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“Functional and Molecular Characterization of Inherited Platelet Disorders”

“Caracterización Funcional y Molecular de Trombopatías Congénitas”

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Introduction

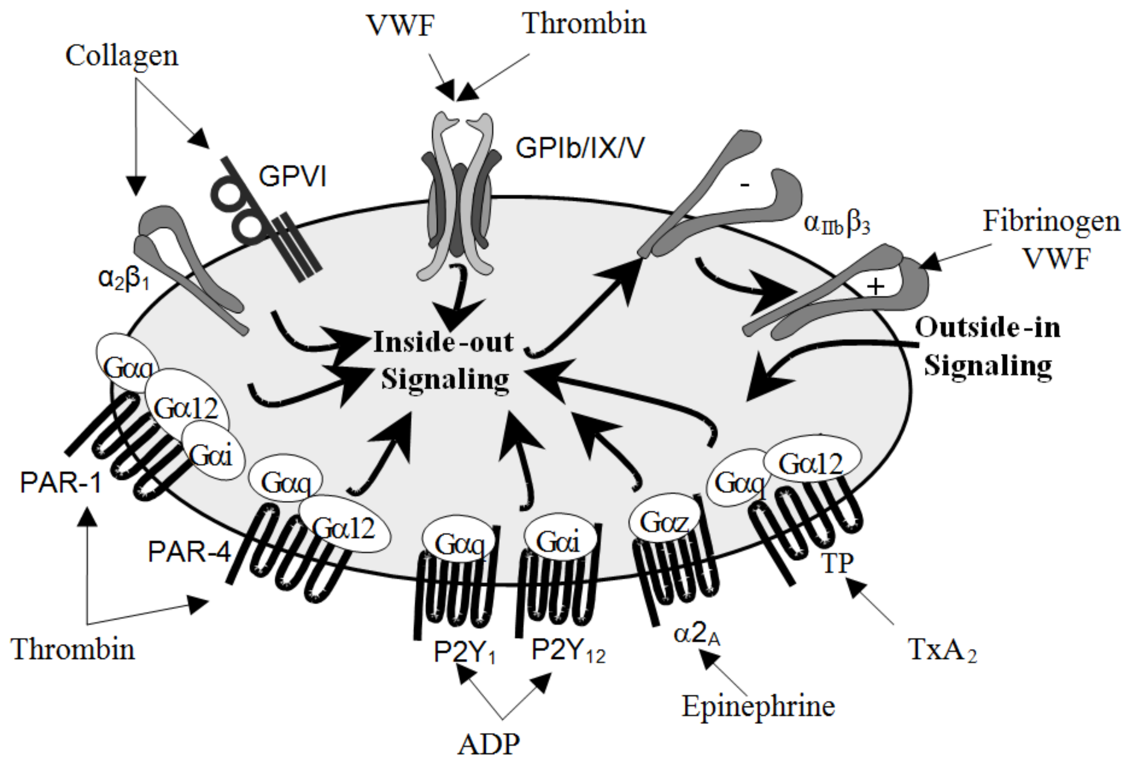
1. Platelet and hemostasis

Platelets are small anucleated cytoplasmic bodies circulating in blood stream. These cellular fragments are derived from megakaryocytes in the bone marrow. In steady state, megakaryocytopoiesis supplies about 10^{11} platelets per day with a new turnover every 8-9 days. This process is influenced by various environmental changes and platelets normally circulate at concentrations of 150–400 × 10^9 L^{-1}. Resting platelets appear small discoid cells (2–4 μm by 0.5 μm), facilitating their margination toward the vessel wall, where they can constantly survey the integrity of the vascular endothelium. The primary function of platelets is to stop blood loss after tissue trauma and exposure of the subendothelial matrix *(George JN 2000, Ruggeri ZM et al. 2007)*.

A wide variety of mobile transmembrane receptors cover the platelet membrane, including many integrins ($\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_V\beta_3$), leucine-rich repeated (LRR) receptors (Glycoprotein [GP] Ib/IX/V, Toll-like receptors), G-protein coupled seven transmembrane receptors (GPCR) (PAR-1 and PAR-4 thrombin receptors, $P2Y_1$ and $P2Y_{12}$ ADP receptors, TP_α and TP_β TxA_2 receptors), proteins belonging to the immunoglobulin superfamily (GPVI, FcγRIIA), C-type lectin receptors (P-selectin), tyrosine kinase receptors (thrombopoietin receptor, Gas-6, ephrins and ephrin kinases) and a miscellaneous of other types (CD63, CD36, P-selectin ligand 1, TNF receptor type, etc) *(Rivera J et al. 2009, Swieringa F et al. 2014)*. Many of these receptors are shared by other cell types, but some are only expressed on platelets, allowing specific interactions and functional responses to vascular adhesive proteins and to soluble platelet agonists (Figure 1). Internally, platelets contain a cytoskeleton, a dense tubular system, few mitochondria, glycogen granules, dense (δ) and alpha (α) storage granules and peroxisomes. The α-granules retain relevant proteins for the hemostatic function of platelets, such as von

Willebrand factor (vWF), fibrinogen (Fg), P-selectin, PECAM-1, CD40 ligand (CD154), platelet factor-4 (PF-4), β-thromboglobulin (β-TG), thrombospondin, platelet derived growth factor (PDGF), Factor V, as well as a backup of GPIIb/IIIa ($\alpha_{IIb}\beta_3$). Delta granules, on the other hand, are rich in nucleotides (ADP and ATP), serotonin, histamine, pyrophosphate, and calcium. Upon activation, granule contents are stepwise released to further promote platelet adhesion and activation *(George JN 2000)*.

Figure 1. Major platelet receptor-ligand interactions (Modified from Rivera J et al. 2009)



1.1. Dynamics of platelet plug formation

Formation of platelet plugs at sites of vascular damage requires a coordinated, both in time and place, series of events leading to: i) platelet arrest onto the exposed subendothelium creating a monolayer of activated cells (*initiation phase*); ii) recruitment and activation of additional platelets through the local release of major platelet agonists (*extension phase*); iii) stabilization of the platelet plug preventing premature disaggregation until wound healing occurs (*stabilization phase*). Currently, the existing static model of thrombus formation has been visualized as a dynamic model of thrombus buildup and stabilization in which continuous signaling is needed to stabilize thrombi and prevent their dissolution *(Cosemans JM et al. 2006)*.

1.1.1. Initiation phase

Thrombus formation in response to tissue trauma initiates with platelet interactions with the extracellular matrix components exposed to blood, particularly vWF, collagen, fibronectin, thrombospondin, and laminin. The rheological conditions largely influence these adhesive interactions. Thus, while at low shear rate, such as that in veins and larger arteries, platelet adhesion to the vessel wall primarily involves binding to fibrilar collagen, fibronectin and laminin, under conditions of elevated shear stress, such as those encountered in the microvasculature or in stenotic arteries, platelet tethering to the damaged subendothelium is critically dependent on their interaction with subendothelial bound vWF*(Savage B et al. 1998, Jackson SP et al. 2003)*. While soluble vWF does not bind to platelets to prevent aggregation in the normal circulation, immobilized vWF onto collagen is highly reactive toward flowing platelets. This may be because immobilized vWF assumes an extended shape under the effect of shear allowing its A1 domain to interact with platelets *(Siedlecki CA et al. 1996, Schneider SW*

et al. 2007). Perfusion assays simulating *in vivo* flow conditions have revealed that platelet adhesion to vWFis a dynamic process in which initial platelet tethering is characterized by transient interactions mediated through GPIbα. This deceleration then allows platelets to form new bonds with slower intrinsic binding kinetics (collagen to platelet collagen receptors, vWF to integrin $\alpha_{IIb}\beta_3$) and to become activated *(Savage B et al. 1998, Jackson SP et al. 2003)*.

It is long known that the GPIb/IX/V complex is the major platelet receptor mediating interaction with vWF. This complex consists of leucine-rich repeat glycoproteins: GPIbα (130 kDa) and GPIbβ (20 kDa) that are disulfide-linked and non-covalently associated with GPIX (20 kDa) and GPV as a 2α:4β:2IX:1V complex *(Rivera J et al. 2000, Luo SZ et al. 2007)*. In addition to vWF, the GPIb/IX/V complex also binds to other adhesive proteins (collagen, thrombospondin-1), α-thrombin and coagulation factors (kininogen, FXI, FXII). It also plays a substantial role in platelet interaction with activated endothelial cells and with leukocytes, through the binding of P-selectin and Mac-1 ($\alpha_M\beta_2$),respectively. The N-terminal globular domain of GPIbα (residues 1-282) contains the non identical but overlapping binding sites for all these ligands. The engagement of GPIbα by immobilized vWF elicits typical activation signals such as transient cytoplasmic Ca^{2+} elevations, protein phosphorylation (PLCγ2, ERK-1/2, Syk), thromboxane (TxA_2) synthesis, ADP release and ultimately activation of $\alpha_{IIb}\beta_3$ *(Gibbins JM 2004, Ozaki Y et al. 2005, Du X 2007)* (Figure 2).

Initiation of platelet adhesion to the injured vessel wall also requires platelet interaction with exposed collagen. Indeed, collagen and vWF form a functional unit for thrombus formation in flowing blood, with vWF contributing to the initial capturing of platelets on the vessel surface and collagen allowing the establishment of stable bonds for firm adhesion and triggering platelet activation. The collagen structure influences platelet adhesion and activation by a poorly understood mechanism and, although platelets can adhere to monomeric collagen, the

more complex structure of fibrilar collagen is required for optimal platelet activation by this adhesive protein *(Savage B et al. 1999)*. Two receptors have been demonstrated in the platelet surface which bind directly to collagen, the GPVI immunoglobulin superfamily member and the integrin $\alpha_2\beta_1$.

GPVI (62kDa) is a platelet–specific low-affinity collagen receptor of high potency in terms of initiating signal generation *(Nieswandt B et al. 2003)*. It has two extracellular immunoglobulin domains, a mucin-like core, a short peptide linker sequence, a transmembrane domain and a short cytoplasmic tail that binds Fyn and Lyn Src kinases. GPVI is also constitutively complexed with FcRγ-chain dimer, which bears an immunoreceptor tyrosine-based activation motif (ITAM) acting as the signal-transducing subunit of the receptor. When the GPVI receptor is cross-linked by binding to collagen or by GPVI specific ligands such as convulxin or alborhagin, the constitutively bound Src kinases phosphorylate the ITAM sequence in the FcRγ-chain allowing the assembly and activation of Syk and initiating activation of a downstream signaling pathway that has many similarities with that employed by immune receptors. Central to this signaling cascade is the formation of the signalosome, composed of various adapter and effector proteins (LAT, SLP-76, Gads), which associates to and activates PLCγ2, thus leading to liberation of 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) and to promotion of full platelet activation *(Watson SP et al. 2005)* (Figure 2).

The $\alpha_2\beta_1$ integrin, commonly referred to as GPIa/IIa, VLA-2 or CD49b/CD29, also plays a role for the adhesion of platelets to collagen and for subsequent optimal activation. The expression level of $\alpha_2\beta_1$, as that of GPVI, is controlled by silent polymorphisms and correlates with the *in vitro* rapidity in platelet adhesion and responsiveness to collagen *(Kritzik M et al. 1998, Corral J et al. 1999)*. The involvement of $\alpha_2\beta_1$ in hemostasis has been anticipated by the mild bleeding tendency and impaired platelet responses to collagen displayed by subjects with

heritable reduced expression of this integrin *(Nieuwenhuis HK et al. 1985)*. Like other integrins, conformational activation of $\alpha_2\beta_1$ increases its affinity for collagen, and seems to require inside-out signaling events that might be driven by engagement of GPVI with collagen *(Nieswandt B et al. 2003)* and/or by activation of $\alpha_{IIb}\beta_3$ *(Kuijpers MJ et al. 2007)*. Moreover, collagen binding to $\alpha_2\beta_1$ also triggers outside-in signaling resembling that induced by GPVI *(Nieswandt B et al. 2003)*, reinforces platelet activation.

Figure 2.Platelet signaling through GPVI and GPIb/IX/V complex. Adapted with permission from Gibbins 2004

1.1.2. Extension phase

After deposition of a platelet monolayer over the exposed vWF and collagen, the next step required for thrombus formation is the recruitment of additional platelets from the flowing blood, which upon activation acquire the ability to stick to each other in a process commonly referred to as platelet aggregation. This is made possible by the local accumulation of soluble agonists that are secreted or produced by adherent-activated platelets, including ADP, TxA_2, epinephrine and thrombin. The final step is activation of $\alpha_{IIb}\beta_3$, causing a conformational change that enables it to bind Fg and vWF, allowing stable bridges between platelets. The great number of $\alpha_{IIb}\beta_3$ copies on the platelet surface, 40,000 to 80,000, allows the assembly of large aggregates at the site of vascular injury. Activation of $\alpha_{IIb}\beta_3$ integrin requires agonist-driven activation events in recruited platelets, referred to as inside-out signaling, including the sequential activation of one or more PLC isoforms yielding a rise in cytosolic Ca^{2+}, activation of PKC and PI-3K, reorganization of the platelet cytoskeleton and activation of cytoskeletal proteins such as talin. The activated talin can bind to the cytoplasmic domain of the β_3 subunit causing dissociation of the cytoplasmic tails and transmembrane domains of α_{IIb} and $\beta_{3,}$ and promoting integrin oligomerization and Fg binding *(Petrich BG et al. 2007)*.

