21, XIIF9 and AP3 to $\beta 3$ (see Figure 2). SZ22 showed that α Ib is clearly detectable in the lysate of the wild type α Ib $\beta 3$ transfectant (lane c) as in the control platelet lysate (lane a).

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For the α IIb-Y38 β 3 mutant transfectant, only a band of slightly greater relative mobility which might correspond to a degradation product of α IIb was detected (lane d). Moab XIIF9 revealed the presence of the Y38 β 3 mutant in the α IIb-Y38 β 3 transfectant (lane d). However, this mutant glycoprotein presents a lower relative mobility when compared to the wild type forms detected in lysates from platelets or wild type α IIb β 3 transfectant (lane c). Both SZ21 and AP3 failed to react with the Y38 β 3 mutant (lanes d) but reacted with normal β 3 expressed in platelet and wild type α IIb β 3 transfectant (lanes a and c respectively). XIIF9 and AP3 but not SZ21 reacted with the endogenous β 3 subunit of the vitronectin receptor expressed in Cos-7 cells as shown by the faint band detected in non transfectant cells (lane b). These results suggested that the p.C38Y mutation should impair the complex formation, or at least its normal processing, as no α IIb of normal mobility could be detected.

Role of the Lys253 of the β -I domain of β 3 in the interaction with the α IIb β -propeller

To understand precisely the impact of the p.K253M mutation, we have analyzed the interface between α IIb (residues 1-452) and β 3 (residues 109-352) subunits using the protein structures obtained by the group of Springer (PDB code 2VDL [Springer et al., 2008]). From a simple visualization, the side chain of the β 3 K253 protrudes from the β -I domain toward the α IIb beta-propeller surface and makes contact with different residues of the α IIb subunit (see Figure 3A). Table 3 summarizes the different results concerning the estimations of the direct electrostatic and desolvation interaction free energy between the α IIb and β 3 chains. Interestingly, K253 has a key role in terms of binding free energy with the most negative contribution (-5.5 kcal/mol) and also in terms of electrostatics (-9.7 kcal/mol). Inversely, desolvation free energy is high (+4.1 kcal/mol). The desolvation of charged and polar amino

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3 acid is unfavorable from an energy point of view. This penalty of desolvation is greatly
4 compensated by Coulomb interactions and by the hydrogen bonds which are formed in the
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6 complex.
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10 Analysis of important interactions of by K253 underlines its weight in the interaction as it
11 has the 1st (-6.7 kcal/mol with D232 of α IIb), the 6th (-2.0 kcal/mol with F231) and the 13th (-
12 0.8 kcal/mol with P238) most negative interactions of the interface. One interaction with
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14 W262 is slightly unfavorable. Concerning free energy, the same observations are made, with
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16 two strong interactions with D232 (-5.5 kcal/mol) and F231 (-1.2 kcal/mol). One unfavorable
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18 interaction is observed again with W262. These results highlight the significance of this
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20 residue, the most important in terms of electrostatic and free energy computation.
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24 A structural model of M253 mutant was constructed. Regarding the whole α IIb and β 3
25
26 interface, the substitution provokes a striking loss of electrostatic energy (+9.1 kcal/mol) but a
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28 gain in desolvation free energy (-5.72 kcal/mol) that cannot counter-balance the decrease of
29
30 electrostatic energy. As shown in Table 3, the diminution of the length of the side-chains and
31
32 the change from a charged polar amino acid to a hydrophobic amino acid has a deep impact.
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34 Residue 253 has no more (negative) interaction with either D232 or F231, or P238. Only a
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36 single interaction with W262 is conserved (see Figure 3B).
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40 From a visual point of view, the decrease of electrostatic energy between K253 (see
41
42 Figure 3C) and M253 (see Figure 3D) is striking. Overall, the most important electrostatic
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44 energy implicated in the interaction becomes negligible. All these contacts are lost in presence
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46 of the methionine, except for W262 that only preserves an electrostatic link. These results
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48 indicate that it is rather the loss of K253 lost than the presence of the methionine which is
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50 responsible for the defect of complex expression.
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DISCUSSION

