Platelet Type von Willebrand Disease (PT-VWD), a rare inherited misdiagnosed bleeding disorder: a multicenter study to increase our knowledge from diagnosis to characterization of new gene mutations.

Introduction

Structure and function of von Willebrand factor (VWF)

Von Willebrand disease (VWD), described for the first time in 1926 by Dr. Erik von Willebrand, is the most common inherited bleeding disorder, with a prevalence of nearly 1% in the general population, and is caused by quantitative or qualitative defects in von Willebrand factor (VWF). VWF is a multimeric plasma adhesive glycoprotein composed of identical subunits of ~250 kDa (monomers). The multimers range in size from dimers of ~500 kDa to species of more than 10000 kDa. VWF plays a critical role in primary haemostasis and in the initiation and progression of thrombus formation at sites of vascular injury. It is fundamental for platelet adhesion to subendothelium and for platelet-to-platelet cohesion, thus promoting the formation of a platelet plug at the site of injury to the vessel wall, as well as for platelet aggregation within the growing haemostatic plug, in particular in vessels with an high shear rate blood flow. VWF binds and transports blood clotting factor VIII (FVIII) in plasma, protecting it from proteolytic degradation and efficiently localizing it at the site of vascular injury, thus indirectly contributing also to the coagulation process. High molecular weight von Willebrand factor (HMW-VWF) multimers bind to platelet receptors better than smaller ones, while binding of VWF to FVIII is not dependent on multimer size.

VWF contributes to pathologic conditions, including venous thromboembolism and atherothrombotic complications, and it displays proinflammatory activity in stroke, atherosclerosis and other inflammatory disorders. The predominant clinical problems of VWD patients are muco-cutaneous bleeding, in particular nosebleeds, bleeding from small skin cuts and from lesions in the mucosa or gastrointestinal tract, menorrhagia and excessive bleeding after trauma, after surgical operations or at childbirth. Patients with severe deficiencies of VWF may bleed into joints or muscles. The bleeding tendency varies from mild to severe with the type and the degree of the VWF defect.

The gene encoding for VWF is located on the short arm of chromosome 12 (12p13.2). It is 178 kilobases arranged into 52 exons. Chromosome 22 carries a partial VWF unprocessed pseudogene that replicates the chromosome 12 sequence between exon 23 and 34.\(^1\)\(^-\)\(^3\) Genetic recombination causing the gene conversion between the VWF gene and its pseudogene is associated with multiple substitutions in the VWF gene and with VWD.\(^4\)\(^-\)\(^6\)

VWF is synthesized in endothelial cells and megakaryocytes as a pre-pro-polypeptide of 2813 aminoacids composed of a pre-sequence (residues 1-22), a pro-peptide (residues 23-763) and the mature subunit (residues 764-2813). The pre-sequence enables entry into the endoplasmic reticulum where pro-VWF
subunits join "tail-to-tail" through disulfide bonds to form dimers. In the Golgi apparatus, pro-VWF dimers link through additional "head-to-head" disulfide bonds to form multimers that may be secreted constitutively in the plasma, or stored in the Weibel-Palade bodies of endothelial cells or the α-granules of platelets, for subsequent regulated secretion. Circulating levels of VWF are increased by several stimuli: upon adrenalin release, as in strenuous exercise or stress, with inflammatory conditions, with severe liver disease, with high levels of thyroid hormones, or with high levels of estrogen and progesterone, as in pregnancy. Levels are also in part genetically determined, in fact blood groups and race influence plasma levels (levels are higher in persons with blood groups A and B compared to blood group O; levels of VWF are higher in persons of black African descent than in Caucasians). The 2050 aminoacid residues mature VWF subunit contains a binding domain for platelet receptor glycoprotein GPIbα (domain A1), for platelet glycoprotein GPIIbIIIa (domain C1), after platelet activation, for collagen (domains A1 and A3) enabling VWF to mediate platelet adhesion to the subendothelium at high fluid shear stress, and for FVIII (domain C1) protecting it from premature proteolysis by activated protein C (Fig. 1).