GPCRs are important 7-transmembrane spanning signaling molecules that play crucial roles in the extension of the platelet plug by most soluble platelet agonists *(Woulfe D et al. 2004, Offermanns S 2006)*. GPCRs can activate associated heterotrimeric guanine nucleotide–binding proteins (G proteins), which in turn act on various effectors (adenylyl cyclase, PLC, PI3K, p115-RhoGEF). In an orchestrated manner, in platelets, agonists acting through GPCRs: i) stimulate PLCβ isoforms via Gqα and Giα subunits, causing an increase in cytosolic Ca^{2+} and activation of PKC and PI-3K; ii) reorganize the actin cytoskeleton via G12α and G13α promoting

the microtubular ring change and the filopodia and lamellopodia formation that drive platelet shape change; iii) suppress cyclic adenosine monophosphate (cAMP) synthesis via Giα family members by inhibiting adenylyl cyclase, which is particularly relevant when intracellular cAMP levels are high due to the action of endothelial cell-derived prostaglandin I_2 (PGI_2) and nitric oxide (NO). There is evidence that Giα-associated G$\beta\gamma$ subunits also activate other signaling pathways such as PI-3Kγ, Src kinases and the Rap1B Ras family protein, which is an important contributor to pathways converging on the activation of $\alpha_{IIb}\beta_3$*(Woulfe D et al. 2004)*.

Platelet agonists with a prominent role in the process of extension of the platelet plug are ADP, TxA_2, thrombin and epinephrine. As mentioned above, ADP is stored in δ-granules and secreted upon platelet activation. Red cells, at sites of vascular injury, also release ADP. This agonist causes a full range of activation events including intraplatelet Ca^{2+} elevation, TxA_2 synthesis, protein phosphorylation, shape change, granule secretion, activation of $\alpha_{IIb}\beta_3$, and aggregation. All these events are mediated by interaction with two classes of purinergic GPCR, $P2Y_1$ and $P2Y_{12}$, which couple to Gqα and Giα, respectively *(Gachet C 2008)*. $P2Y_{12}$ has been identified as the major receptor to amplify and sustain ADP-mediated platelet activation initiated via $P2Y_1$. Indeed, $P2Y_{12}$ is the target of thienopyridine drugs (ticlopidine, clopidogrel, prasugrel) widely used in the prevention of vascular events in patients with cardiovascular disease, especially those having stent insertion *(Barrett NE et al. 2008)*. Despite their likely categorized roles, co-activation of both $P2Y_1$ and $P2Y_{12}$ seem to be required for optimal ADP-induced aggregation and ADP-promoted thrombus growth. In addition to $P2Y_1$ and $P2Y_{12,}$ human platelets express a third purinergic receptor $P2X_{1,}$ with a significant role in platelet function. $P2X_1$ is an ATP-driven calcium channel unable to trigger platelet aggregation by itself, but under high shear conditions acts as a positive regulator of platelet responses to collagen and thus plays a significant role in thrombus formation.

Thromboxane A_2 is a labile prostanoid synthesized by activated platelets through the sequential actions of cyclooxygenase (COX) and TxA_2 synthase enzymes. TxA_2 is a vasoconstrictor and a potent platelet agonist causing shape change, phosphoinositide hydrolysis, Ca^{2+} mobilization, protein phosphorylation, secretion, and aggregation *(Nakahata N 2008)*. Once synthesized, it diffuses across the platelet membrane and activates other recruited platelets, thus favoring the growth of the platelet plug. The TxA_2 receptor (TP) exists in two splice variants (TPα and TPβ) which differ only in their C-terminal cytoplasmic domains and that are encoded by a single gen located at 19p13.3. In human platelets, TPα is the only translated isoform, although mRNA for TPβ is also present *(Meadows TA et al. 2007)*. Biochemical studies have shown that TPs in platelets couple to Gqα and G13α activating pathways, but not to Giα. Loss of Gqα abolishes IP3 formation and Ca^{2+} mobilization, but not the G12/13α mediated activation of Rho GTPases and shape change, after activation with U46619, a TxA_2 analogue. In contrast, platelets deficient in both Gqα and G13α do not respond to TxA_2. There have been reported several homozygous and heterozygous patients suffering from lifelong mucosal bleeding due to genetic changes in TP or in other elements of the TxA_2 signaling pathway*(Watson S et al. 2010)*.

It is well established that thrombin rapidly generated at sites of vascular injury plays a major role in promoting and stabilizing thrombi under all shear conditions *(Jackson SP et al. 2003, Crawley JT et al. 2007)*. Generation of thrombin requires several surface-mediated reactions mediated by the tenase complex (FIXa in complex with FVIIa, which activate FX) and by the prothrombinase complex (FXa in complex with FVa, which activate FII). The activated platelet membrane, with phosphatidylserine translocated from the inner to the outer leaflet, provides such procoagulant surface, as well as endothelial cells or microparticles. Thrombin is perhaps the most effective platelet activator, and provokes a full range of responses (shape

change, secretion, TxA_2 generation, Ca^{2+} mobilization, protein phosphorylation and aggregation). It is capable of activating platelets at very low concentrations (0.1 nM) and no other platelet agonist seems to be as efficiently coupled to PLCβ activation. Within seconds, thrombin increases ten-fold the cytosolic level of Ca^{2+}, triggering downstream events as activation of PLA_2 *(Woulfe D et al. 2004)*. The thrombin-induced platelet responses are mediated at least partially by the GPIb/IX/V complex *(Rivera J et al. 2000, Du X 2007)*, and mainly by two protease activated receptors (PAR), namely PAR-1 and PAR-4 in humans and PAR-3 and PAR-4 in mice *(Coughlin SR 2005, Swieringa F et al. 2014)*.

The GPIbα subunit of the GPIb/IX/V complexcontains a high affinity binding site for α-thrombin, accounting for as much as 90% of the total protease that can bind to platelets *(Mazzucato M et al. 1998)*. This binding site is located within residues 268-287 at the N-terminal globular domain of GPIbα and can bind two separate thrombin molecules by interacting with both exosite I and II of the protease *(Celikel R et al. 2003, Dumas JJ et al. 2003)*. Binding of α-thrombin to GPIbα induces platelets adhesion and spreading, secretion and aggregation *(Mazzucato M et al. 1998, Adam F et al. 2003)*. Despite recognizing that an intact GPIb/IX/V complex may be required for optimal thrombin responsiveness *(Lopez JA et al. 1998)*, there is little doubt that PARs are sufficient to activate platelets and account for most, if not all, of the thrombin-induced signaling. Unlike other GPCRs as those for ADP or TxA_2 which signal through standard receptor/ligand interactions, PAR-1 and PAR-4 are activated by a unique irreversible proteolytic cleavage within the first extracellular loop exposing a new N-terminus that serves as a tethered ligand. Short synthetic peptido-mimetics of the new N-terminus sequences generated by thrombin (SFLLR and GYPGQV for PAR-1 and PAR-4, respectively) can activate these receptors and reproduce most of the platelet action of thrombin in platelets *(Coughlin SR 2005)*. Although activation of either PAR-1 or PAR-4 can trigger platelet secretion

and aggregation, PAR-1 is probably the most important receptor in thrombin responsiveness. To our knowledge, no patient has been identified with congenital deficiencies of PAR receptors.

Although the contribution of catecholamines to the hemostatic/thrombotic process is generally thought to occur through its constrictive action on the vascular wall, circulating or locally secreted epinephrine also favors platelet activation in the growing platelet plug. Reduced number of epinephrine receptors has been related with mild bleeding disorders in few patients *(Rao AK et al. 1988)*. In contrast to other agonists, epinephrine is considered to be a weak agonist unable to directly activate PLC or to cause shape change. However, it acts sinergically with many other agonists at low concentrations significantly increasing their activatory effect. This potentiating action of epinephrine is due to its capacity to inhibit cAMP formation throughout the coupling of its platelet α_2A-adrenergic receptor to the Giα family member Gzα *(Yang J et al. 2000)*. Variable responsiveness to epinephrine in relation to factors such as age, strenuous exercise or pathologic conditions such as heart disease or myeloproliferative syndromes has been related to changes in the platelet expression level of α_2A-adrenergic receptors *(Kaywin P et al. 1978, Wang JS et al. 1999)*. Some polymorphisms in the α_2A-adrenergic receptor influence *in vitro* shear mediated platelet function *(Yabe M et al. 2006)*. All these findings strengthen the relevance of signaling initiated by epinephrine through the α_2A-adrenergic receptor on thrombus formation.

1.1.3. Stabilization phase

The last phase in the formation of an effective thrombus that arrests blood loss at the site of vascular injury has been named stabilization or perpetuation. It refers to the late wave of signaling events promoted by the close contact between recruited platelets once aggregation

has started. Despite there is no evidence that these platelets form tight junctions, activated platelets within the forming plug come into sufficiently close contact with gaps below 50 nm-to allow direct or indirect bridges between adjacent platelets and to allow paracrine action of platelet released molecules, favoring the transfer of information as in a neurological or immunological synapse. This narrow contact also restricts the diffusion of plasma factors within the gaps, preventing for instance a premature fibrinolytic action of plasmin over the growing thrombus. These late events consolidate the stability of the forming thrombus avoiding early disaggregation and/or embolization.

The most relevant, or at least the best known, contact-dependent signaling events during this stabilization phase is outside-in signaling through integrins, particularly $\alpha_{IIb}\beta_3$. It comprises the signals emanating from $\alpha_{IIb}\beta_3$ once ligand binding, predominantly Fg, has occurred, which trigger essential events for thrombus growth and stabilization, such as cytoskeletal reorganization, formation and stabilization of large platelet aggregates, development of a procoagulant surface and a clot retraction that helps to narrow the gaps between platelets and to increase the local concentration of soluble platelet agonists *(Woulfe D et al. 2004, Watson SP et al. 2005)*.

Apart from $\alpha_{IIb}\beta_3$ clustering, much evidence indicates that integrin outside-in signaling relays on tyrosine phosphorylation of $\alpha_{IIb}\beta_3$ upon inside-out signals, and on the formation of large protein signaling complexes between the cytoplasmic domains of $\alpha_{IIb}\beta_3$ and intraplatelet proteins such as FAK, talin, myosin, β_3-endonexin, CIB1, Shc, Src and Syk, the PTP1B tyrosine phosphatase and PKCβ, among others.

The CD40 ligand (CD40L, CD154), a protein member of the TNF family present on the surface of activated platelets, also seems to be important in this context. This protein, progressively shed from platelet surface producing a soluble form, sCD40L, can bind to its

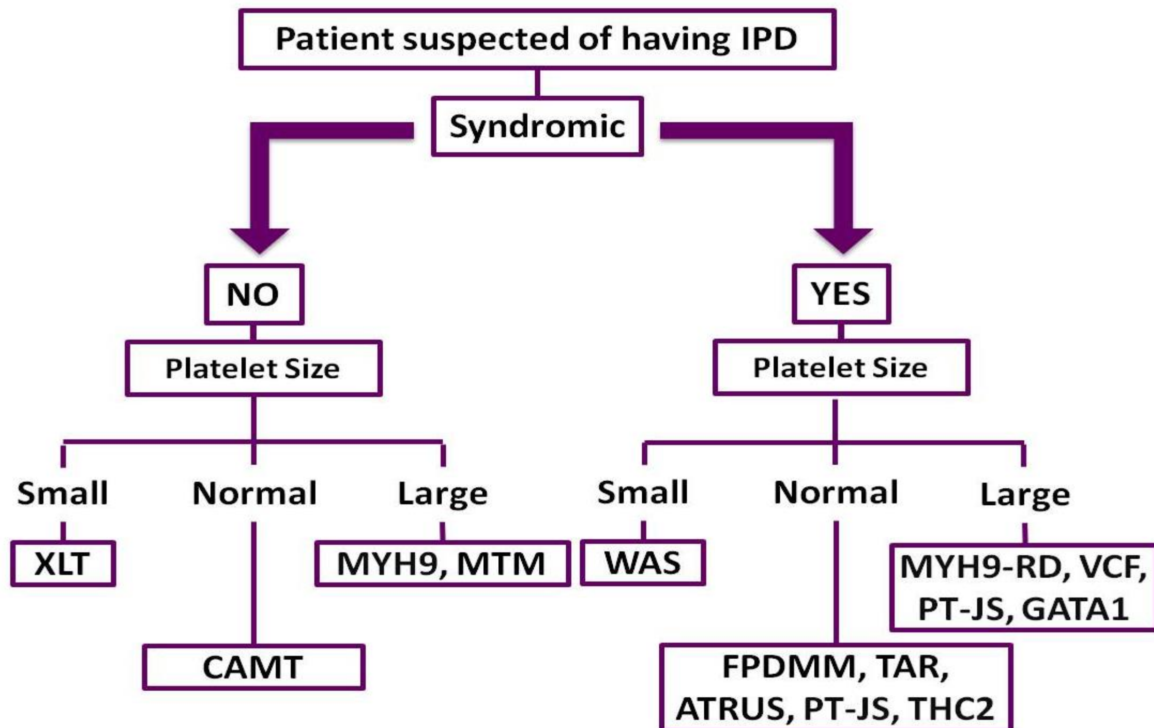
receptor CD40 but also to $\alpha_{IIb}\beta_3$ through a KGD (or RGD domain in mice) favoring the outside-in integrin signaling *(Hermann A et al. 2001)*. Additional ligand/receptor interactions generated by close platelet-platelet contact are family of Eph receptor tyrosine kinases and their ligands, known as ephrins, binding of semaphorin 4D (Sema4D) and Gas-6 to their platelet receptors, and CD84, a protein homologous to SLAM, and PEAR-1 (platelet endothelial aggregation receptor 1).