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3 The aim of the study was to identify mutations responsible for GT platelet phenotype.
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5 SDS-PAGE, western-blot and flow cytometry analyses carried out on patients' platelets
6
7 confirmed GT clinical diagnosis. Twenty-nine mutations were identified in both α IIb and β 3
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9 genes. Most of these mutations (47 %) correspond to missense mutations but nonsense,
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11 insertion, deletion, and single point mutations affecting splice sites were also found.
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15 Mutations previously reported in the literature (see tables 1 and 2) have not been further
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17 studied in-vitro for their involvement in GT. It can be noted that their presence in the patients
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19 studied herein and in one or more additional unrelated patients would confirm their role in GT
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21 phenotype. New mutations directly affecting splice sites, creating nonsense codon or insertion
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23 / deletion associated with frame shift anomaly were considered as known cause of GT. The
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25 pathological effects of new allelic variations responsible for missense substitutions and one
26
27 new allelic variation leading to a splicing anomaly are discussed below.
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31 The substitutions p.V903F (patient 6 and propositus 15) and p.S926L (patient 16) affect
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33 the extra-cellular N-terminal part of the α IIb β -subunit that composes the calf-2 domain (see
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35 Figure 4). In both cases they prevent α IIb β 3 complex expression at the Cos-7 cell surface.
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37 They are predicted to impair the correct folding of the Calf-2 domain as the amino-acids V903
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39 and S926 are located in its core (see Figure 4). To estimate their structural importance, the
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41 human α IIb primary sequence has been aligned with several corresponding integrin sequences
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43 - marsupial, fish, amphibian, prochordates and insect (Supp Figure S1A). These sequences
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45 share at least 45 % of identity with the human α IIb (Psi-blast data from
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47 <http://www.ncbi.nlm.nih.gov/BLAST>) and only 17 % of these residues are conserved through
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49 all the seven organisms tested. It is noteworthy that V903 and S926 belong to these few
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51 amino-acids, indicating that their conservation could be linked to a strong structural
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53 evolutionary constraint. Furthermore, their poor conservation in other human integrin α -
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55 subunits (data not shown) reinforces their α -IIb specificity.
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3 The missense mutation c.1878G>C (patient 10), contiguous with the donor splice site of
4 intron 18, was predicted to be responsible for a p.Q595H substitution in α IIB. However this
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8 p.Q595H mutation identified in a type I GT patient does not prevent the expression of the
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11 mutant complex at the surface of transfected Cos-7 cells. Study of the patient's platelet
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13 mRNA revealed full normal β 3 mRNA synthesis but α IIB mRNA remained undetectable. A
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15 splicing anomaly can lead to mRNA decay and explain the lack of detection of the mRNA
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17 coding for α IIB in patient 10. *In silico* splicing testing by using the Genscan and_NNSPLICE
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19 0.9 web servers effectively predicted splicing anomalies. Similarly Jayo and coworkers [Jayo
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21 et al., 2006] reported that the α IIB c.2829C>T mutation (exon 27) predicted to induce a
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23 p.L912P substitution, partially leads to exon 28 skipping. The authors pointed out that rather
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25 than the disruption of an ESE site, the aberrant mRNA processing would result from the
26
27 strengthening of the SR protein SC35 binding score by 0.65 units (ESEFinder analysis).
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29 Interestingly the ESEFinder web server predicted the mutation c.1878G>C to increase the
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31 score by 0.61 of an existing SF2/ASF binding site and to create two new binding sites for
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33 SF2/ASF and SRp55 factors with significant high scores (respectively 2.5 and 4.33). It can
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35 therefore be hypothesized that the 1878G>C mutation alters the normal splice factor pattern
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37 necessary to accurately splice intron 18. This was confirmed by a minigene study that showed
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39 the c.1878G>C alteration to provoke exon 18 skipping during mRNA splicing (data not
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41 shown).
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49 In the β 3 gene, the homozygous 4 bp deletion c.1125+3_1125+6del (patient 25) was the
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51 single intronic mutation identified that does not directly affect a splice site but its contiguous
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53 intronic sequence. Genscan analysis predict an altered splicing that correlates with the partial
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55 loss of a sequence necessary to hybridize a small nuclear ribonucleoprotein involved in the
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57 splicing process [Wang and Cooper, 2007].
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The mutation c.191G>A (patient 21) responsible for a p.C38Y substitution, is located in the PSI domain of the $\beta 3$. Transient co-transfection of the $\beta 3$ mutated cDNA and with normal α IIb cDNA showed the mutation to reproduce a GT type I phenotype as no complex was detected on the cell surface by any of the anti- α IIb $\beta 3$ Moabs tested herein and including testing with a panel to $\beta 3$ (XIIF9, AP3 and Y2-51, data not shown). Computational analysis (Genscan, ESEFinder and NNSPLICE 0.9) indicated that the c.191G>A mutation would not induce splicing modification of the pre-mRNA. However, western-blot analyses showed that α IIb and the mutated $\beta 3$ were synthesized in transfected COS cells. The mutated Y38 $\beta 3$ presents a lower relative mobility than the wild type form which could result from structural modifications induced by the C16 – C38 disulfide bond disruption. A similar observation has been reported by Valentin and co-workers [Valentin et al., 1995], who have shown that alanine substitution of C38 lowered the relative mobility of $\beta 3$ expressed in COS cells. The structural modification of the PSI domain induced by the p.C38Y mutation was evidenced by the lack of reactivity of Moabs AP3 and SZ21 whose epitopes are respectively located in amino-acids stretches 49-98 [Peterson et al., 2003] and 28-35 [Honda et al., 1995]. So the structural anomaly of the PSI domain impairs the complex association and / or its post-translational maturation leading to its retention inside the cell.

The missense mutations p.M118R, p.G221D and p.K253M are located in the β -I domain of $\beta 3$ and are responsible for Type I GT. M118 and G221 are highly conserved in most integrin β subunits (data not Shown), and through invertebrates to vertebrates (see Supp Figure S1B). Most of the β -I domain mutations previously described, are responsible for Variant [Bajt et al., 1992; D'Andrea et al., 2002; D'Andrea et al., 2008; Lanza et al., 1992; Loftus et al., 1990] or type II GT [Jackson et al., 1998; Morel-Kopp et al., 2002; Nair et al., 2004; Tadokoro et al., 2002; Ward et al., 2000]. For comparison, Figure 5 locates the mutated amino-acids in the primary sequence (panel A) and in the β -I domain structure (panel B). Whatever the variant or

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3 type II GT phenotype, the mutated complexes are located completely or partially at the
4 surface of the β -I domain, and some of them (see Figure 5B, underlined amino-acids) present
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6 significant electrostatic or / and free energies interactions with the β -propeller (data not
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8 shown). Variant mutations concern the MIDAS or SyMBS sites [Zhu et al., 2008] directly
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10 affecting amino-acids involved in metal coordination (p.D119Y, p.S123P) or their very
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12 nearby environment (p.R217W or Q) (see Figure 5A). Both variant and GT Type II mutations
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14 support at least a 10 % expression of α IIB β 3 at the platelet surface. This contrasts with the
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16 failure to detect the β 3-R118 and -D221 forms of the complex. Both the p.M118R and the
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18 p.G221D mutations, located inside the β -I domain, should deeply alter its proper folding,
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20 preventing complex expression at the platelet surface. Due to the nature of the amino-acid
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22 involved, these mutations induce important changes of steric hindrance and charge. However,
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24 in one case, the mutation p.L117W which affects a 100 % buried amino-acid still permits a 10
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26 % expression of α IIB β 3 at the platelet surface [Basani et al., 1997]. We could hypothesize that
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28 the characteristics of these residues, both hydrophobic and aliphatic, could explain this
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30 behavior. In contrast the p.G221D mutation, changes of volume and charge of the side-chains
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32 has a too deep impact. Similarly, such a steric hindrance change could explain the type I
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34 phenotype induced by the p.M124V mutation [Gonzalez-Manchon et al., 2004], as the M124
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36 side chain pointed inward the β -I domain.
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47 Unlike for M118 and G221, K253 is not conserved in other integrin β subunits and if it is
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49 present in β 3 from fish to primates it is replaced by an arginine in amphibian and absent from
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51 invertebrate integrins (see Supp Figure S1B). However the p.K253M substitution results in
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53 type I GT. The 3-dimensional structure of the α IIB β 3 headpiece revealed that the side chain of
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55 K253 protrudes from the β -I domain toward a pocket at the surface of the α IIB β -propeller
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57 (see Figure 3A and 5B). Analyses of electrostatic, desolvation and free energies revealed that
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59 K253 is one of the most important amino-acids involved in the β -I domain interaction with
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the β -propeller of α IIb. Furthermore, a mutant model of the α IIb β 3 headpiece shows that the p.K253M substitution does not introduce steric and energy clashes but only suppresses important contacts with the α IIb β -propeller. So, the p.K253M mutation revealed K253 as a key amino-acid for the association of the β -I domain with the β -propeller. The last mutation reported so far of the β -I domain that leads to type I GT was a substitution of the I325-P326-G327 sequence by a methionine. This mutation impairs the α IIb β 3 association [Morel-Kopp et al., 1997]. However, the mutated amino-acids locate between the α 6 helix and the β 7 sheet and do not directly face the α IIb β -propeller surface (see Figure 5B). Furthermore the I325-P326-G327 sequence does not seem to have significant if any, interaction with the β -propeller as indicated by free and electrostatic energy analysis (data not shown). It might indirectly impair the interaction(s) of the α 5 and / or α 6 helix with the β -propeller. The last four mutations described, p.K137Q [Peterson et al., 2008], p.T140I (HPA-16bw) [Jallu et al., 2002], p.R143Q (HPA-4) [Wang et al., 1992] and p.T195N (HPA-17bw) [Stafford et al., 2008] only lead to allelic variants involved in alloimmune thrombocytopenia that are not related to GT phenotype. All these amino-acids locate at the surface of the β -I domain with their side chains directed outside.