![Fig. 1: Primary structure of pre-pro-VWF and its ligand](image)

After secretion in plasma, HMW-VWF multimers are rapidly cleaved into smaller multimers by a metalloprotease, ADAMTS-13, and cleared from blood to prevent excessive thrombus formation (VWF has an half-life of 12-20h). The concentration and the multimers distribution of plasma VWF reflect the balance between multimers assembly, secretion, proteolysis and clearance from circulation. Mutations that affect one or more of these processes result in von Willebrand disease (VWD).von Willebrand disease (VWD) classification

VWD is classified into three primary categories, type 1, 2 or 3. Type 1 and 3 are quantitative defects of VWF that is partially or completely reduced respectively, while type 2 is a qualitative defect of VWF.

- Type 1 (70-80% of VWD cases): mild to moderate reduction in qualitatively normal VWF, caused by reduced secretion, accelerated clearance or enhanced proteolysis of VWF
- Type 2 (20% of VWD cases): qualitatively abnormal VWF
• Type 3: it's the most rare variant of VWD and it's characterized by a severe deficiency of VWF and FVIII.

Type 2 VWD is divided into four variants:

• Type 2A: depending on the mutations (dominant missense mutations, usually in the A2 domain, and occasionally in the A1 domain of VWF gene), HMW-VWF multimers are absent, or they are vulnerable to rapid proteolysis in circulation and, thus, only low molecular weight VWF (LMW-VWF) multimers remain. Deficiency of HMW multimers greatly reduces VWF binding to platelet GPIbα, whereas the binding of LMW multimers to FVIII is normal.

• Type 2B: it is caused by dominant gain-of-function mutations, usually missense, in the A1 domain of the VWF gene and it is characterized by an increased affinity of mutant VWF for platelet GPIbα. This higher affinity leads to spontaneous binding of HMW-VWF to circulating platelets, with their subsequent clearance from circulation. These patients may have moderate to severe thrombocytopenia which may aggravate bleeding.

• Type 2M: uncommon type of type 2 VWD caused by mutations in the A1 domain and characterized by a defective binding of VWF to platelet GPIbα, despite a relatively normal size distribution of VWF multimers.

• Type 2N: it is caused by recessive mutations in the D’ to D3 domains which markedly decrease the affinity for FVIII, despite a normal distribution of VWF plasma multimers.

The purpose of the above classification is primarily clinical, to facilitate the diagnosis and treatment of patients with VWD.

Given its heterogeneity, the diagnosis of VWD is often difficult and no single clinical or laboratory assessment allows to formulate a definitive diagnosis of VWD. In fact, a panel of different laboratory assays, that investigate the presence and the functionality of VWF, is required for a precise diagnosis. On the other hand, the differentiation between the various types of VWD is fundamental to individualize the most appropriate therapeutic approach. In fact, treatments which are the standard of care for some types of VWD, like desmopressin, may be dangerous or detrimental for other types, like 2B VWD.

Laboratory assays for VWD diagnosis
Screening assays for VWD include the activated partial thromboplastin time (aPTT), the bleeding time (BT) and the automatic platelet function analyzer (PFA-100®), but they are non-specific because an alteration of these tests can be the consequence of many different conditions. Ristocetin cofactor test (VWF:RCo) and ristocetin-induced platelet aggregation (RIPA) are functional assays of VWF based on the evaluation of this multimeric protein's ability to interact with the platelet GPIb/IX/V complex in the presence of ristocetin, an antibiotic, causing platelet agglutination. VWF:RCo measures ristocetin-induced agglutination of formalin-fixed platelets in the presence of patient's plasma (ristocetin is constant, but plasma dilution varies) and is
reduced both in quantitative and in qualitative defects of VWF. RIPA measures ristocetin-induced agglutination of patient's own platelets in platelet rich plasma (PRP is constant and ristocetin dilutions vary) and it was until recently the only available laboratory test for the diagnosis of type 2B VWD, because able to detect the enhanced affinity of mutant VWF for GPIbα.