2. Inherited platelet disorders

The hemostatic function of platelets may be compromised by acquired or congenital defects affecting either the production/destruction of these pseudocells or the expression or the functionality of any of the elements involved in platelet physiology, leading to bleeding diathesis*(Salles, II et al. 2008, Scharf RE et al. 2011, Nurden AT et al. 2014)*

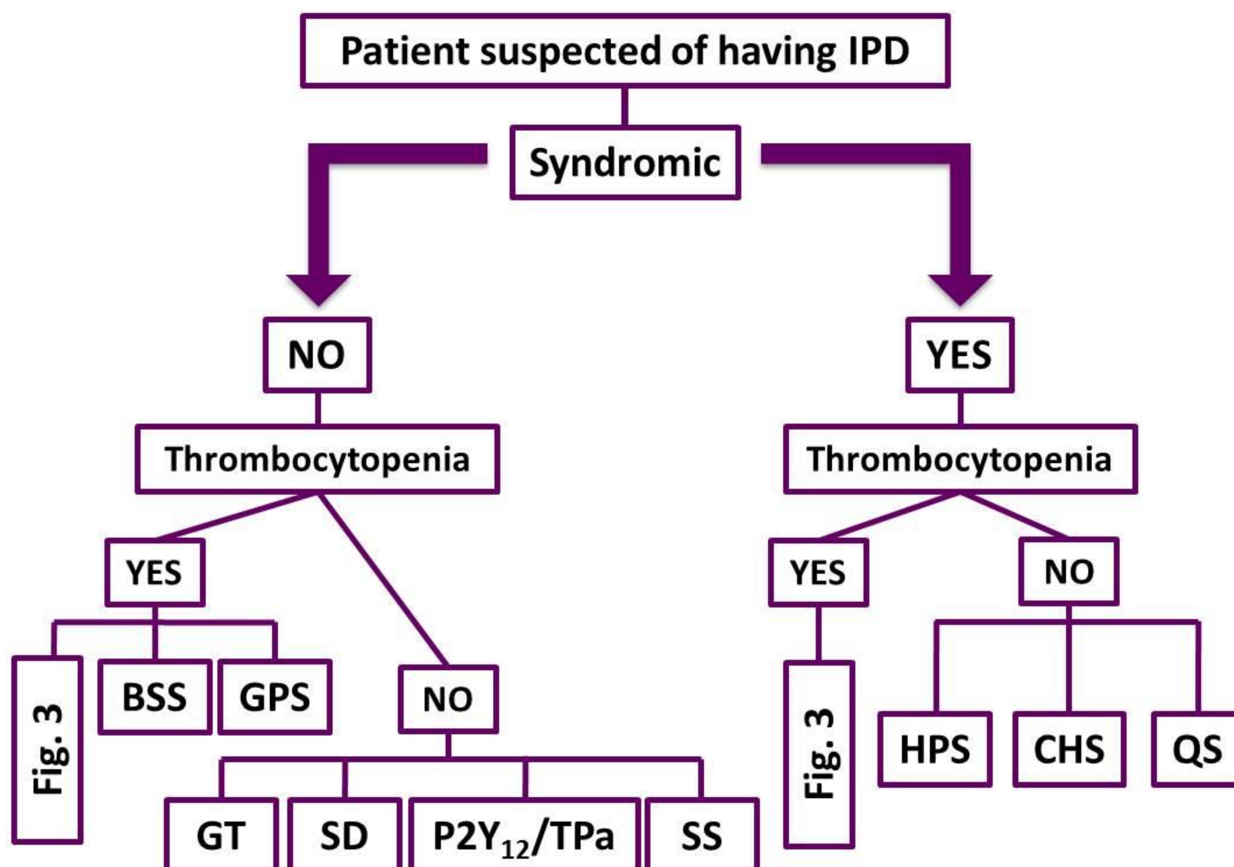
For the purpose of this work, the inherited platelet disorders (IPDs) will be grouped in: i) hereditary thrombocytopenias, where defects result in a decrease of platelet number in blood; and ii) platelet function disorders leading to dysfunctional platelets (with platelet number being also possibly affected). (Figures 3 and 4).

Figure 3.Classification of Inherited Platelet Disorders (I).



Abbreviations: ATRUS, Amegakaryocytic Thrombocytopenia with Radio-Ulnar Synostosis; CAMT, congenital amegakaryocytic thrombocytopenia; FPDMM, Familial platelet disorder with associated myeloid malignancy; GATA1,Thrombocytopenia associated with mutations in GATA-1; MTM, Mediterranean Macrothrombocytopenia ; MYH9-RD, MYH9 related diseases; PT-JS, Paris-Trousseau or Jacobsen Syndrome; TAR, Thrombocytopenia with Absent Radius; THC2, thrombocytopenia 2; VCF, Velocardiofacial/DiGeorge Syndrome; WAS, Wiskott-Aldrich Syndrome; XLT, X-linked Thrombocytopenia

Figure 4. Classification of Inherited Platelet Disorders (II).



Abbreviations: BSS, Bernard-Soulier Syndrome; CHS, Chediak-Higashi Syndrome; Fig. 3, Figure 3; GPS, Gray Platelet Syndrome; GT, Glanzmann Thrombastenia; HPS, Hermansky-Pudlak Syndrome; P2Y12/TPa, ADP or TxA2 Receptor Defect; QS, Quebec Syndrome; SD, Signaling Defect; SS, Scott Syndrome; TxA2, Thromboxane A2

2.1. Inherited Thrombocytopenias

2.1.1. Syndromic thrombocytopenias

2.1.1.1. Wiskott-Aldrich Syndrome (WAS)

The Wiskott-Aldrich syndrome is an X-linked recessive condition consequence of mutations in the gene *WAS*, encoding for the WAS protein (WASP) which is expressed primarily in lymphocytes and megakaryocytes (MKs) *(Mahlaoui N et al. 2013, Nurden AT et al. 2014)*. WASP is an adaptor protein critical to intermolecular interactions that plays a role in signal transduction. This protein is essential for MK differentiation and is involved in proliferation and

differentiation of hematopoietic progenitors.

This disease is characterized by moderate to severe thrombocytopenia, humoral and cellular immune deficiency, eczema, allergies, recurrent infections an increased risk for autoimmunity and malignancy*(Aldrich RA et al. 1954)*. The hallmark of this syndrome is the small size of its platelets (mean platelet volume (MPV): 3.5 to 5 fl, normal: 7-11 fl) and the decreased platelet number (5-50 × 10^9L^{-1}).

2.1.1.2. Thrombocytopenia with Absent Radius (TAR) and Amegakaryocytic Thrombocytopenia with Radio-Ulnar Synostosis (ATRUS)

TAR is a syndromic form of thrombocytopenia with normal MPV, associated with absent radius, often an allergy to cow's milk during childhood, and an increased risk for other bone defects, cardiac, or renal problems *(Freson K et al. 2014)*.

High thrombopoietin (TPO) levels and defective TPO signaling are detected in this syndrome. Exome sequencing revealed that compound inheritance of a low-frequency noncoding SNP and a rare null allele in *RBM8A*, a gene encoding the exon-junction complex subunit member Y14, cause TAR syndrome *(Albers CA et al. 2012)*. The reason why decreased levels of Y14 are associated with altered TPO signaling still remains unknown.

Amegakaryocytic thrombocytopenia with radio-ulnar synostosis is a very uncommon syndrome, characterized by fusion of the proximal radius and ulna, and congenital thrombocytopenia with normal MPV. It is transmitted as an autosomal dominant trait caused by mutations in the *HOXA11* gene (with less than 10 cases described in the literature so far) *(Bolton-Maggs PH et al. 2006)*.

2.1.1.3. MYH9-related Disorders (MYH9-RD)

MYH9-related disease (MYH9-RD) is an inherited disorder with a complex phenotype characterized by giant congenital thrombocytopenia (30-100 × $10^9 L^{-1}$), leucocyte inclusions, and possible subsequent manifestations of hearing loss, impaired renal function and cataracts. MYH9-RD is characterized by a considerable variability in clinical evolution and the degree of the thrombocytopenia is greatly variable among different subjects; usually the bleeding tendency is moderate.

The previously designed anomalies, the May-Hegglin, Fechtner, Sebastian syndrome and Epstein syndromes *(Althaus K et al. 2009)*, are in fact a clinical continuum now called MYH9-RD. The mutated gene in this autosomal dominant macrothrombocytopenia is *MYH9*, located on chromosome 22, which encodes the heavy chain of the non-muscle myosin-IIA (NMM-IIA) involved in cytoskeletal contractility. Molecular genetic testing can identify the causative *MYH9* mutation that can predict the clinical evolution in 85% of MYH9-RD cases *(Pecci A et al. 2014)*, which is essential to patients' clinical management and genetic counseling.

2.1.1.4. Velocardiofacial/DiGeorge Syndrome (VCF)

This syndrome is associated with cardiac defects, thymic and parathyroid hypoplasia, cognitive delays, learning disabilities, facial dysmorphia, and immune deficiency. It is caused by a monoallelic interstitial microdeletion (del22q11.2) due to a meiotic recombination during spermatogenesis or oogenesis. In 10-20% of cases the microdeletion is transmitted in an autosomal dominant manner; however, in most cases it occurs *de novo (Nurden AT et al. 2014)*.

Patients with the classic deletion have not only the deletion of the *TBX1*gene, associated with the syndromic abnormalities, but also that of *GP1BB*; therefore, these patients show a

reduction in the expression of platelet surface GPIb/IX/V. Between 12% and 25% of these individuals have also macrothrombocytopenia, although increased bleeding tendency is not frequent *(Bolton-Maggs PH et al. 2006)*.

2.1.1.5. Paris-Trousseau (PT) or Jacobsen Syndrome (JS)

Paris-Trousseau (PT) (also known as Jacobsen syndrome) is an autosomal dominant macrothrombocytopenia associated with a deletion of *FLI1*, an important transcription factor in megakaryopoiesis located on chromosome 11q23 *(Salles, II et al. 2008)*.

All patients have thrombocytopenia and platelet dysfunction with giant alpha (α) granules. In this syndrome there are two megakaryocyte populations: one is morphologically normal, while the other consists of small immature megakaryocytes suffering massive lysis *(Mattina T et al. 2009)*. Other phenotypes include dysmorphic facial and congenital heart defects, mental retardation, ophthalmologic, gastrointestinal, and genitourinary problems, gross and fine motor delays, and infections of the upper respiratory system *(Freson K et al. 2014)*. The deletion size goes from ~ 7 to 20 Mb, occurring *de novo* in 85% of reported cases.

2.1.2. Asyndromic Thrombocytopenias

2.1.2.1. X-linked Thrombocytopenia (XLT)

It is a less severe form of WAS in which immunodeficiency and eczema are not clinically significant. Microthrombocytopenia is always present *(Thrasher AJ 2009)*.

2.1.2.2. Mediterranean Macrothrombocytopenia (MTM)

In Southern Europe there is a relatively common form of macrothrombocytopenia (70-130 X 10^9L^{-1}) and preserved platelet function, which is usually detected in routine analysis. In some cases, the genotype and phenotype are equivalent to that of a carrier of the Bernard Soulier syndrome (BSS) *(Drachman JG 2004)*.

2.1.2.3. Thrombocytopenia 2 (THC2)

It is an autosomal dominant disorder caused by mutations in the 5' UTR of *ANKRD26.* Affected individuals usually present with moderate thrombocytopenia, reduction in platelet α-granules, normal *in vitro* platelet aggregation and MPV, and predisposition to leukemia and myeloid malignancies *(Noris P et al. 2011)*.