In conclusion, we have identified 29 mutations in a panel of 24 GT patients and 2 GT carriers. Seventeen of these mutations have not been described so far. We have confirmed by *in vitro* studies that the missense mutations identified impaired normal expression of the mutant complex (except for one case). Nonsense and splice site mutations were considered to be related to the GT phenotype. This study emphasizes the high allelic heterogeneity of both the α IIb and β 3 genes involved in GT phenotype. Finally the GT variant p.K253M of β 3 unveiled the key role of K253 in the β -propeller / β -I association of the α IIb β 3 headpiece.

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For Peer Review

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Figure legends :**Figure 1 : Flow cytometry analysis of the expression of the mutant forms of α IIB β 3.**

Transiently transfected COS-7 cells expressing the wild type (WT) or the following mutants of α IIB β 3, p.S926L, p.Q595H, p.V903F (α IIB) and p.C38Y, p.M118R, p.G221D, p.K253 (β 3) were tested in flow cytometry by using Moabs SZ22 (hatched bar), SZ21 (dot bar) and P2 (white bar) or irrelevant mouse IgG (black bar). As control, untransfected cells (NT) were also tested. Following Moab incubation, bound IgGs were detected by using a polyclonal goat anti-mouse IgG conjugated to FITC. Washed cells were analyzed on an Epic-XML flow cytometer. Except for the α IIB p.Q595H one, all mutants showed a markedly decreased expression of the complex when compared to the wild type. Results represent the mean \pm SD of 3 experiments.

Figure 2 : Western blot analysis of the Y38 β 3 form of the α IIB β 3 complex expressed in COS-7 cells. SDS lysates from normal platelets (lane a), non-transfected COS-7 cells (lane b), or COS-7 cells expressing the wild-type (lane c) or the Y38 β 3 (lane d) forms of the complex were subjected to SDS-polyacrylamide gel electrophoresis under non reduced conditions and transferred to nitrocellulose membrane. Following incubation with Moabs SZ22, XIIF9, SZ21 and AP3, membrane bound IgGs were revealed by using anti-mouse IgG conjugated to peroxidase and the enhanced chemiluminescence detection technique.

Figure 3 : Location and modeling of the electrostatic effect of the p.K253M substitution. (A) and (B) Ribbon diagram of the α IIB β -propeller (in blue) and β 3 β I (in red) complexed domains. For the β I domain, The residue 253 is shown in green with (A) a lysine and (B) a methionine, while for β -propeller partners the P228 is shown in yellow, the F231 in

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grey, the W262 in pink and the D232 in orange. (C) and (D) Electrostatics visualization of the single point mutation p.K253M with (C) wild K253 $\beta 3$ and (D) mutated M253 $\beta 3$ subunits. The visualizations are done using PyMol software (<http://www.pymol.org>).

Figure 4 : Localization of the V903 and S926 on a ribbon diagram of α IIIb**.** This α I**IIb** cartoon shows the internal location of the V903 and S926 side chains (orange, ball and stick representation) in the calf-2 composed by the N-terminal part of the β -subunit of α I**IIb** (blue) and the C-terminal part of the α -subunit (yellow and red respectively for β -sheet and α -helix). This ribbon diagram was obtained from the resolved structure of α I**IIb** (PDB code 3FCS) using the Swiss-PdbViewer software (version 4.0.1).

Figure 5 : Localization of the allelic variants of the β -I domain of $\beta 3$. Residues identified in this study and from the literature as involved in GT or neonatal alloimmune thrombocytopenia and that locate in the β -I domain of $\beta 3$ are reported on its primary sequence (panel A) and a ribbon diagrams showing the β -I domain and a partial view of the β -propeller of α I**IIb** (panel B). Allelic variants of residues highlighted in magenta, blue, green are respectively responsible for variant, II and I GT phenotypes or yellow for those concerning alloimmunisation. The GT type induced by the allelic variant of the H192 (grey) is not reported [Peretz et al., 2006]. Amino-acids identified by the energy analyze as in contact with the α I**IIb** β -propeller are underlined. The primary sequence (panel A) identifies the secondary structures [Xiao et al., 2004] and amino-acids responsible for MIDAS (\star), ADMIDAS (\bullet) and SyMBS (\blacklozenge) sites [Springer et al., 2008]. As known, variant GT mutations GT specifically affect metal ion binding sites but other mutants concern residues evenly distributed along the primary sequence. Boxed residues have been identified in this study. The ribbon diagram (panel B) was obtained from the resolved structure of α I**IIb** (PDB file 2VDL) using the Swiss-