Enzyme-linked immunosorbent assays (ELISA) include collagen binding assay for von Willebrand factor, VWF:CBA, that is a quantitative and qualitative assay which measures the ability of VWF to bind collagen, VWF:FVIIIB, that analyzes VWF binding to FVIII, and von Willebrand factor antigen, VWF:Ag, that measures the total amount of the protein using an antibody against VWF.

The size distribution of VWF multimers can be analyzed using sodium dodecyl sulfate (SDS)-agarose electrophoresis followed by immunostaining.

Gene sequencing can also be performed to confirm the subtype of VWD.\(^{(3, 7-9)}\)

Platelet-type von Willebrand disease (PT-VWD) and type 2B VWD

Platelet-type von Willebrand disease (PT-VWD) or pseudo-VWD, is a rare inherited autosomal dominant bleeding disorder described for the first time by Weiss et al (1982) and Miller et al (1983). It is caused by mutations of the platelet glycoprotein Ibα (GPIbα) gene located on chromosome 17. Five different mutations have been described: four (Gly233Val, Met239Val, Gly233Ser, and Asp235Tyr) in the C-terminal disulfide loop of the VWF-binding domain of GPIbα and one (421-429del) in the macroglycopeptide, a structural domain of the protein. These gene alterations cause a gain of function mutation of GPIbα, the largest component of the four subunits platelet membrane receptor complex GPIb/IX/V which contains a VWF-binding domain in the N-terminal extracytoplasmic region, and result in an abnormally enhanced affinity of GPIbα for HMW-VWF. As a consequence, platelets from patients with PT-VWD bind spontaneously normal high HMW-VWF multimers, with their accelerated clearance from the circulation and consequent bleeding.

Patients with PT-VWD are characterized by mild thrombocytopenia with increased Mean Platelet Volume (MPV), interpreted as the consequence of enhanced platelet turnover, by platelet aggregates in blood smears, and by a prolonged bleeding time associated with mucocutaneous bleeding or hemorrhage after surgery. Ristocetin-induced platelet aggregation (RIPA) is enhanced in response to low dose of ristocetin, HMW-VWF multimers are reduced and plasma von Willebrand factor antigen (VWF:Ag) is normal or mildly reduced while von Willebrand factor activity is reduced, resulting in a low VWF:activity/VWF:Ag ratio.

Given the clinical and laboratory similarities (Tab. 1), patients with PT-VWD are often wrongly diagnosed as type 2B VWD (the VWD variant in which the enhanced affinity between GPIbα and VWF is caused by a mutation on the VWF molecule), and the real prevalence of PT-VWD is therefore probably underestimated. 15% of patients with a type 2B VWD diagnosis seem to be actually affected by PT-VWD.\(^{(10, 11)}\) Recently,
Enayat et al. have described 14 cases of PT-VWD in individuals with a precedent diagnosis of type 2B VWD, in which a mutation in VWF gene was not found.\textsuperscript{(12)}