Although the prevalence is unknown, THC2 seems to be a frequent disorder, responsible for at least 10% of hereditary thrombocytopenia with normal MPV *(Noris P et al. 2011)*.

2.1.2.4. Congenital Amegakaryocytic Thrombocytopenia (CAMT)

Congenital amegakaryocytic thrombocytopenia (CAMT) is a rare autosomal recessive bone marrow failure syndrome characterized by severe thrombocytopenia which can evolve into aplastic anemia and leukemia. The disorder appears in childhood and occasionally abnormalities of the central nervous system, skeleton or cardiac defects have been reported. It is often recognized on the first month of life *(Al-Qahtani FS 2010)*.

It is caused by mutations in the *c-MPL* gene provoking a dysfunctional expression of the TPO receptor. Unlike other thrombocytopenias detected in the perinatal period, the plasma levels of TPO on this defect can be more than 10 times higher than normal.

There is a genotype-phenotype correlation, so that mutations predicted to result in a complete loss of function of the receptor exhibit a more aggressive pattern with a rapid progression to hypocellular bone during the course of the disease, while patients with missense mutations still experience a residual activity of MPL, and might show a temporary increase in platelet counts and develop later on pancytopenia *(Bolton-Maggs PH et al. 2006)*.

2.1.2.5. Familial platelet disorder with associated myeloid malignancy (FPDMM)

This is an autosomal dominant disorder caused by mutations in the gene *RUNX1* that encodes for a hematopoietic transcription factor. Many of the patients with FPDMM have low platelet counts or platelet activation defects. In a significant number of cases they are initially diagnosed with immune thrombocytopenia and subsequently classified as myelodysplastic syndrome (MDS). The incidence of MDS/AML in patients with FPDMM is around 40%, with a median age of appearance of 35 years. Large intragenic deletions in FPDMM ensure *RUNX1* haploinsufficiency as a mechanism for this disease, but also causative mutations have been identified in the Runt domain of this gene. Of note, while *RUNX1* mutations are insufficient to cause MDS/AML, however, they establish a preleukemic state that predisposes to AML *(Nurden AT et al. 2014)*.

2.1.2.6. Thrombocytopenia associated with mutations in GATA-1

Defects in the X-linked transcription factor GATA-1, which controls the development of erythroid and megakaryocytic cells, causes familial macrothrombocytopenia that can be associated with dyserythropoiesis, anemia and reticulocytosis resembling mild beta-thalassemia.Generally platelets are large, have a strongly reduced number of α–granules and

are dysfunctional with reduced aggregation responses and abnormal membrane expression of the GPIb/IX/V and GPVI receptors *(Nurden AT et al. 2014)*.

2.2. Inherited Platelet Function Defects

This heterogeneous group of rare diseases is characterized by abnormal platelet function and sometimes with thrombocytopenia. They can be classified according to the functional element being affected in:

2.2.1. Defects of platelet receptors

2.2.1.1. Defects of the GPIb/IX/V complex

The GPIb/IX/V complex comprises four glycoproteins Ibα, Ibβ, IX and V in a 2α:4β:2IX:1V ratio *(Luo SZ et al. 2007, McEwan PA et al. 2011)* (Figure 2). This receptor is exclusive of platelets and megakaryocytes and is the product of the *GP1BA*, *GP1BB*, *GP9* and *GP5* genes, that assemble within maturing megakaryocytes in the bone marrow *(Li R et al. 2013)*. Furthermore, it also acts as a thrombin receptor, and interacts with the cytoskeleton (with actin and filamin), contributing to the maintenance of platelet morphology.

2.2.1.1.1. Bernard-Soulier Syndrome (BSS)

Bernard-Soulier Syndrome is an autosomal recessive bleeding disorder, characterized by moderate to severe thrombocytopenia, giant and dysfunctional platelets, abnormal prothrombin consumption and prolonged bleeding time *(Andrews RK et al. 2013)*. It is caused

by mutations in *GP1BA*, *GP1BB* and *GP9* genes; so far 112 different variants have been characterized causing the absence of GPIb/IX/V complex in platelets (classic BSS) or, in exceptional cases, the expression of a nonfunctional receptor (BSS variant) *(Savoia A et al. 2014)*.

Its distinctive feature in the laboratory is the absence of platelet agglutination with ristocetin, which is not corrected by normal plasma, unlike severe von Willebrand disease (vWD). Instead, aggregation with other agonists (ADP, collagen, epinephrine, thrombin, etc.) is normal. Flow cytometry enables rapid demonstration of selective GPIb/IX/V receptor deficit and increased mean platelet volume (MPV) *(Nurden AT et al. 2014)*.

2.2.1.1.1. Platelet-type von Willebrand Disease (PvWD)

Platelet-type von Willebrand Disease, also known as Pseudo von Willebrand Disease, is a rare autosomal dominant platelet disorder caused by an abnormal function of the GPIbα protein, due to mutations in the gene *GP1BA*. To date, only six mutations have been described: five missense variants and one 27bp deletion *(Othman M 2011, Othman M et al. 2014, Woods AI et al. 2014)*. The common effect of these mutations is an abnormally high affinity of GPIb/IX/V receptor for vWF resulting in platelet hypersensitivity to ristocetin, and in a low ratio between the functional activity and the antigenic level of vWF in plasma (vWF: RCo/vWF: Ag<0.6), reflecting the loss of high molecular weight vWF multimers *(Salles, II et al. 2008, Nurden AT et al. 2014)*. Clinically, this leads to an increased removal of vWF multimers and platelets for the circulation, resulting in a bleeding diathesis.

The differential diagnosis of this disorder and vWD type IIB is very important, since both diseases have similar phenotyping parameters and clinical symptoms; however, these two

conditions have different etiology and require different treatment modalities *(Bolton-Maggs PH et al. 2006)*.

2.2.1.2. Abnormalities In The Fibrinogen Receptor: Glanzmann Thrombasthenia (GT)

Glanzmann Thrombastenia is a rare autosomal recessive disorder caused by qualitative or quantitative defects of the Fg receptor, the $\alpha_{IIb}\beta_3$ integrin. GT is associated with clinical variability: some patients have only minimal bruising, while others have frequent, severe and potentially fatal hemorrhages.

Diagnosis should be suspected in patients with mucocutaneous bleeding with normal platelet count and morphology, and impaired aggregation responses to physiologic stimuli. However, agglutination with ristocetin is normal or show a decreased second-wave aggregation *(Nurden AT et al. 2013)*. There are 3 different types of GT: type I, with complete absence of $\alpha_{IIb}\beta_3$ (<5%); type II, with 10-20% of residual $\alpha_{IIb}\beta_3$,and GT variant with expression of a non-functional complex (> 50%) *(Nurden AT et al. 2013)*. The *ITGA2B* and *ITGB3* genes involved in this pathology are located very proximal to each other on 17q21-23 *(Thornton MA et al. 1999)*. More than a hundred different mutations (deletions, insertions, point mutations, etc.) have been described in 200 patients so far, and these numbers are constantly growing.

2.2.1.3. Defects in Other Platelet Receptors

The heritable disorders of platelet receptors, other than GPIb/IX/V and $\alpha_{IIb}\beta_3$, arise from abnormal expression/function of platelet membrane receptor or their associated signaling pathways and result in defects in aggregation to one or more agonists *(Rivera J et al. 2009)*.

Affected individuals usually show a mild bleeding tendency.

Seven patients with selective defects in platelet adhesion and activation by collagen have been identified due to double mutations in *GP6* (gene encoding for the collagen receptor GPVI) *(Watson S et al. 2010, Matus V et al. 2013)*. Also, 14 patients with bleeding diathesis associated with genetic defects in the $P2Y_{12}$ ADP receptor have been reported *(Cattaneo M 2011, Patel YM et al. 2014)*. Defective TxA2 receptor function was identified in other 10 patients presenting with mucocutaneous bleeding, and point mutations (W29C, N42S, R60L or D304N) were detected in the gene encoding for the platelet TPa receptor *(Mumford AD et al. 2013, Nisar SP et al. 2014, Nurden AT et al. 2014)*

2.2.2. Storage Pool Disease (SPD)

Storage pool disease (SPD) is a heterogeneous group of disorders that have in common a deficiency of α and/or dense (δ) granules or their constituents, that result in a defect in ADP released from activated platelets and abnormal secretion-dependent platelet aggregation, which comprises the extension phase of clot formation *(Thon JN et al. 2012)*.

Various congenital disorders are caused by a quantitative or qualitative deficiency of platelet granules (α, δ, or more rarely, both). Patients with these conditions have a moderate bleeding diathesis and variable abnormalities of platelet aggregation responses to low doses of different agonists. Electron microscopy provides the best indication of granular deficiencies *(Bolton-Maggs PH et al. 2006, Salles, II et al. 2008, Masliah-Planchon J et al. 2013, Nurden AT et al. 2014)*.

2.2.2.1. Alpha Granule Defects

2.2.2.1.1. Gray Platelet Syndrome (GPS)

Gray platelet syndrome is a severe congenital deficiency in the number of platelet α-granules and their content due to mutations in the *NBEAL2* gene *(Albers CA et al. 2011)*. Patients with this rare autosomal recessive disorder present moderate mucocutaneous bleeding diathesis, thrombocytopenia with large and pale platelets, myelofibrosis and in some cases, splenomegaly. Onset of clinical symptoms is severe in the neonatal period or in early childhood; however thrombocytopenia and myelofibrosis are progressive in nature. Diagnosis is based on clinical findings and requires demonstration of absence or marked the reduction of α-granules by electron microscopy. Most patients have high serum vitamin B12 levels.

2.2.2.1.2. Quebec Platelet Syndrome (QS)

Quebec platelet syndrome is a platelet disorder with dominant inheritance, characterized by normal or reduced numbers of platelets, and an excess of urokinase plasminogen activator (u-PA) in α-granules, generating excessive plasmin and abnormal proteolysis of other proteins, such as FV, vWF, Fg or P-selectin. The QS is caused by a mutation in the gene *PLAU* encoding u-PA *(Hayward CP et al. 2011)*. Moderate to severe bleeding is usually observed after trauma, surgery or obstetric interventions. Both mucocutaneous and muscle/joint bleeds are characteristic of these disorders.

2.2.2.2. Dense Granule Defects

Congenital δ-granule deficiencies may occur as an isolated phenomenon of unknown genetic cause or in association with defects of intracellular traffic of the lysosome-related organelles (LRO). The pattern in these disorders is normal platelet count but impaired platelet functionality. Such diseases include three rare autosomal recessive diseases *(Gunay-Aygun M et al. 2004, Dotta L et al. 2013, Masliah-Planchon J et al. 2013, Nurden AT et al. 2014)*.

2.2.2.2.1. Hermansky-Pudlak Syndrome (HPS)

Hermansky-Pudlak syndrome is an autosomal recessive disorder characterized by oculocutaneous albinism, ceroid accumulation in mononuclear phagocytic-cells, variable pulmonary fibrosis, inflammatory bowel disease and hemorrhagic diathesis *(Wei AH et al. 2013)*.

The disease has a heterogeneous molecular basis, with mutations in up to 9 different genes (*HPS1*, *AP3B1*, *HPS3*, *HPS4*, *HPS5*, *DTNBP1*, *BLOC1S3* and *BlOC1S6*), all involved in intracellular vesicle trafficking *(Wei AH et al. 2013)*.

2.2.2.2.2. Chediak-Higashi Syndrome (CHS)

This syndrome is also characterized by partial oculocutaneous albinism, silvery hair, giant lysosomal granules, inclusion bodies in neutrophils and other cells, frequent pyogenic infections, peripheral neuropathy, and accelerated phase in up to 85% cases. CHS is exclusively due to mutations in the gene *LYST/CHS1*, encoding the LYST protein, a regulator of lysosomal trafficking *(Kaplan J et al. 2008)*. It is thought to be a genotype-phenotype relationship with loss-of-function mutations associated to the childhood-onset severe clinical forms of the

disease, and missense mutation with milder adolescent- or adult-onset forms *(Karim MA et al. 2002)*.

2.2.2.2.3. Griscelli Syndrome (GS)

Griscelli Syndrome is a rare autosomal recessive disorder characterized by mild bleeding, partial albinism and silvery hair. It can be associated to neurological impairment (type I), immunodeficiency (type II), or be isolated (type III) *(Nurden AT et al. 2014)*. GS type I is caused by a mutation myosin Va gene (*MYOSA*); type II is caused by mutations in Rab27a (*RAB27A*), while GS type III is due to alterations in or melanophilin (*MLPH*).

2.2.2.3. Congenital defects of α and δ granules

These patients have normal (or mildly reduced) platelet count and morphology. By electron microscopy platelets are moderately deficient in both α and δ-granules, and very few cases with a severe deficiency in both structures are described.Platelet α granules and dense bodies lose their content through the open canalicular system and therefore the disease mechanism is different from other disorders affecting granules *(White JG et al. 2007)*.