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3 PdbViewer software (version 4.0.1). α -helix (red) and β -sheet (yellow) are shown. All
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5 residues whose mutants are involved in type II GT lay on the β -I surface except for the L117,
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7 some of them (underlined amino-acids) directly contacting the β -propeller. The Met 118 and
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9 Gly 221 locate inside the β -I domain and the Lys 253 points out to the β -propeller surface
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11 however their identified allelic variants completely prevent the α IIb β 3 complex expression at
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13 the platelet surface.
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Table 1 : α IIB mutations identified by genomic analysis^a

N ^o	Proband	GT ^b	Ex/IVS ^c	Sequence alteration ^d	Status ^e	Restriction enzyme	Predicted HGVS ^f	Predicted Classical ^g	Accession number ^h
1	CabGT-1	I	Ex 4	c.531T>A	Hom.	Hga I	p.C177*	p.C146*	ss131010618
2	CabGT-2	I	Ex 10	c.917dupA	Het.	Alw I	p.R307Efs*22	p.R276E + FS ^k	ss131007603
3			Ex 19	c.1912dup T	Het.	No enzyme	p.C639Mfs*22	p.C608M + FS ^{k,l}	ss 131007606
4	CabGT-3	I	Ex 13	c.1214T>C	Het.	Sfc I	p.I405T	p.I374T ^l	ss 131007609
5			IVS14	c.1442-13_1442-1del	Het.	Hph I	Splice ⁱ	Splice	ss 131007612
6	CabGT-4	I (car ^w)	IVS14	c.1442-13_1442-1del	Het.	Hph I	Splice	Splice	ss 131007612
7	CabGT-5	I	IVS14	c.1442-13_1442-1del NI ^j	Het.	Hph I	Splice	Splice	ss 131007612
8	CabGT-6	I	Ex 14	c.1413C>G	Het.	Dde I	p.Y471*	p.Y440* ^m	ss 131007615
9			Ex 27	c.2800G>T	Het.	Mae III / Tsp45 I	p.V934F	p.V903F	ss 131007617
10	CabGT-7	I	Ex 16	c.1563T>A NI	Het.	No enzyme	p.C521*	p.C490*	ss 131007620
11	CabGT-8	NC ^x	Ex 17	c.1672C>T	Hom.	Mse I	p.Q558*	p.Q527*	ss 131007623
12	CabGT-9	I	IVS18	c.1879-2A>G	Hom.	Xba I	Splice	Splice	ss 131007625
13	CabGT-10	I	Ex 18	c.1878G>C	Hom.	Hpy CH IV	p.Q626H / Splice	p.Q595H / Splice	ss 131007628
14	CabGT-11	I	Ex 18	c.1787T>C	Hom.	No enzyme	p.I596T	p.I565T ⁿ	ss 131008441
15	CabGT-12	II	Ex 21	c.2113T>C	Het.	Fok I	p.C705R	p.C674R ^{l,o,p}	ss 131007632
16			IVS29	c.3060+2T>C	Het.	Hph I	p.V982_K1020del	p.V951_K989del ^q	ss 131007634
17	CabGT-13	I	Ex 23	c.2333A>C	Hom.	Bsr I / TspR I	p.Q778P	p.Q747P ^r	ss 131007637
18	CabGT-14	I	Ex 27	c.2748_2757del	Hom.	Rsa I	p.T917Sfs181	p.T886S + FS	ss 131007639
19	CabGT-15	I (car)	Ex 27	c.2800G>T	Het.	Mae III / Tsp45 I	p.V934F	p.V903F	ss 131007617
20	CabGT-16	I	Ex 28	c.2870C>T	Hom.	Hpy CH4 V	p.S957L	p.S926L	ss 131007642
21	CabGT-17	II	Ex 28	c.2929C>T	Het.	Ava I	p.R977*	p.R946* ^{l,s,t}	ss 131007644
22			NI						
23	CabGT-18	NC	Ex 29	c.2944G>A	Het.	Bst NI	p.V982M	p.V951M ^{u,v}	ss 131007647
24			Ex 29	c.2965G>A	Het.	Nci I	p.A989T	p.A958T ^{u,v}	ss 131007649
25				NI					

^a cDNA ITGA2B GenBank accession number NM_000419.3^b GT : Glanzmann type

^c Ex : Exon ; IVS : intronic sequence

^d Nomenclature according to HGVS : +1 corresponds to the A of ATG translation initiation codon

^e Status : Het. : heterozygous; Hom. : Homozygous

^f Predicted effect, nomenclature according to HGVS : +1 corresponds to the initiating Met (signal peptide included)

^g Predicted effect, classical nomenclature : +1 corresponds to the first amino acid of the mature α Ib

^h NCBI dSNP accession number

ⁱ Splice defect based upon modeling with GENSCAN / NNSPLICE and ESEFinder

^j NI : 2nd mutation not Identified

^k FS : Frame shift

References : ^l D'Andrea et al., 2002 [D'Andrea et al., 2002]; ^m Scott et al., 1998 [Scott et al., 1998]; ⁿ Ruan et al., 1998 [Ruan et al., 1998]; ^o Gonzalez-Manchon et al., 1999 [Gonzalez-Manchon et al., 1999]; ^p Mitchell et al., 2003 [Mitchell et al., 2003]; ^q French et al., 1997 [French and Coller, 1997]; ^r Tadokoro et al., 1998 [Tadokoro et al., 1998]; ^s Basani et al., 2000 [Basani et al., 2000]; ^t Peretz et al., 2006 [Peretz et al., 2006]; ^u Nurden et al., 2004 [Nurden et al., 2004]

^v V951M is responsible for GT but not A958T [Nurden et al., 2004]

^w Car : carrier

^x NC : Not communicated

Table 2 : $\beta 3$ mutations identified by genomic analysis^a

N°	Proband	GT ^b	Ex/IVS ^c	Sequence alteration ^d	Status ^e	Restriction enzyme	Predicted HGVS ^f	Predicted Classical ^g	Accession number ^h
19	CabGT-19	NC ^j	IVS2	c.166-2A>G	Hom.	Apa I	Splice ⁱ	Splice	ss 131007652
20	CabGT-20	I	Ex 2	c.100C>T	Hom.	Hpy CH4 IV	p.R34*	p.R8* ^l	ss 131007655
21	CabGT-21	I	Ex 3	c.191G>A NI ^k	Het.	Tsp 45I	p.C64Y	p.C38Y	ss 131007657
22	CabGT-22	I	Ex 4	c.431T>G	Hom.	Bsm F1	p.M144R	p.M118R	ss 131007660
23	CabGT-23	II	Ex 5	c.665 T>C	Hom.	Acy I	p.L222P	p.L196P ^m	ss 131007662
24	CabGT-24	I	Ex 5 Ex 6	c.740 G>A c.836 A>T	Het. Het.	Fok I Fat I	p.G247D p.K279M	p.G221D p.K253M	ss 131007665 ss 131007667
25	CabGT-25	NC	IVS8	c.1125+3_1125+6del NI	Het.	No enzyme	Splice	Splice	ss 131007670
26	CabGT-26	I	Ex 9 Ex 9	c.1143C>A c.1260G>A	Het. Het.	Taq I Hph I	p.K376_T420del	p.K350_T394del ⁿ	ss 131007672 ss 131007674