<table>
<thead>
<tr>
<th>Platelet-type von Willebrand disease</th>
<th>Type 2B von Willebrand disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autosomal dominant platelet disorder</td>
<td>Autosomal dominant VWF defect</td>
</tr>
<tr>
<td>Caused by mutations in the GPIBA gene on chromosome 17 (4 mutations identified so far)</td>
<td>Caused by mutations in exon 28 of the VWF gene on chromosome 12 (52 mutations identified so far)</td>
</tr>
<tr>
<td>Gain of function mutation – increased affinity of abnormal platelet GPIbα complex for normal plasma VWF</td>
<td>Gain of function mutation – increased affinity of abnormal plasma VWF for normal platelet GPIbα complex</td>
</tr>
<tr>
<td>Mucocutaneous bleeding or excessive bleeding after surgeries or invasive procedures</td>
<td>Mucocutaneous bleeding or excessive bleeding after surgeries or invasive procedures</td>
</tr>
<tr>
<td>Laboratory: mild thrombocytopenia, platelet macrocytosis, platelet clumping on blood smears, prolonged BT, enhanced RIPA, reduction of HMW VWF multimers in plasma, reduced ratio between VWF : RCo/VWF : CB and VWF : Ag, normal or reduced plasma FVIII levels</td>
<td>Laboratory: mild thrombocytopenia, platelet clumping on blood smears, prolonged BT, enhanced RIPA, reduction of HMW VWF multimers in plasma, reduced ratio between VWF : RCo/VWF : CB and VWF : Ag, normal or reduced plasma FVIII levels</td>
</tr>
<tr>
<td>Differential diagnosis: RIPA not enhanced using patient’s plasma and normal platelets; platelet aggregation in the ‘cryoprecipitate challenge’ test (see text); analysis of specific mutations in the GPIBA gene</td>
<td>Differential diagnosis: RIPA enhanced using patient’s plasma and normal platelets; no platelet aggregation in the ‘cryoprecipitate challenge’ test (see text); analysis of specific mutations in the VWF gene</td>
</tr>
<tr>
<td>Therapy: platelet transfusion</td>
<td>Therapy: DDAVP, plasma-derived VWF/FVIII concentrates</td>
</tr>
</tbody>
</table>

Tab. 1: Similarities and differences between PT-VWD and VWD-2B\textsuperscript{(10)}

Given the possible under-diagnosis of PT-VWD, it is highly possible that, apart from the 5 already described mutations, other mutations exist. Indeed, preliminary results of our laboratory suggest the presence of a novel mutation (Arg127Gln) in a PT-VWD patient in the LRR, a region of the VWF-binding domain where mutations were not described so far.

Moreover, because of the difficulty in the identification of PT-VWD and the very few cases so far described, little is known about the consequences of the constitutively active GPIb/IX/V receptor-VWF interaction on platelet genesis and function. In particular, whether VWF binding to the GPIb subunit transduces relevant activation signals has remained controversial for a long time. The short cytoplasmic domains of the different subunits of GPIb/V/IX are not associated to GTP-binding proteins and do not possess intrinsic enzymatic activities but they interact constitutively with a number of intracellular proteins, including filamin (actin-binding protein)\textsuperscript{(13)}, 14-3-3ζ\textsuperscript{(14)}, and calmodulin\textsuperscript{(15)}. The interaction of these proteins with GPIb/V/IX plays a role in the correct localization and exposure of the complex on platelet surface. Recently, GPIb/V/IX mediated intracellular signalling leading to platelet activation and aggregation, and involving α\textsubscript{IIb}β\textsubscript{3} activation has become evident. GPIb/V/IX can be co-immunoprecipitated from platelets with signalling molecules, including Src family kinases, PI 3-kinase and SHIP-2 that can be involved in the cross-
talk between these two platelet glycoproteins. Alterations of $\alpha_{\text{IIb}}\beta_3$ activation due to a defective GPIb/V/IX-mediated signalling, such as in PT-VWD, have never been described.

Fig. 2: Cross-talk between GPIb/IX/V and $\alpha_{\text{IIb}}\beta_3$ \(^{(17)}\)

Preliminary results obtained in our laboratory suggest defective activation of $\alpha_{\text{IIb}}\beta_3$ in PT-VWD. Indeed, we have observed defective binding of PAC-1 (a monoclonal antibody that recognizes the active conformation of $\alpha_{\text{IIb}}\beta_3$) after stimulation with ADP or TRAP-6 in platelets from a patient with PT-VWD carrying the Met239Val mutation. Moreover, GPIb/IX/V is an important regulator of proplatelet formation (PPF), the process by which megakaryocytes (Mks) generate platelets within the bone marrow and release them in the bloodstream. Indeed, defective PPF has been described in Mks of patients with Bernard Soulier Syndrome (BSS), a hereditary platelet disorder characterized by the absence or dysfunction of the GPIb/IX/V complex and by blocking the GPIb$\alpha$-VWF interaction. The direct role of VWF in PPF is debated, because it was shown to be important but not necessary for this process in mice. In humans, the role of the GPIb$\alpha$-VWD interaction in megacaryopoiesis has also been studied by culturing megakaryocytes from type 2B-VWD patients, showing defective PPF. However, the role of an hyper-active GPIb$\alpha$ receptor, like in PT-VWD, has never been described. In our laboratory we have previously demonstrated that altered signalling triggered by an hyper-active $\alpha_{\text{IIb}}\beta_3$ receptor negatively influences PPF.
in human Mks\textsuperscript{25}. Given the role of the GPIb/IX/V receptor in PPF and its possible interaction with $\alpha_{IIb}\beta_3$, studying PPF by Mks in patients with PT-VWD would be of interest.