The bleeding tendency of these patients is absent or very low, normally associated with a severe hemorrhagic challenge.

2.2.3. Congenital Defects in Signal Transmission of Platelet Activation

A substantial number of patients with an inherited abnormality in platelet function and a bleeding diathesis, present with decreased aggregation and secretion of granules content upon activation, and are lumped in the category of primary secretion defects or platelet

activation defects. This group includes congenital enzyme deficiencies (such as nucleotide cyclases, phospholipases, cyclooxygenases, kinases), alterations in G proteins (Gαq, Gαi, Gαs), defects in transcription factors and abnormalities in cytoskeletal proteins (such as talin or kindlin-3), involved in outside-in signaling of integrins such as $\alpha_{IIb}\beta_3$ *(Rao AK 2013, Nurden AT et al. 2014)*.

2.3. Other Congenital Platelet Defects

This group includes defects in the procoagulant activity of platelets.

2.3.1. Scott Syndrome

It is a rare hemorrhagic disorder characterized by bleeding following invasive procedures caused by deficient phosphatidylserine externalization during platelet activation, resulting in impairment in the activation coagulation process in the area of vascular injury. Its molecular cause is not clear and could be due to mutations in genes such as *ABCA1* or *TEMEM16F*, encoding proteins involved in the externalization of negative phospholipids *(Solum NO 1999, Castoldi E et al. 2011, van Kruchten R et al. 2013)*.

3. Diagnosis of IPDs

The diagnosis of an IPD should be attained by a combination of an appropriate personal and family clinical history, a physical examination, standard laboratory studies, including an assortment of platelet morphologic and functional studies, and ideally, molecular analyses. Figure 5 shows a stratification of diagnostic tests for the study IPDs.

Initial evaluation of the patient must include a personal and familiar history with specific

attention to mucocutaneous bleeding, ethnicity and consanguinity, a bleeding history in the presence/absence of hemostatic challenges, associated medical complications consistent with some of the more common IPDs, as well as a physical examination to detect possible syndromic features (hearing loss, immunodeficiency, renal function, cardiac function, mental retardation, facial dysmorphism, eyes, bone, skin, etc.) *(Bolton-Maggs PH et al. 2006, Lambert MP 2011, Gresele P et al. 2014)*. A drug history should be taken, including herbal remedies, non-steroidal anti-inflammatory drugs (NSAIDs) *(Lambert MP 2011)*, as well as previous requirement for blood transfusions.

Laboratory assessment of patients with a sustained suspicion of an IPD should include full blood count and blood smear evaluation to assess platelet size and morphology and any white cell or red cell changes, and coagulation tests (prothrombin time, activated partial thromboplastin time and fibrinogen). All patients with symptoms suggestive of an IPD in the absence of severe thrombocytopenia should be investigated for vWD *(Quiroga T et al. 2012)*.

3.1. Platelet Function Testing

Many laboratories are utilizing a panel of basic screening tests when a patient presents with a clinical suspicion of defects in hemostasis. If these screening tests are all normal but the clinical indication is strong for platelet defects, it is imperative that a complete diagnostic workup is still performed. If appropriate, a complex panel of specialized tests including platelet aggregometry, a measure of platelet release, flow cytometry analysis of signal transduction pathways, and genetic studies will be done.

Platelet function testing presents many challenges in ensuring that accurate and meaningful results are obtained *(Gresele P et al. 2014)*. First, platelet function tests remain

poorly standardized, and there are no widely available internal or external quality control materials. Thus, laboratories have to establish normal ranges for each test in a series of healthy volunteer and/or assay a normal control in parallel with the patient in order to ensure that each test/reagent is well working. Moreover, many platelet function tests require performance on fresh blood within a narrow time window (<3 h) after extraction, and their result can be significantly affected by the quality and handling of the blood samples. To minimize processing-induced activation and desensitization, during phlebotomy it is important to use a light tourniquet, a needle of at least 21 gauge, discarding the first few milliliters of blood drawn, ensuring immediate gentle mixing with anticoagulant, keeping all tubes at room temperature, checking that the blood tube is not over or under-filled and avoiding unnecessary manipulation of the sample.

3.1.1. Screening Tests

3.1.1.1. Platelet Count and Blood Smear

In all patients, the platelet count should be measured firstly by means of automatic hematological counter. In addition, a peripheral blood film should be examined to confirm thrombocytopenia and to assess potential alterations in platelet size and morphology and any white cell or red cell changes *(Bolton-Maggs PH et al. 2006)*.

3.1.1.2. Bleeding Time

Traditionally, platelet function testing begun by performing an *in vivo* bleeding time, which consists of causing a small incision in the skin of the forearm, or the earlobe, and

recording the time required for a clot to form at the site of the incision and to stop the flow of blood. Until the early 1990s, this technique was regarded as a useful screening test of platelet function, with the advantage of simplicity, low cost and measurement of the physiological hemostasis.

However, the bleeding time is invasive, poorly reproducible, time-consuming, insensitive to many mild platelet defects, and fails to correlate with the bleeding tendency *(Rodgers RP et al. 1990)*. Because of these limitations, the widespread use of the bleeding time test has rapidly declined over the past 15 to 20 years, to be replaced by other less-invasive and more reproducible platelet function assays *(Harrison P et al. 2013, Gresele P et al. 2014)*.

3.1.1.3. Platelet Function Analyzer (PFA)-100

In the last 25 years, the growing clinical need has strongly driven the development of devices, point-of-care instruments that allow rapid, simple, and non-specialized platelet function testing in clinical hemostasis laboratories.

Among these, the PFA-100 has been the most extensively used and evaluated machine. The PFA (Siemens, Marburg, Germany), is a relatively simple bench top instrument that simulates high shear platelet function within disposable test cartridges, and has been considered a surrogate *in vitro* bleeding time. The instrument monitors the drop in flow rate, and the time required to obtain full occlusion of the aperture is reported as closure time (CT), up to a maximum of 300 seconds. The test is simple to perform, rapid and can test relatively small volumes (0.8 mL/cartridge) of citrated blood up to 4 h from sampling. Clinical applications have included screening for vWD and its treatment monitoring, identification of inherited and acquired platelet defects, monitoring antiplatelet therapy, and assessment of surgical bleeding

risk *(Hayward CP et al. 2006, Favaloro EJ 2008)*. However, the CT is influenced by platelet count and hematocrit, and seems to have poor specificity for any particular disorder. Thus, normal PFA CT results can be used with some confidence to exclude severe vWD or severe platelet dysfunction, but would not exclude a possible mild vWF deficiency or mild platelet disorder *(Hayward CP et al. 2006)*. Any presumptive platelet function defect detected by abnormal CT needs to be confirmed by more specific tests.

3.1.1.4. Other Point-of-Care Testing (POCT)

Apart from PFA-100, other point-of-care instruments have been variably introduced in the platelet function testing field, such as automatic thromboelastographs, VerifyNow, Impact-R (cone and plate analyzer), etc. *(Shah U et al. 2007, Michelson AD 2009, Harrison P et al. 2013)*.

Thrombelastography (TEG) (Hemoscope, Niles, IL) and rotational thrombelastometry (ROTEM) (Pentapharm GmbH, Munich, Germany) measure the physical properties of forming clots by the use of an oscillating cup that holds whole blood samples. TEG provides various data related to fibrin formation, clot development, the ultimate strength, and stability of fibrin clot as well as fibrinolysis. The particular advantages of TEG/ROTEM are to provide a complete profile of clot formation and allow for interactions between whole blood elements, including platelets and the coagulation and fibrinolytic systems. There is inter-laboratory variation and it is a time-consuming analysis (at least 30 min).

Verify Now® (Accumetrics, San Diego, CA) is a whole blood point-of care assay based on optical detection of changes in light transmittance upon aggregation of platelets inside cartridges containing fibrinogen-coated beads and distinct agonists *(Smith JW et al. 1999)*. Essentially, in this test citrated blood tubes are inserted into the device holding P2Y12

cartridges that contained fibrinogen-coated beads and 20 µM ADP and 22 nM Prostaglandin E1 (PGE1) in order to measure platelet aggregation mediated by the ADP receptor P2Y12, but avoiding the platelet response to ADP trough the P2Y1 receptor. Changes in light transmittance as activated platelets bind and aggregate to fibrinogen-coated beads are reported in P2Y12 Reaction Units (PRU). Cartridges also bear a second chamber containing iso-TRAP, PAR-4 peptide, and fibrinogen-coated beads that provide an internal positive control of platelet aggregation status (PRU BASE) that is not influenced by drugs such as aspirin or thienopyridines. The Verify Now system is being widely used in the context of antiplatelet therapy monitoring *(Jover E et al. 2014)*, but scarcely for the purpose of investigating IPD.

3.1.2. Diagnostic Tests

3.1.2.1. Platelet Aggregometry

Platelet aggregometry was developed in the early 1960s and it still is regarded as the "gold standard" of platelet function testing. Light transmission aggregometry (LTA) is still the most widely used test for identifying and diagnosing platelet function defects and can be performed within commercially available multichannel aggregometers. Platelet rich plasma (PRP) is stirred within a cuvette located between a light source and a detector. After addition of a various panel of agonists, such as collagen, ADP, thrombin, ristocetin, epinephrine, and arachidonic acid, the platelets aggregate and light transmission increases. The biphasic response pattern consists on a primary response to an exogenous agonist, followed by a secondary response to the release of dense granule contents, which can be masked if high concentrations of agonists are added. The main disadvantages of LTA are the use of PRP instead

of whole blood under relatively low shear conditions, the requirement of large sample volumes, and that it is time-consuming. In addition, there are many pre-analytical and analytical variables that can affect LTA results and therefore the technique remains not well standardized. In order to diminish variability among laboratories, in recent years many surveys and several guidelines in LTA procedure have been published *(Cattaneo M et al. 2009, Dawood BB et al. 2012, Cattaneo M et al. 2013)*.

Light transmission aggregometry can be also combined with luminometry to simultaneously measure dense granular nucleotide release, thus allowing evaluation of platelet dense granules content and the platelet secretion process *(Cattaneo M 2009)*.

Platelet aggregation, also in combination with platelet secretion, can be measured in whole blood by impedance methods. This may have the advantage of testing platelet responses in their native milieu, containing RBCs and other blood cells *(McGlasson DL et al. 2009)*. The use of whole blood platelet aggregation in clinical laboratories has been recently facilitated by the development of a semiautomatic device called Multiplate Analyzer that uses disposable cartridges with different agonists. Yet, this procedure does not fully reflect the LTA responses *(Seyfert UT et al. 2007, Valarche V et al. 2011, Chen F et al. 2012, Wurtz M et al. 2013)*.

3.1.2.2. Flow Cytometry

There is no doubt that one of the biggest advances in platelet function analysis has been the application of flow cytometry. This technique has several advantages including the need of a small volume of whole blood and relatively low number of platelets, thus making possible analysis in pediatric samples and in thrombocytopenic patients. Yet, it requires an expensive instrument, specialized personnel training, and the available monoclonal antibodies or related

agents are still expensive. The most commonly used flow cytometry test in platelet analysis is the quantification of platelet receptors, and this assay has been most useful to identify $\alpha_{IIb}\beta_3$ and GPIb/IX/V deficiencies in GT and BSS, respectively. Flow cytometry has also been devised to evaluate platelet granules status *(Harrison P et al. 2013)*, microparticles formation and exposure of anionic phospholipids (procoagulant activity) *(Montoro-Garcia S et al. 2012)*, the expression of platelet activation markers on individual platelets, or the quantization of aggregates between platelets and other blood cells. The most common platelet activation markers assessed by flow cytometry are P-selectin expression on the platelet surface (as a marker of α-granule secretion), the conformational change of $\alpha_{IIb}\beta_3$ into its active state (measured with monoclonal antibody PAC-1), platelet-leukocyte conjugates, microparticle examination, exposure of anionic, negatively charged phospholipids on the platelet surface (procoagulant activity), and phosphorylation of vasodilator stimulated phosphoprotein-phosphorylation (VASP-P) (Bio-Cytex, Marseille, France), as a marker of P2Y12 receptor activation-dependent signaling *(Dahlen JR et al. 2013)*.

3.1.3. Other Tests

3.1.3.1. Biochemical Assays

Classically several biochemical assays have been used to study different aspects of platelet function. Thus, activation and secretion may be assessed with immunoassays, enzyme linked immunosorbent assay (ELISA) or radio immuno assay (RIA), which measure the concentration of bioactive compounds such as thromboxane A_2 or granular proteins (β-TG, PF-4, PDGF) in the supernatant of the stimulated platelets. The ability of platelet δ-granules to

uptake and release can also be evaluated by using radioactive serotonin ([^{14}C]-5-hydroxytryptamine; [^{14}C]-5HT) *(Mezzano D et al. 2009)*.