^acDNA ITGB3 GenBank accession number NM_000212.2

^bGT : Glanzmann type I or II (< 5% or 10 – 25 % of normal platelet α IIB β 3[Caen, 1989]). See Supp_mat tableS1 for detailed data.

^cEx : Exon ; IVS : intronic sequence

^dNomenclature according to HGVS : +1 corresponds to the A of ATG translation initiation codon

^eStatus : Het. : heterozygous; Hom. : Homozygous

^fNomenclature according to HGVS : +1 corresponds to the initiating Met (signal peptide included)

^gClassical nomenclature : +1 corresponds to the first amino acid of the mature $\beta 3$

^hNCBI dSNP accession number

ⁱSplice defect based upon modeling with GENSCAN, NNSPLICE and ESEFinder

^jNC : Not communicated

^kNI : 2nd mutation not Identified

^lReferences : ^lNegrier et al., 1998 [Negrier et al., 1998]; ^mNurden et al., 2002 [Nurden et al., 2002]; ⁿJin et al., 1996 [Jin et al., 1996]

Table 3 : Energetic effects of the Lys 253 Met substitution in the α I**II**b/ β 3 interaction

Energy of :		Lys 253			Met 253		
		Rank	Value (kcal/mol)	α I II b contact	rank	Value (kcal/mol)	α I II b contact
αIIIbβ3 Interface	Electrostatic (4r) Energy	/	-25.14	/	/	-16.08	/
	Desolvation Free Energy	/	-1.09	/	/	-6.81	/
	Van der Waals (CHARMm19)	/	-4894.63	/	/	-4903.95	/
Residue	Binding free energy	1 st negative	-5.541	/	12 th negative	-1.309	/
	Desolvation free energy	1 st positive	4.124	/	6 th negative	-1.347	/
	Electrostatic energy	1 st negative	-9.666	/	/	<i>low</i>	/
Contact	Electrostatic	1 st negative	-6.715	D232	/	/	/
		6 th negative	-2.076	F231	/	/	/
		13 th negative	-0.788	P228	/	/	/
		10 th positive	0.670	W262	6 th negative	-0.935	W262
	Free energy	1 st negative	-5.517	D232	/	/	/
		8 th negative	-1.166	F231	/	/	/
2 th positive		2.057	W262	/	/	/	

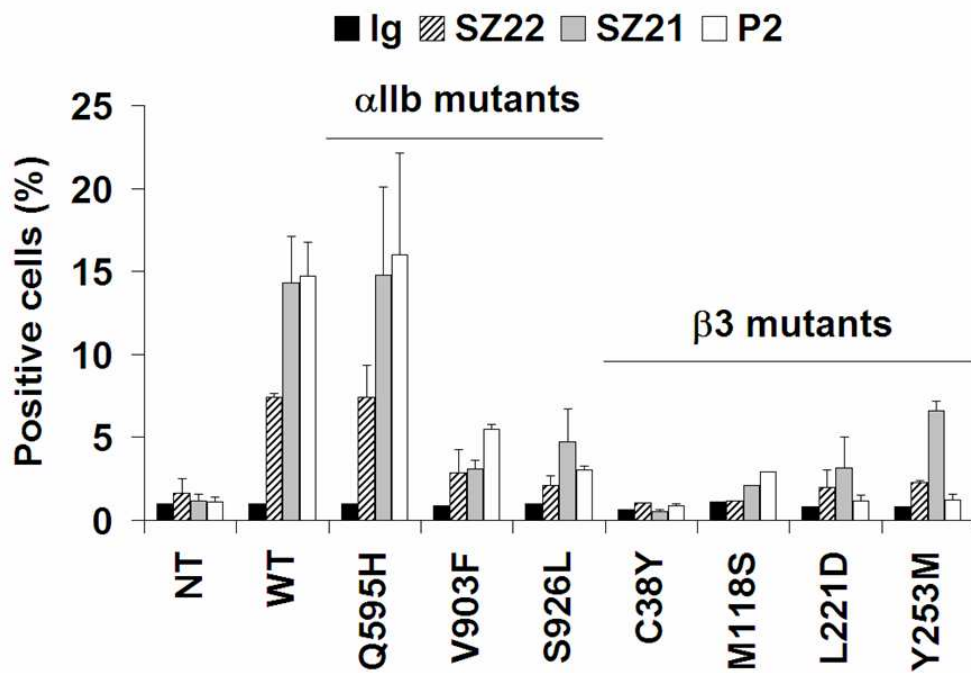


Figure 1 : Flow cytometry analysis of the expression of the mutant forms of α IIb β 3.
235x165mm (96 x 96 DPI)