However, differential diagnosis between PT-VWD and type 2B-VWD is crucial because the two diseases must be treated differently: plasma-derived VWF/FVIII concentrate for patients with type 2B VWD, and platelet transfusions for patients with PT-VWD. The appropriate therapy is fundamental because treating patients with PT-VWD with VWF concentrates or desmopressin induces platelet agglutination in vivo and increases thrombocytopenia.

The differential diagnosis between these two conditions relies on genetic analysis, which is technically demanding and not available to most laboratories. Alternatively, differential diagnosis requires several assays, including RIPA mixing tests and cryoprecipitate challenge, which are cumbersome and can give false positives.\textsuperscript{[10]}

Recently, in our laboratory, a new diagnostic flow cytometric assay to quantify the ristocetin-induced VWF binding to platelet GPIb$\alpha$ has been developed for the diagnosis of VWD (Fig. 3).

The binding of VWF to platelets was assessed using two different approaches, one using patient’s autologous PRP (direct VWF binding), the other using formalin-fixed platelets in the presence of patient’s plasma (undirect VWF binding). Results obtained with flow cytometric assay correlate well with the ones obtained with the other most common assays used for the diagnosis of VWD. Differently from most other tests for VWD, except RIPA, this assay reveals the increased affinity between VWF and platelets, and thus allows to identify patients with type 2B VWD.\textsuperscript{[26]} Moreover, using simple mixing tests (Fig. 4A), this assay differentiates PT-VWD from type 2B VWD, demonstrating the platelet or plasma origin of the defect (Fig. 4B).\textsuperscript{[27]}
This novel assay can be a useful method to better differentiate between PT-VWD and type 2B VWD thanks to its higher sensitivity and reproducibility (VWF binding induced by low dose of ristocetin in type 2B VWD patients was increased about 14.6 fold while using the aggregometer test was increased 3.6 fold compared to healthy subject; in patients with PT-VWD, VWF binding was increased about 34.5 fold, while using the aggregometer test was increased about 3.7 fold compared to healthy subjects), to the simple and rapid performance of the assay and to the small volume of sample required to perform the test (about 200ul) compared to the volume required for the mixing test performed by light transmission aggregometry, making it especially useful for pediatric patients.\(^{(27)}\)

Aim of the project

The overall aim of this project is to improve our knowledge on platelet-type-Von Willebrand disease (PT-VWD). To this end, we will perform a large screening to identify new cases and then perform molecular and cell biology studies to unravel the mechanism that starting from a mutation of the gene GPIBA leads to bleeding and thrombocytopenia.

Therefore:

- We propose a multicentric study to validate the flow cytometer test described above, thus making it a new simple and high sensitive routine laboratory assay that can distinguish between PT-VWD and 2B VWD, thus revealing the real prevalence of this little known inherited bleeding disorder and so giving all the instruments to choose the appropriate clinical treatment of patients with type 2B VWD and PT-VWD.
- We will characterize novel mutations involved in PT-VWD (genetic and functional characterization).
- We will shed new light on the mechanisms underneath platelet dysfunction on this rare disease, particularly on the role of GPIb/IX/V-\(\alpha_{IIb}\beta_3\) interaction.
• We will study PPF by Mks from PT-VWD patients to better understand the macrothrombocytopenia associated with PT-VWD.