Platelet signaling pathways can also be evaluated by measuring protein phosphorylation/dephosphorylation of specific proteins by classical western blotting or immunoprecipitation assays *(Miller JL 2009)*. Assaying plasma TPO levels is helpful in evaluating certain inherited thrombocytopenias and it can suggest abnormalities of the c-Mpl receptor *(Al-Qahtani FS 2010)*.

3.1.3.2. Electron Microscopy and Other Platelet Imaging Procedures

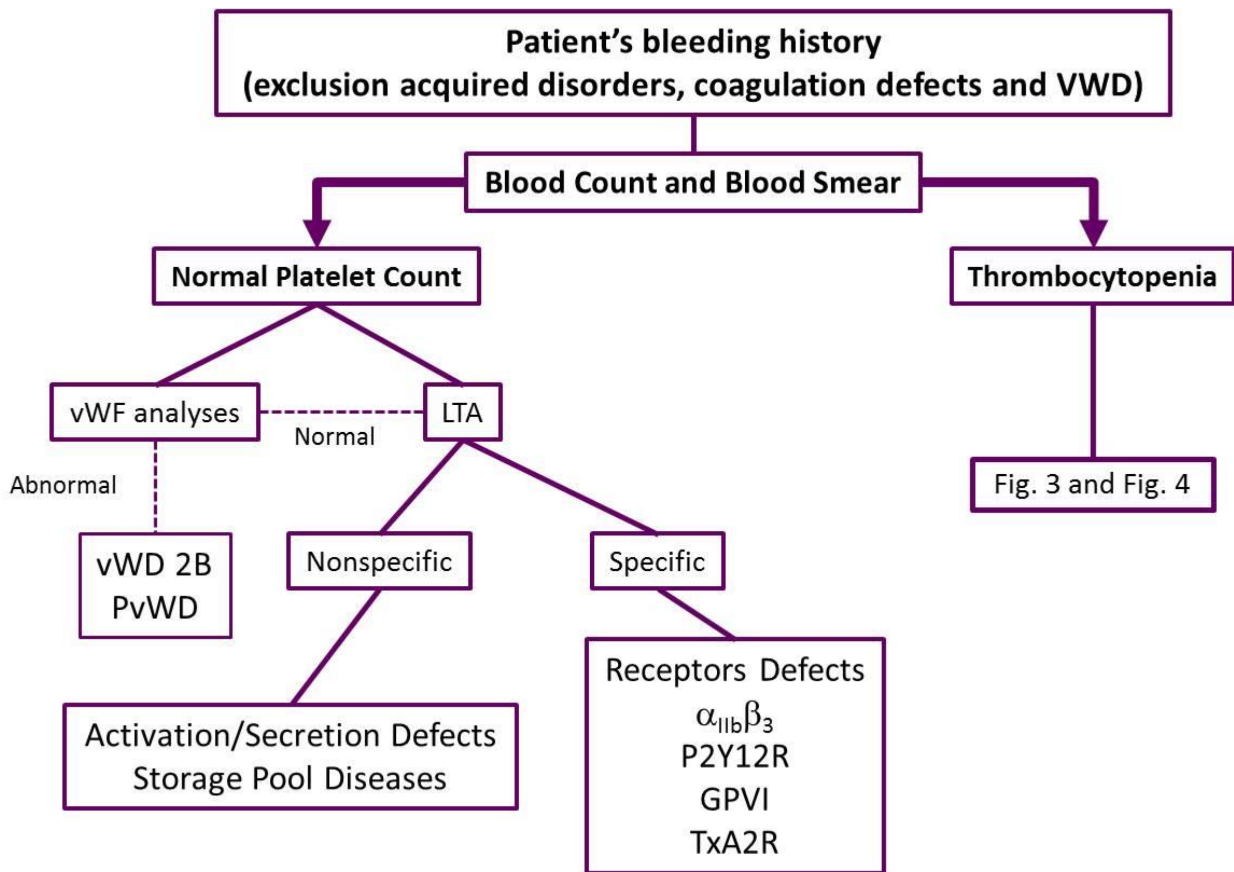
Electron microscopy is valuable in the study of patients with defects that affect platelet ultrastructure or the number or content of platelet granules *(White JG 2004, Clauser S et al. 2009)*. Generally, platelet electron microscopy protocols are laborious and complex. However, some organelles such as δ-granules can be distinguished easily using unfixed and stained preparations, simplifying the procedure and facilitates its use for the study of patients with suspected deficiencies in δ-granules *(White JG 2008, Hayward CP et al. 2012)*.

Macrothrombocytopenia evaluation, especially if there is normal response to ristocetin, should be accompanied by classical May–Grünwald–Giemsa staining to look for Döhle bodies in leukocytes (MYH9-RD) and a simple immunofluorescence test may be more sensitive to point to MYH9-RD *(Althaus K et al. 2010)*.

Finally, apart from conventional scanning electron microscopy, atomic force microscopy and other complex platelet microscopy procedures are under development, such as non-contact hopping probe ion conductance microscopy, that aim to monitor morphological changes of platelets in real time that could help to unravel the complex platelet physiology *(Liu*

X et al. 2014).

Figure 5. Diagnostic algorithm for IPDs



Abbreviations: LTA, Light Transmission Aggregometry; PvWD, Pseudo von Willebrand Disease; vWD, von Willebrand Disease; vWF, von Willebrand Factor

3.2. Molecular Characterization

Despite platelet function testing is first-line investigation in most laboratories for diagnosis of IPD *(Gresele P et al. 2014)*, a full and unequivocal diagnosis of these diseases can only be complete when the genetic defect has been defined for each patient *(Nurden AT et al. 2009, Daly ME et al. 2014)*. Detecting the molecular alteration is important for the medical management of affected patients, to avoid inappropriate treatments, to provide genetic counseling, and to satisfy the increasingly higher information demand of patients about their diseases.

However, the large number of structural proteins, receptors and other proteins involved in signaling pathways that can be affected in platelets, together with minimal guidance offered by the genotype-phenotype correlations, provides a significant challenge for the molecular diagnosis of these IPDs. Nowadays, access to molecular techniques for diagnosis of IPDs is still limited to most clinical laboratories *(Gresele P et al. 2014)*.

To date, the approach of molecular study of IPDs depends on whether previous platelet function testing point to a particular diagnosis with known candidate(s) gen(es) *(Watson SP et al. 2013)*. However, this picture may change in the near future due to the rapid evolution and cost reduction of massive sequencing procedures.

3.2.1. Candidate Gene Approach

For a substantial number of IPD, the clinical presentation and laboratory platelet function testing provide clues to the identification or suspicion of the platelet protein that is affected. Best examples of this scenario are the severe IPDs BSS and GT where accurate diagnosis can be obtained from LTA assays and flow cytometry studies of platelet membrane glycoproteins. In the late 1980s the genes encoding key platelet receptors, such as $\alpha_{IIb}\beta_3$ or GPIb/IX/V, began to be identified, and shortly afterwards the first genetic defects in patients with GT and BSS were identified by Sanger sequencing of these genes.

Nowadays, there is a wide list of genetic loci known to be associated with platelet traits and platelet disorders, and so a strategy of candidate gene can be theoretically used in the molecular diagnosis of many patients with an IPD *(Kunicki TJ et al. 2010, Kunicki TJ et al. 2012, Balduini CL et al. 2013, Bunimov N et al. 2013, Freson K et al. 2014, Nurden AT et al. 2014)*. For most of the severe IPDs (SBS, GT, MYH9, HPS, CHS) investigated in this thesis, we have used this

approach of first identifying candidate genes based on clinical suspicion and platelet functional studies, and then performing PCR/RT-PCR amplification of DNA/RNA with specific primers and Sanger sequencing to discover the underlying molecular alterations.

However, in many patients with common IPDs, such as platelet secretion or signaling defects, identification of a candidate gene is not that obvious due to the non-specific clinical and laboratory phenotype, the complexity of traits that are influenced by a combination of inherited and acquired defects, and the incomplete penetrance of bleeding between family members. In these cases, novel alternative molecular approaches are required to identify the underlying genetic defects.

3.2.2. Linkage Analysis

Linkage analysis is a powerful tool to detect the chromosomal location of disease genes. It is based on the observation that genes that reside physically close on a chromosome remain linked during meiosis.

3.2.2.1. Autozygosity Mapping

In consanguineous families, there is a high probability that an affected individual inherits both copies of the mutated gene from a common recent ancestor; as a consequence of this principle, the chromosomal region surrounding the mutated gene may be expected to be homozygous; such region is said to be identical by descent or autozygous. In order to identify disease-causing genes, therefore, homozygous chromosomal segments can be searched for in inbred affected individuals, and this mapping is facilitated by specific sequences of DNA called microsatellites or short tandem repeats (STRs).

Two successful examples of this approach are the discovery of the *HPS8* gene in a large consanguineous family with oculocutaneous albinism and platelet dysfunction *(Morgan NV et al. 2006)* and the identification of the second mutation in the *HPS7* gene, described in this thesis *(Lowe GC et al. 2013)*.

3.2.2.2. Genome-Wide Linkage Analysis

Genome-wide linkage analysis is an effective tool for defining candidate *loci* in those IPDs where candidate genes are not obvious. It is particularly useful when it is possible to study large families with several affected and unaffected members, or groups of patients with IPDs that share common features. This molecular approach permitted the identification of the *MHY9* gen as the genetic loci responsible for the MYH9-RD *(Seri M et al. 2000)*, and more recently to localize the chromosomic regions bearing the gen causing TAR and GPS *(Klopocki E et al. 2007, Gunay-Aygun M et al. 2010)*.

3.2.3. Next Generation Sequencing (NGS)

Next generation sequencing refers to non-Sanger-based high-throughput DNA sequencing technologies. NGS platforms perform massively parallel sequencing, during which millions of fragments of DNA from a single sample are sequenced in unison *(Grada A et al. 2013)*.

This new technology is becoming the “gold standard” approach for the identification of underlying genetic defects in patients with rare inherited bleeding disorders since it provides an increasingly cheaper, quicker and higher throughput alternative to sequencing DNA than traditional Sanger’s method. It also reduces the time needed to perform the mapping studies in

the search for the gene(es) involved in the disorder *(Daly ME et al. 2014)*.

For better outcomes, it is important to know the pattern of inheritance of the IPD. If the defect shows an autosomal recessive pattern of inheritance and there is evidence of consanguinity within the affected family, it could be restricted to homozygous changes. On the other hand, if the defect is dominantly inherited, DNA analysis of other affected and unaffected family members will allow further reductions to be made. Alternatively, when two or more families share very similar phenotypes, mutations in the same or related genes might be expected *(Daly ME et al. 2014)*. The possibility that an IPD is due to a *de novo* mutation should also be considered in those sporadic cases where there is no family history of a bleeding tendency. Likewise, the possible contribution of mosaicism to differences in expression of a bleeding tendency between affected individuals of the same family should not be overlooked *(Gresele P et al. 2013)*.

There are various recent examples of NGS successfully identifying new genes and mutations of platelet disorders, such as the identification of the *NBEAL2* as the causative gene for GPS, identified by three groups at the same time *(Albers CA et al. 2011, Gunay-Aygun M et al. 2011, Kahr WH et al. 2011)*. In this Thesis we used NGS and whole exome strategy in one patient with severe macrothrombocytopeia of unknown cause. In one of them this approach permitted the identification of a mutation in the *MYH9* gen and a diagnosis of MYH9-RD in this girl that was not previously suspected.

Despite the advantages of using NGS to study the unknown causative mutation or gene of specific IPDs, NGS has several limitations that can hamper the final outcome. First, although it is rapidly becoming less time-consuming and cheaper, it is still too expensive for clinical laboratories. Moreover, the high number of sequence variations in any human exome (about 25000) challenges to distinguish the causative mutation or mutations that contribute to the

platelet dysfunctional phenotype. Analysis of the huge amount of information of whole exome provided by NGS is time-consuming and require special bioinformatics equipment and knowledge *(Daly ME et al. 2014)*.

A more simple application of NGS to IPDs, and bleeding disorders in general, is to select a panel of specific genes or genomic regions, previously known to be involved in these diseases. This targeted NGS is more affordable, produces much higher coverage of genomic regions of interest, and reduces sequencing cost and time *(Xuan J et al. 2013)*. Examples of the recent use of this approach are the ThromboGenomics project (https://haemgen.haem.cam.ac.uk/thrombogenomics) included in the biggest Bridge Project (https://bridgestudy.medschl.cam.ac.uk/index.shtml), and the GAPP project (http://www.birmingham.ac.uk/research/activity/mds/domains/cardio-resp-neuro/vita/platelet-gapp/index.aspx). With this strategy, the causative alterations in the *RBM8A* gene that lead to TAR phenotype were identified by participants of the ThromboGenomics project *(Albers CA et al. 2012),* investigators of the GAPP project discovered a novel a *HPS4* mutation *(Jones ML et al. 2012)*, and mutations in *FLI1* and *RUNX1* with defects in platelet granule secretion and excessive bleeding *(Stockley J et al. 2013)*.