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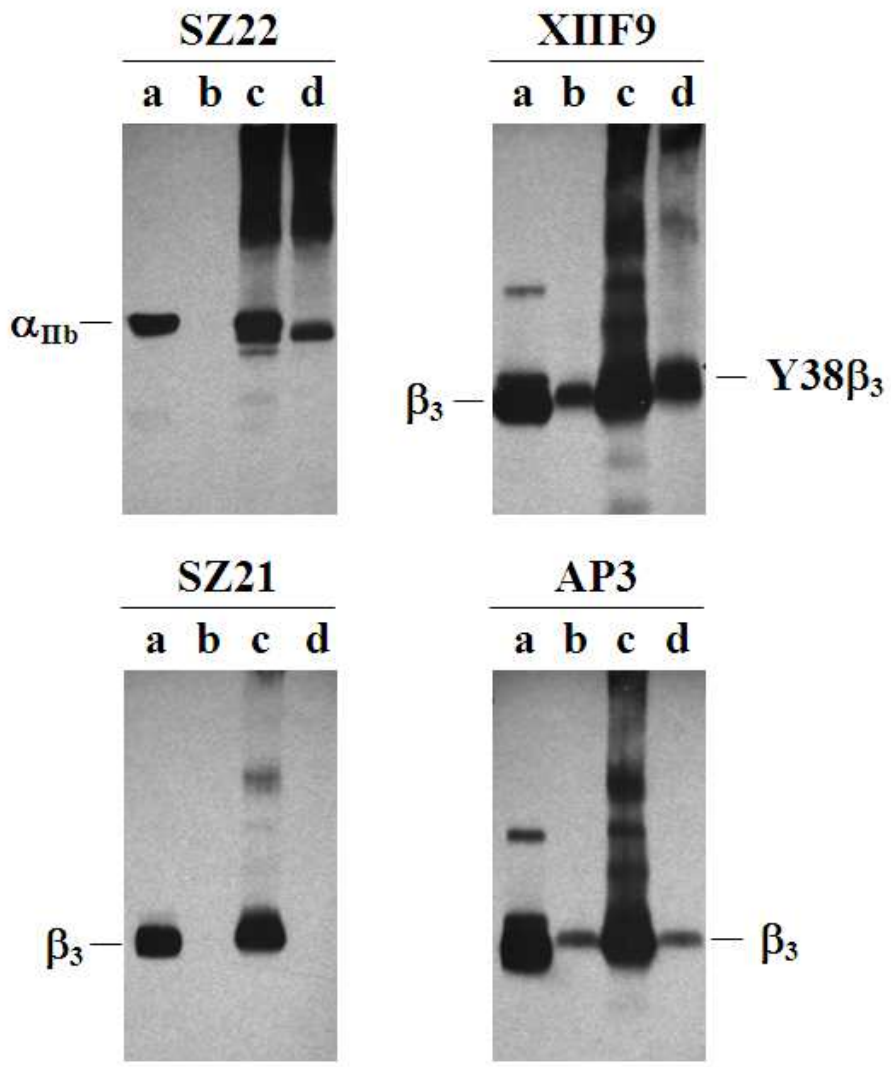
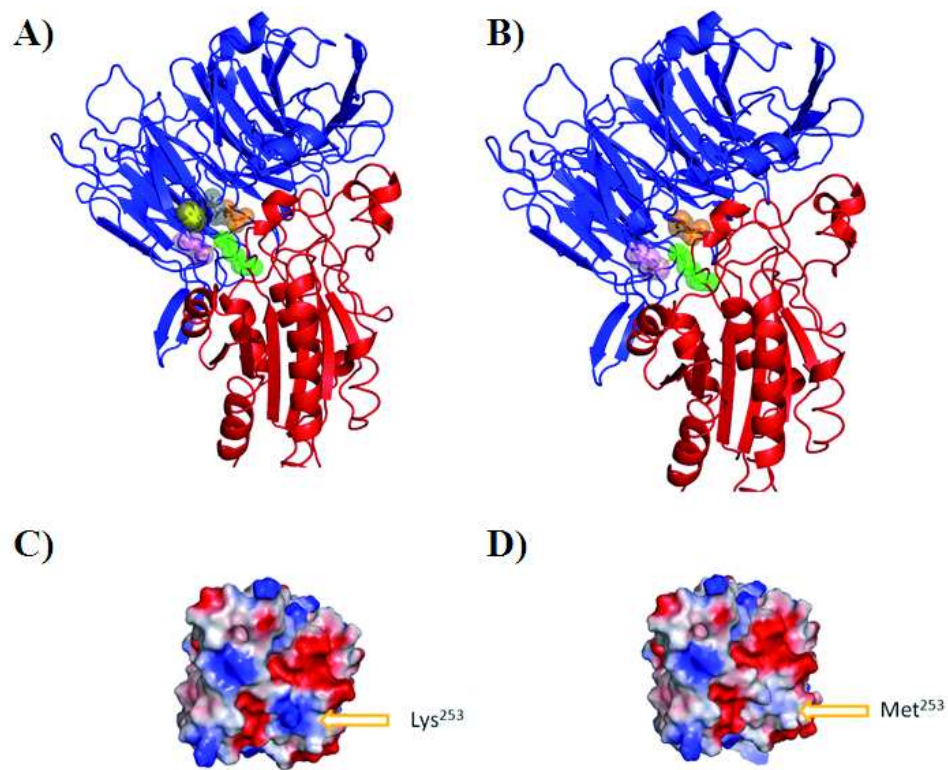


Figure 2 : Western blot analysis of the Y38 β_3 form of the $\alpha_{IIb}\beta_3$ complex expressed in COS-7 cells.
155x177mm (96 x 96 DPI)



CMYK color scale

Figure 3 : Location and modeling of the electrostatic effect of the p.K253M substitution.
193x158mm (96 x 96 DPI)