Methods of the study

Patients
We will enroll patients with an increased ristocetin-induced platelet aggregation (suspicious diagnosis of type 2B VWD) and we will centralize frozen plasma aliquots from patients with a suspicious or confirmed diagnosis of type 2B VWD. On these samples, we will assess the mixing test performed by flow cytometer and we will compare this test with already evaluated tests (mixing test performed by light transmission aggregometry, genetic test). For this purpose, all the interested laboratories will send to us a small amount of frozen plasma (200µl are enough) and, if it is possible, also a small aliquot of whole blood for DNA extraction and genotypization.

Flow cytometry
Using flow cytometry and plasma aliquots from patients with diagnosis of suspect type 2B VWD sent by the laboratories involved in this multicenter study, we'll carried out the following mixing tests:

1. platelets from an healthy subject and plasma from patients
2. platelets and plasma from an healthy subject
to evaluate the ristocetin-induced VWF binding.

Platelets used for the two mixing tests will be both fresh platelets from an healthy subject and formalin-fixed platelets.

To obtain a pellet of fresh platelets from healthy subjects, we will prepare PRP (platelet rich plasma) centrifuging citrate whole blood at 120g for 10minutes at room temperature, we will add disodium-ethylenediamine-tetraacetic acid (EDTA) and we will aliquot it in working aliquots that will be centrifuged. After removing the supernatant plasma, to prepare the two different mixes described above, we will resuspend the obtained platelet pellet, to the final concentration of 50000/µl, or with the plasma from an healthy subject or with the plasma from the patients with a suspect diagnosis of 2B VWD. Samples will be incubated for 3 minutes at 37°C without stirring, with ristocetin (0, 0.75, 1, 1.5, 2 mg/mL) and subsequently treated for the flow cytometric analysis as described below.

Alternatively, 200 µL of formalin-fixed platelets (50000/µL) will be incubated for 5 minutes, without stirring, with ristocetin (1 mg/mL) in the presence of 25 µL of three different plasma dilutions from healthy subjects or patients. A standard curve will be created using four scalar dilutions of a control plasma containing a known amount of VWF, and the percentage of platelets positive for VWF will be plotted as a function of the plasma dilutions.
For flow cytometric assay, 10 µl of both the suspensions prepared as described above will be fixed for 15 min with PFA 1%, then diluted 1:5 with PBS and labeled with a saturating concentration of the anti-VWF monoclonal antibody and subsequently with FITC-conjugated goat anti-mouse IgG (1:400). To block the reaction, samples will be further diluted with 1 mL of PBS and analyzed in an FC500 flow cytometer (Beckman Coulter).

Platelets will be identified by their morphology (light and side scattering characteristics) and a total of 5000 platelets will be analyzed for VWF binding. A negative control consisting of a sample labeled with an isotopic control antibody, will be studied in parallel.\(^\text{[26]}\)

Other laboratory assays
All the plasma samples from patients will be analyzed with the normal set of assays used for the diagnosis of VWD: VWF:Ag, VWF:RCo and VWF:CB (ELISA tests) and mixing tests performed by light transmission aggregometry

Molecular genetic analysis
For the identification of the molecular defect, the patient’s genomic DNA will be extracted from whole blood and the entire coding region of GPIBA will be amplified by PCR, using four oligonucleotide primer pairs. PCR products will be purified and the complete sequence of the GPIBA coding region will be obtained by direct sequencing.

Characterization of the novel mutations
Novel mutations in GPIBA that we will eventually discover will be expressed in Chinese hamster ovary (CHO) cells to be functionally characterized.