Recently, our group has just joined a project using targeted NGS of a limited number of genes to identify molecular alterations in patients with bleeding disorders *(Bastida JM et al. 2014: "Diseño y validación de un panel de secuenciación masiva dirigida al diagnóstico de las diátesis hemorrágicas hereditarias"; abstract presented in the national congress of the Spanish society of Thrombosis and Hemostasis as a plenary session)*

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Objectives

As summarized in the Introduction section, platelet physiology is rather complex and inherited platelet disorders comprise a wide and heterogeneous group of diseases in terms of clinical symptoms and platelet defects. Indeed, early and accurate diagnosis of the patients with congenital platelet disorders would benefit their clinical management and quality of life, and could help to develop new treatment strategies for these diseases. However, full characterization of inherited platelet disorders is hampered by the variability and low specificity of common platelet function tests, the complexity and yet limited accessibility of molecular diagnosis, and, in Spain, by the lack of highly specialized reference centres for the study of these diseases.

Accordingly, the objectives of this Thesis have been:

1. To facilitate access to the specialized functional and molecular diagnosis of inherited platelet disorders, for the medical community of the Iberian Peninsula, by promoting and initiating a collaborative project for the recruitment and study of patients with a previous diagnosis or clinical suspicion of either inherited thrombocytopenias or congenital disorders of platelet function.
2. To perform a quantitative re-evaluation of bleeding diathesis in the recruited patients by means of a common bleeding scale.
3. To complete a detailed and standardized analysis of platelet function in the samples from recruited patients, aiming to confirm or discard a functional diagnosis.
4. To achieve a molecular diagnosis in the individuals with a confirmed laboratory diagnosis.
5. To establish potential relationships between clinical and/or laboratory phenotype and genotype, in different types of inherited platelet disorders.

Chapter I

Functional and Molecular Characterization of Inherited Platelet Disorders in the Iberian Peninsula: Results from a Collaborative Study

1. Introduction

Inherited platelet disorders (IPDs) constitute a group of relatively uncommon isolated diseases usually characterized by a lifelong mild to moderate bleeding diathesis, with a true incidence still unknown. Although individual IPDs are rare and a population based study on the overall prevalence of these disorders has never been undertaken, in aggregate they may be as prevalent as von Willebrand disease (vWD), the most common inherited bleeding disorder *(Quiroga T et al. 2007, Quiroga T et al. 2009, Lambert MP 2011)*.

A few classic IPDs such as Bernard–Soulier Syndrome (BSS) and Glanzmann Thrombasthenia (GT) have been molecularly characterized for over 20 years, while others such as inherited disorders of signaling pathways downstream from membrane receptors are still in many cases difficult to diagnose since the molecular and genetic mechanisms are in the vast majority of patients unknown *(Rao AK 2013)*. Following the suspicion of an IPD, the diagnosis should be attained by a combination of an appropriate personal and family history, a physical examination, standard laboratory studies, including an assortment of platelet morphologic and functional studies, and ideally, confirmatory, targeted-gene analyses *(Balduini CL et al. 2003, Harrison P et al. 2011, Dawood BB et al. 2012, Glembotsky AC et al. 2012)*. However, the identification of many platelet disorders has been hampered by the lack of a common international consent for a diagnostic algorithm, and also by the limited access to specific platelets studies and to molecular tests which are usually performed only by a relatively small number of highly specialized laboratories *(Glembotsky AC et al. 2012)*. Additionally, it´s been long established that platelet functional studies are to be performed within a limited time after blood collection, although the feasibility of performing those tests at a more extended time lapse when evaluating severe platelet defects has rarely been addressed *(Dovlatova N et al.*

2014). Consequently, diagnosed cases are frequently clustered around such centers, while elsewhere >50% of patients who are likely to be affected remains partially characterized and without a molecular diagnosis *(Pereira J et al. 2008, Lambert MP et al. 2013)*. As information develops from the study of IPDs, more patients will be identified, expanding our understanding about normal platelet biochemistry and physiology, and our knowledge of the functional network and critical domains of specific platelet elements. These investigations will provide patients with clearer clinical diagnoses, and will permit to identify affected family members, as well as benefit patients from genetic counseling. Moreover, these studies are essential to distinguish these diseases from acquired platelet disorders to avoid unnecessary and potentially harmful treatments, and to educate patients on when to seek for specific therapies and about the effect of lifestyle on hemostasis *(Drachman JG 2004, Daly ME et al. 2014)*. Although sporadic IPDs from the Iberian Peninsula have been described, until recently, there was no reference laboratory to assist physicians on the diagnosis of patients with suspected IPDs. Therefore, to respond to such a challenge, and with the aims to (1) evaluate the accuracy of previous diagnoses of IPD by non-specialized diagnostic laboratories, (2) analyze the feasibility of performing reliable platelet functional studies within the 24 hours from phlebotomy, under specific conditions, (3) and to confirm a diagnosis of IPD at a molecular level we undertook this study. Presented information includes data from the "Functional and Molecular Characterization of Patients with Inherited Platelet Disorders" a collaborative project that was undertaken under the scientific sponsorship of the Spanish Society of Thrombosis and Hemostasis. The main part of this manuscript focuses on describing our findings from patients diagnosed with two severe autosomal recessive IPDs affecting the platelet adhesion and aggregation, BSS and GT respectively *(Andrews RK et al. 2013, Nurden AT et al. 2013)*.

2. *Materials & Methods*

2.1. Patient selection and blood drawing

In the course of the last five years, samples from 70 unrelated patients with a clinical suspicion of IPD from different Spanish and Portuguese hospitals have been analysed for this study. Venous blood was drawn from each patient and a parallel healthy control that had not taken antiplatelet medication for at least 2 weeks, and samples from both individuals were delivered within 18–24 hours by express courier at room temperature to Murcia, Spain, where all further studies were done. Moreover, fresh blood was taken from a healthy volunteer from our facility each day that functional studies were performed, and served as an additional control. Blood samples were collected into commercial 7.5% K3 EDTA tubes (for complete blood count [CBC], and DNA & RNA isolation) and into buffered 3.2% sodium citrate (for platelet functional studies) using a 20-gauge needle. Immediately after blood collection, all tubes were mixed by gentle inversion at least 6 times (and discarded if there was any evidence of clotting), and samples were maintained at room temperature (20-25°C) during shipping.

All individuals gave informed consent; the sampling from the children was carried out with the permission of the parents. Controls, patients, and relatives were fully informed of the aim of this study, which was performed according to the declaration of Helsinki, as amended in Edinburgh in 2000. This study obtained approval from the Reina Sofia Hospital Ethics Committee.

The bleeding tendency of patients and their relatives was recorded by the medical doctors using a bleeding score (BS) questionnaire that recorded the most frequent and typical symptoms from 0 to 3 *(Rodeghiero F et al. 2005, de Moerloose P et al. 2009)*. The recorded data

were then processed providing a numerical assessment of bleeding severity. Before any additional testing, it was mandatory to have results from the following tests: basic biochemical tests including renal and liver function, prothrombin time, activated partial thromboplastin time, CBC, mean platelet volume (MPV), and morphological examination of a blood smear. In patients not exhibiting thrombocytopenia, additionally Clauss fibrinogen, factor VIII: C activity, von Willebrand factor (vWF) antigen, and ristocetin cofactor activity assay were studied. Patients were excluded from further laboratory testing after the diagnoses of acquired defects or of vWD. Subject diagnoses were based on the opinion of the referring physician, and the BS questionnaire and medical records were reviewed by two physicians from our facility who also reviewed laboratory data. Definite bleeding disorders were compared to the clinical suspicion by the referring physician, and sub-classified as previously being accurately diagnosed or misdiagnosed/unconfirmed based on platelet functional studies as well as on molecular findings. Initial evaluation also included platelet aggregation, glycoprotein expression, and analysis of activation markers by flow cytometry, and according to initial suspicion, specialized tests were performed, including clot retraction, and molecular studies.

2.2. Platelet functional studies

Platelet rich plasma (PRP) was prepared by centrifugation of citrated whole blood at 150 x g for 10 min and the top two-thirds removed by careful pipetting into a separate tube. Platelet poor plasma (PPP) was prepared by a further centrifugation step of the same tube at 1000 x g for 20 min. PRPs from citrated blood samples that appeared macrothrombocytopenic on blood smears were prepared by means of tilting the tube to an angle of 45° for 1-2h *(Bolton-Maggs PH et al. 2006)*.

Light transmission aggregometry (LTA) was performed as described *(Tello-Montoliu A et al. 2012)* using undiluted PRP when the platelet concentration of patient´s and control´s PRP samples were in the range of 200 and 600 × 10^9L^{-1} *(Cattaneo M et al. 2007)*, and diluting the control´s sample with autologous PPP, when the patient´s PRP count was below 150 x 10^9L^{-1}, to have similar optical densities (±10%). Time course changes in the maximal percentage of light transmission of PRP over baseline (PPP) were recorded for 300s using an Aggrecorder II aggregometer (Menarini Diagnostics, Florence, Italy). Agonists used for LTA included: 2 mg mL^{-1} collagen, 10 μM ADP; 1.6 mM arachidonic acid, 1.25 mg mL^{-1} ristocetin, and 25 μM Thrombin Receptor Activating Peptide (TRAP). In patients with clinical suspicion of GT, responses to 10 mM dithiothreitol (DTT) and 100 nM phorbol myristate acetate (PMA) were also analysed; in those with suspicion of BSS, aggregation responses to ristocetin and TRAP were only assessed. Some subjects were not tested with all agonists. LTA samples were preincubated (37º C, 3 min), and all tracings were inspected by the same person (JR), who provided an overall interpretative comment. Platelet aggregation was considered abnormal if it was reversible, or if the maximum amplitude was <50% of the paired parallel control drawn the same day and shipped under similar conditions than samples from each patient. If the samples from such paired control from the referring centre exhibited considerable weaker responses to 25 μM TRAP than those of the fresh control (<30%), the tubes were discarded for functional analysis, and further samples were requested for analysis.

For flow cytometry, citrated whole blood diluted 1:10 in saline buffer was assessed for expression of major platelet membrane glycoproteins (GP) that support platelet adhesion and aggregation (GPIa, GPIbα, GPIIb, GPIIIa,) through a direct standard flow cytometry technique with appropriate labelled monoclonal antibodies in a FACScalibur platform (Becton Dickinson, San Jose, CA). PRP was used to study platelet activation markers (P-Selectin, granulophysin

[stimulated by 25 μM TRAP]), and activated $\alpha_{IIb}\beta_3$ complex (unstimulated and activated by 100 nM PMA or 25 μM ADP). Monoclonal antibodies (Becton Dickinson) used were FITC*CD49b (GPIa), FITC*CD42b (GPIbα), PE*CD41a (GPIIb), FITC*CD61 (GPIIIa), FITC*PAC-1 (activation-specific anti-GPIIb/IIIa antibody), PE*CD62 (P-selectin), and PE*CD63 (granulophysin). For each sample run, data acquisition of 10,000 events was gated on forward and side-angle light scatter with gains adjusted to include the platelet population. Then, the fluorescence of stained platelets was analysed (CellQuest software, Becton Dickinson) to obtain both the percentage of positively stained cells and the mean fluorescence intensity (MFI).

For patients with the clinical suspicion of GT, we additionally tested the *in vitro* haemostatic response through the "clot retraction assay". PRP samples were diluted in homologous PPP to final platelet counts of 300 x $10^9 L^{-1}$ and incubated in glass tubes for 5 minutes at 37°C. Clot formation was induced by calcium chloride (8 mM L^{-1} final concentration), and samples were photographed and weighed after 1 hour of incubation at 37°C *(Ward CM et al. 2000)*.

Samples from patients showing reduced/absent GPIIb/IIIa or GPIb/IX expression underwent Western blot analysis of platelet glycoproteins, by standard procedures using specific antibodies, GPIIb (132.1, Blood Research Institute, Milwaukee, WI), GPIIIa (AP3, Blood Research Institute, Milwaukee, WI), and GP Ibα (LJ-Ib10, La Jolla, CA).

2.3. Molecular characterization

DNA and RNA sere isolated from each patient by standard procedures, and stored frozen until use. Molecular analysis of candidate genes was done in patients with a laboratory diagnosis of GT or BSS (see below), and also in patients with clinical and laboratory suspicion of MYH9 related disorder, Chediak-Higashi Syndrome (CHS), Hermansky-Pudlak Syndrome (HPS), Platelet von Willebrand disease (PvWD), congenital amegakaryocytic thrombocytopenia (CAMT), and thrombocytopenia 2 (THC2).