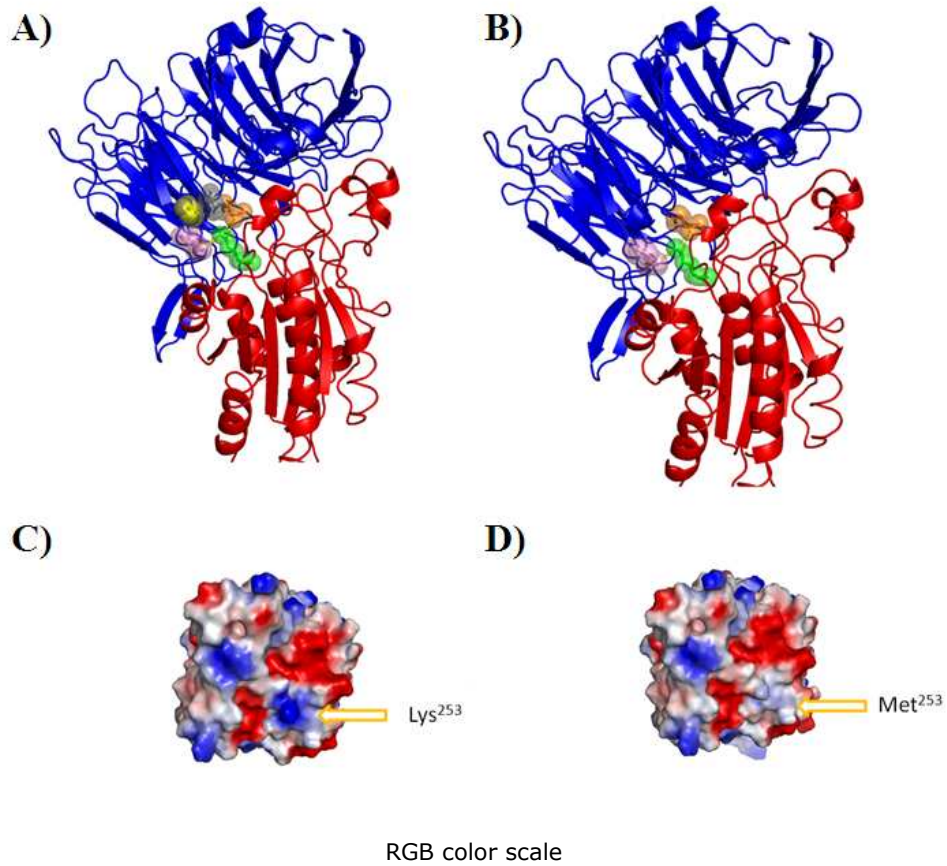
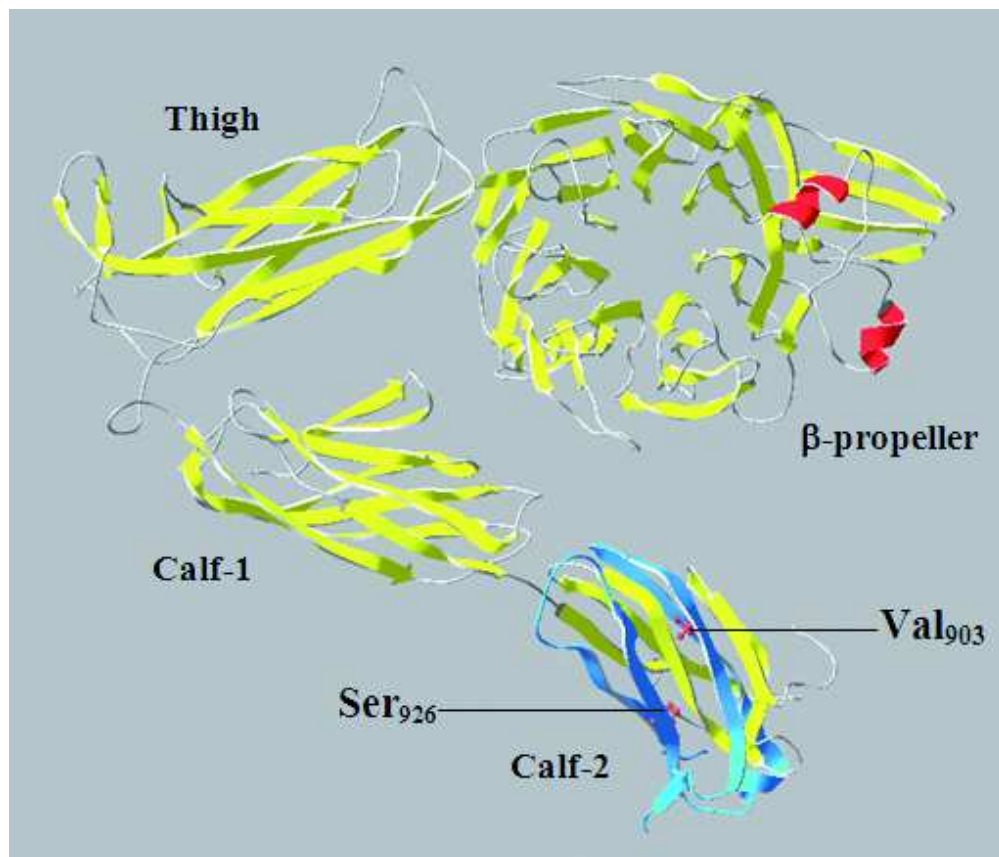


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193x158mm (96 x 96 DPI)

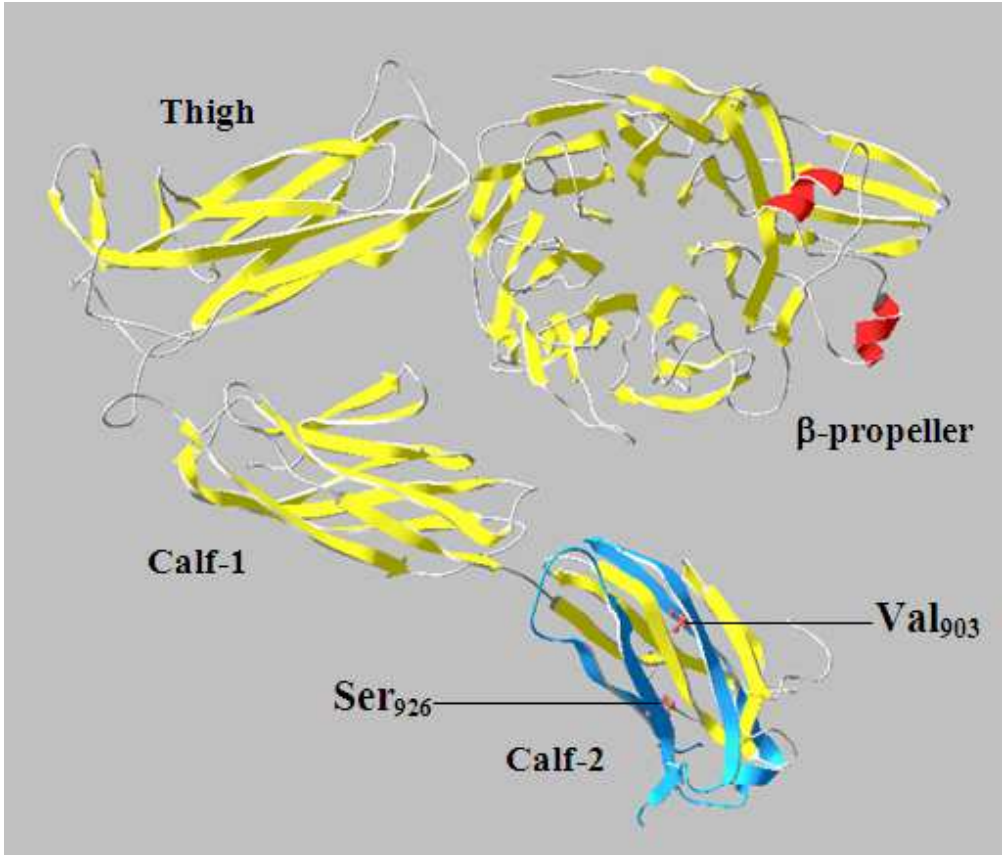


CYMK color scale

Figure 4 : Localization of the V903 and S926 on a ribbon diagram of aIIb.
135x115mm (96 x 96 DPI)