The full length GPIBA cDNA will be amplified from an healthy control and from patients’ platelets and inserted in the multiple cloning site of the pcDNA3 mammalian expression vector in order to obtain plasmids coding for the wild type (pcDNA3-GPIbaWT) or the mutant (pcDNA3-GPIbaMUT) GPIbα subunits. The CHO β/IX cells (developed from Dr Jose Lopez, Baylor College of Medicine, Houston, TX) stably expressing the GPIbβ and GPIX subunits of the receptor complex\(^\text{[28]}\), will be transfected with the pcDNA3-GPIbaWT or the pcDNA3-GPIbaMUT plasmids using Lipofectamine, as previously described\(^\text{[unpublished observations, 29]}\).

To determine the effect of the mutation on surface expression of the receptor, we will perform flow cytometry, 48 hours after transfection, using specific mAbs that bind different epitopes on the GPIbα molecule as previously described\(^\text{[26]}\).

To determine the effect of the mutation on GPIbα function, ristocetin-mediated binding of VWF to CHO cells expressing WT and mutant GPIbα will be studied by flow cytometry as previously described\(^\text{[26]}\).
GPIb/IX/V mediated signalling

To assess if a constitutively active GPIb/IX/V can mediate a defective signalling leading to a defective cross-talk with α\(_{\text{IIb}}\)β\(_3\), we will evaluate by Western Blotting the activation status of the main signalling proteins that have been described to interact with GPIb/IX/V and α\(_{\text{IIb}}\)β\(_3\): Src-family kinases, PI 3-kinase and SHIP-2. Washed platelets from patients and controls will be lysed in lysis buffer in resting conditions or after activation and an equal quantity of proteins for each sample will be analyzed by SDS-PAGE using the appropriate moAb.

Study of α\(_{\text{IIb}}\)β\(_3\) function

We will assess the function of α\(_{\text{IIb}}\)β\(_3\) in PT-VWD by three laboratory tests:
- PAC-1 binding to assess the ability of α\(_{\text{IIb}}\)β\(_3\) to be activated. PAC-1 binding will be studied by flow cytometry after activation with ADP (10 μM) and TRAP (20 μM) as previously described\(^{30}\).
- Light transmission aggregometry (LTA) to assess if α\(_{\text{IIb}}\)β\(_3\) can mediate platelet aggregation in response to different stimuli. LTA will be performed on patients’ platelet rich plasma (PRP) challenged with ADP (2 μM) adrenaline (5 μM), TRAP-6 (4 μM), arachidonic acid (1 mM), collagen (2 μM), aggregation will be followed in a four-channel aggregometer (APACT4, Helena Biosciences, UK) as previously described\(^{30}\).
- Spreading assay to assess the ability of α\(_{\text{IIb}}\)β\(_3\) to mediate the adhesion of platelets to different substrates. Washed platelets will be layered on coverslips coated with fibrinogen (100 μg/ml), type-I collagen (25 μg/ml) and Von Willebrand factor (10 μg/ml) for 30 minutes. Adherent platelets will be fixed, permeabilized, stained with FITC-conjugated phalloidin and analyzed by fluorescence microscopy.

Megakaryocyte differentiation and proplatelet formation

CD45+ cells will be separated from peripheral blood of the patients and cultured in Stem Span SFEM medium supplemented with 10 ng/ml Thrombopoietin, Interleukin-6 and Interleukin-11 for 14 days as previously described\(^{25}\). Megakaryocyte differentiation will be evaluated at day 14 of culture on cells cytospun on poly-L-lysine-coated glass coverslips and stained with anti CD41 antibody. To evaluate proplatelet formation onto adhesive substrates megakaryocytes at day 14 of culture will be separated on a BSA gradient (3-4%), plated onto glass coverslips coated with 100 μg/ml fibrinogen or 10 μg/ml VWF, and allowed to adhere for 16 h at 37°C and 5% CO2. To evaluate proplatelet formation in suspension, megakaryocytes will be seeded in 24 well plates and incubated for 16 h at 37°C and 5% CO2. Samples will be analyzed by immunofluorescence as previously described\(^{25}\).

Statistical analysis

Results will be analyzed using GraphPad Prim statistic software (Student t test or ANOVA as appropriated). Correlation analyses were carried out using the Spearman’s test.
Bibliography