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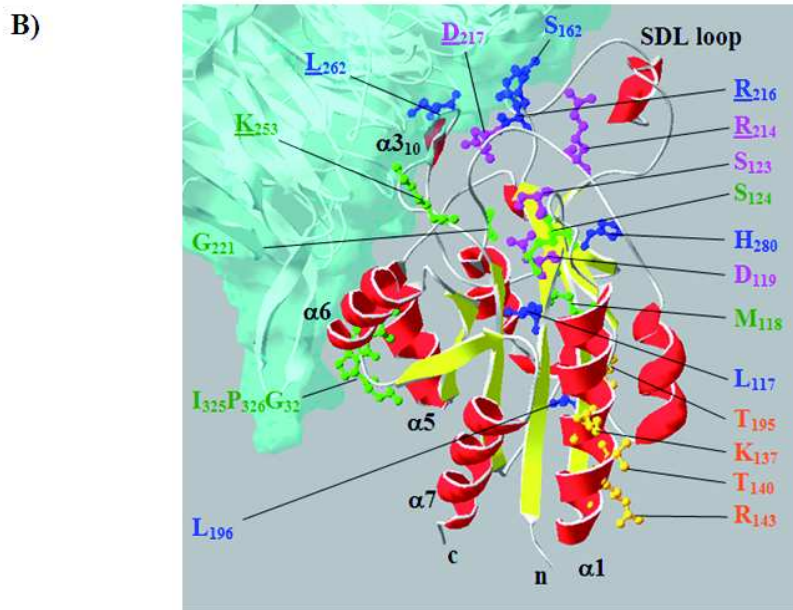
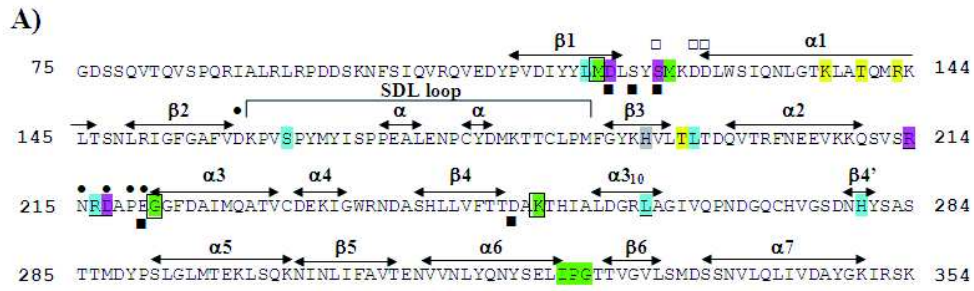
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RGB color scale

Figure 4 : Localization of the V903 and S926 on a ribbon diagram of aIIb.
135x115mm (96 x 96 DPI)

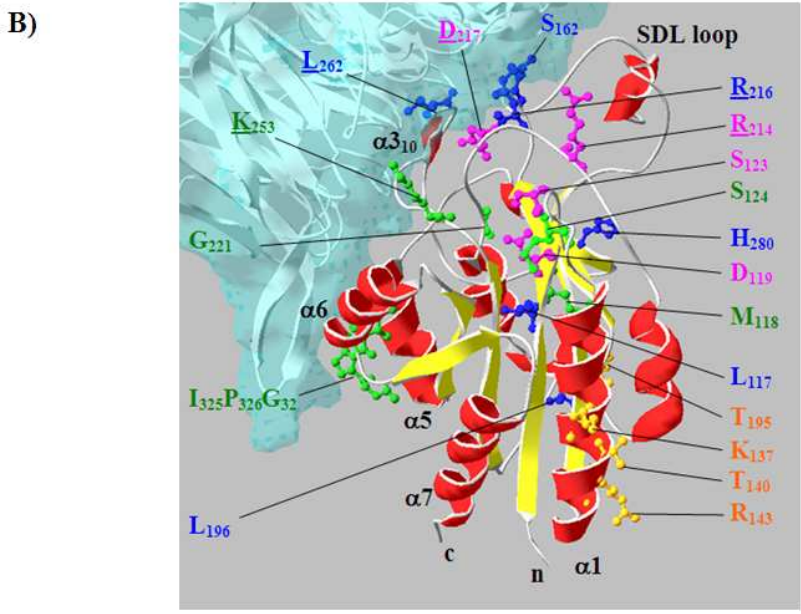
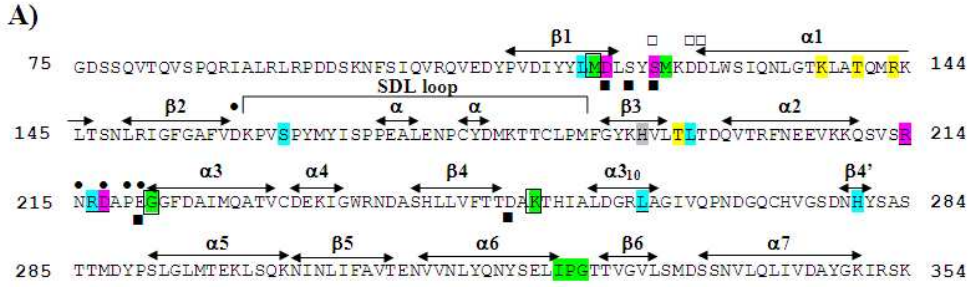




CMYK color scale

Figure 5 : Localization of the allelic variants of the β -I domain of β 3.
212x200mm (96 x 96 DPI)

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RGB color scale

Figure 5 : Localization of the allelic variants of the β -I domain of β 3.
212x200mm (96 x 96 DPI)