To detect mutations in *ITGA2B* (ENSG00000005961) or *ITGB3* (ENSG00000259207), total RNA from peripheral blood was used for cDNA synthesis using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen, Paisley, UK), following the manufacturer’s protocol. Using specific oligonucleotides and conditions (primer sequences and conditions are available upon request) we amplified and sequenced *ITGA2B* and *ITGB3* by Sanger’s approach. Every mutation found in cDNA was confirmed on DNA samples.

The *GP1BA* (ENSG00000185245), *GP1BB* (ENSG00000203618) and *GP9* (ENSG00000169704) genes were screened for mutations using genomic DNA. Genomic fragments were analysed by polymerase chain reaction (PCR) using primers designed specifically to amplify the coding regions (primer sequences and conditions are also available upon request). PCR products were sequenced in the ABI 3130xl genetic analyser (Applied Biosystems, Carlsbad, CA).

Patients with the clinical suspicion of other IPDs were studied and screened for mutations in the specific candidate gene. Thus, all exons and intron-boundaries of *HPS1* (ENSG00000107521) were sequenced by Sanger’s method in two patients with the clinical suspicion of HPS; in one of these patients *HPS4* (ENSG00000100099) was additionally

sequenced. RNA from patients with the suspicion of CHS and MYH9 related disorders was extracted and their cDNA was amplified and sequenced seeking mutations in *LYST/CHS1* (ENSG00000143669) and *MYH9* (ENSG00000100345), respectively. When the clinical suspicion was PvWD, CAMT, or THC2, the molecular approach contemplated the direct analysis of the most affected DNA regions in these disorders: exon 2 of *GP1BA* in PvWD *(Salles, II et al. 2008)*, the first 3 exons of *MPL* (ENSG00000117400) gene in CAMT *(Germeshausen M et al. 2006)*, and the 5'UTR region of *ANKRD26* (ENSG00000107890) in THC2 *(Pippucci T et al. 2011)*. In one patient with clinical suspicion of BSS not confirmed by functional and molecular studies, exome sequencing was performed (Agilent SureSelect Capture v4 + UTRs and Genome Analyzer IIx platform, Agilent Technologies, Santa, Clara, CA). Using RUbioSeq software *(Rubio-Camarillo M et al. 2013)* sequencing data were aligned to the human reference genome (GRCh37) and exome variant analysis and functional prediction were performed with the default parameters for somatic variation analysis.

3. Results

Of the 70 patients enrolled in this study, molecular characterization of GT was achieved in 12 patients, while the causative mutation/s was recognized in 8 cases of BSS, in 2 with CHS, 1 with HPS, 3 with MYH9 related disease, 1 with CAMT, and 1 with THC2 (9 men and 19 women with a median age of 33 years). Overall, 12 novel mutations have been identified in this study. Our functional and molecular approach did not confirm a definitive diagnosis of IPD in approximately one-third of patients that had been referred with such a clinical diagnosis. Nineteen patients with defects of platelet functionality were categorized as having signaling and/or secretion disorders (Table 1).

Table 1. Molecular and functional characterization of patients with IPDs.

Diagnostic suspicion at referral	n	Cases Confirmed	Cases Misdiagnosed	Molecular Diagnosis
Signaling and/or secretion disorders	25	19 signaling and/or secretion disorders (76%)	6 (24%)	0
Glanzmann Thrombasthenia	20	13 (65%)	7 (35%)	12
Bernard-Soulier Syndrome	13	8 (61.5%)	5 (38.5%)	8
Chediak-Higashi Syndrome	2	2 (100%)	0	2
Hemansky-Pudlak Syndrome	2	1 (50%)	1 (50%)	1
MYH9 disorders	4	2 (50%)	2 (50%)	3*
Platelet von Willebrand Disease	2	0	2 (100%)	
Congenital Amegakaryocytic Thrombocytopenia	1	1	0	1
Thrombocytopenia 2	1	1	0	1
All IPDs Referred	**70**	**47 (67.1%)**	**23 (32.9%)**	**28 (40%)**

Abbreviation: IPDs, inherited platelet disorders

*One of the patients referred with a clinical suspicion of BSS was diagnosed as having MYH9-related disorder

3.1. Glanzmann Thrombasthenia

Thirteen patients were diagnosed with GT (4 men and 9 women, with a median age of 31 years) (Table 2). The disorder was readily identifiable in the presence of a normal platelet number (109-280 x 10^9 platelet L^{-1}) and size, and by platelet function testing, consisting in severely impaired aggregation in response to all physiological agonists, and also to DTT and PMA (Table 3). Ristocetin-induced platelet agglutination (RIPA) was normal or reduced to some extent (74 ± 24%), and GPIbα and GPIX expression was normal in all patients, while clot retraction was consistently abnormal. On flow cytometry 8 patients (GT-2, GT-4, GT-5, GT-8, GT-9, GT-10, GT-12 and GT-13) were diagnosed as type I GT (<5% of GPIIb/IIIa expression), 4 (GT-1, GT-3, GT-6 and GT-7) as type II GT (GPIIb/IIIa expression between 5% and 20%), while 1

patient (GT-11) was classified as variant GT (50% expression but receptor being not functional according to LTA and clot retraction). All patients exhibited no binding of PAC-1 activation-specific anti-GPIIb/IIIa antibody upon stimulation with ADP, PMA, or TRAP. In platelet lysates from type II patients, faint but detectable $\alpha_{IIb}\beta_3$ bands of the expected apparent molecular weight were seen, while platelets from the patient with variant GT showed reduced (50% of normal) levels of these proteins (data not shown).

Patients presented with a variable bleeding diathesis as measured by the BS. Considering grades 2 and 3 as severe bleeding, most of the patients had gross blood loss due to epitasis (54%), cutaneous bleeding (54%) and menorrhagia (100% of women), while other common symptoms (oral cavity, gastrointestinal, following dental extraction or mild injuries, postpartum, or hematomas) were in general milder (mean BS of 0.9).

Table 2. Molecular diagnosis of patients with Glanzmann Thrombasthenia.

Patients		Gene	Inheritance	Mutation	Reference
9F, 4M, median age of 31 yrs; mean BS: 10.23					
GT-1	GT II	*ITGB3*	Compound Heterozygous	p.Met124Val	*(Gonzalez-Manchon C et al. 2004)*
				c.774-775delTG	new
GT-2	GT I	*ITGA2B*	Compound Heterozygous	p.Leu183Pro	*(Grimaldi CM et al. 1998)*
				c.2473_2481delinsTCACCTG GTC	new
GT-3	GT II	*ITGA2B*	Homozygous	p.Cys674Arg	*(Gonzalez-Manchon C et al. 1999)*
GT-4	GT I	*ITGB3*	Homozygous	p.Tyr190Cys	new
GT-5	GT I	*ITGA2B*	Compound Heterozygous	p.Glu507stop	new
				c. 2637delC	new
GT-6	GT II	*ITGA2B*	Compound Heterozygous	p.Val951Met	*(Nurden AT et al. 2004)*
				p.Ala958Thr	*(Nurden AT et al. 2004)*
				p.Glu507stop	new
GT-7	GT II	*ITGB3*	Homozygous	p.Met118Arg	*(Jallu V et al. 2010)*
GT-8	GT I	*ITGA2B*	Homozygous	c.2965delG	new
GT-9	GT I	*ITGA2B*	Homozygous	c.692insT	*(D'Andrea G et al. 2002)*
GT-10	GT I	*ITGA2B*	Homozygous	c.1599delAT	new
GT-11	GT Variant	*ITGB3*	Compound Heterozygous	p.Tyr190Cys	new
				p.Leu196Pro	*(Nurden AT et al. 2002)*
GT-12	GT I	*ITGB3*	Homozygous	-	-
GT-13	GT I	*ITGA2B*	Homozygous	p.Trp51Stop	new

Abbreviation: GT, Glanzmann Thrombasthenia.

Table 3. Maximal aggregation (%) of patients diagnosed with Glanzmann Thrombastenia, Bernard Soulier Syndrome, parallel controls , and controls drawn at the time of analysis

	Platelet Count ($x10^9L^{-1}$)	TRAP (25 μM)	ADP (10 μM)	Ristocetin (1. 25 mg mL^{-1})	Araquidonic acid (1.6 mM)	Collagen (2 μg mL^{-1})	DTT (10 mM)	PMA (100 nM)
GT (n=13)	171.7±42.5	18.3±4.5	3.0±5.2	74.1±24.7	9.8±5.0	8.2±9.6	7.9±12.8	8.8±4.4
BSS (n=8)	*	49.4 ± 21.1	-	4.4 ± 2.5	-	-	-	-
Parallel controls (n=21)	226.3±48.4	81.1±14.8	70.8±18.9	75.2± 21.5	71.8±28.7	72.6±20.7	47.5±10.4	42.8 ± 30.2
Fresh controls (n=21)	209.8±29.7	91.2±9.5	85.6±9.7	91.3±9.7	89.4±8.2	88.7±9.2	56.1±10.2	54.7±25.7

All values are mean ± Standard deviation. *always <$20x10^9$ platelet L^{-1}

Abbreviations: BSS, Bernard Soulier Syndrome; GT, Glanzmann Thrombasthenia; TRAP: thrombin receptor activating peptide; ADP: Adenosine diphosphate; DTT: dithiothreitol; PMA: phorbol myristate acetate.

In order to determine the causative mutations, *ITGB3* and *ITG2B* genes were analyzed, allowing us to identify mutations in all patients but one (Table 2). Overall, 11 mutations were found in *ITGA2B* and 5 in *ITGB3*. Of the 16 alterations, 8 were novel mutations, including 2 nonsense mutations (leading to premature stop codons for amino acids 51 and 507 in α_{IIb}), 3 frameshift changes in *ITGA2B* (c.2637delC, c.2965delG, and c.2473_2481delinsTCACCTGGTC) and one in *ITGB3* (c.774-775delTG), one mutation affecting a splice site c.1599delAT in *ITGA2B*, and one missense p.Tyr190Cys in *ITGB3*; the other 8 mutations were already described in the literature *(Grimaldi CM et al. 1998, Gonzalez-Manchon C et al. 1999, D'Andrea G et al. 2002, Nurden AT et al. 2002, Gonzalez-Manchon C et al. 2004, Nurden AT et al. 2004, Jallu V et al. 2010)*. As GT is an autosomal recessive disease, patients are mostly compound heterozygotes for *ITGA2B* or *ITGB3* mutations in the absence of consanguinity. Homozygous mutations were found in three patients (GT-3, GT-9, and GT-11) from non-consanguineous families -to the best of our knowledge-, and in all patients with consanguineous family history. Despite complete

sequencing, no potential pathological mutations were located in either gene of patient GT-12, who exhibited a family history of consanguineous union, and presented no detectable levels of *ITGB3* mRNA by qRT-PCR (data not shown).

Segregation analysis confirmed the presence of heterozygous mutations in all available carriers (parents) except of patient GT-6. Interestingly, patient GT-6 exhibited a triple heterozygosity in the integrin α_{IIb} subunit, as a result of a nonsense mutation (p.Glu507Stop) of paternal origin, and two *de novo* missense changes, p.Val951Met, and p.Ala958Thr, that had been previously described in another patient *(Nurden AT et al. 2004)*. Noteworthy, the already reported missense mutations- found together with a splice site mutation in the α_{IIb} gene inherited from the mother- were paternal in origin and cosegregated in the same allele across three generations *(Nurden AT et al. 2004)*. In our patient, however, throughout investigation of other family members (father, mother, brother) did not detect the two missense mutations in any of the individuals, and DNA profiles generated from blood from the patient and parents revealed double paternal/maternal contribution at different loci in the propositus, confirming the maternal and parental relationship.

To define the structural impact of the new missense mutation p.Tyr190Cys, identified in two of our patients (patients GT-4 and GT-11), the role of the amino acid substitutions in β_3 was analyzed *in silico*. The three dimensional diagram of the Cys190 suggested a disruption of the hydrogen bound created in between Tyr190 and Tyr115. This change would also generate new hydrogen bound with Gly189, predicting a destabilizing effect on the fold (data not shown).

3.1. Bernard Soulier syndrome

This syndrome was diagnosed in eight individuals (2 men and 6 women with a median

age of 41 years) (Table 4). In these patients, platelet count was consistently less than 20 x $10^9 L^{-1}$ by commercial cell counters, with persistent findings of giant platelets in the peripheral blood smear. Patients had slightly diminished aggregation responses to TRAP compared to their parallel controls, but RIPA was absent or severely defective in all patients (Table 3). Flow cytometry revealed that the GPIbα and GPIX expression was severely reduced (<10% compared to controls). MFI of other platelet GPs were normal or increased due to the increased size of the cells. Further studies using Western-blot analysis revealed an absent or severely reduced GPIbα band in all patients (data not shown).

More than half of the patients (patients BSS-2, BSS-5, BSS-6, BSS-7 and BSS-8) had been splenectomized without having any benefit, as they had been previously misdiagnosed with primary immune thrombocytopenia (ITP). The severity grade, measured by BS for epistaxis and menorrhagia was 2 & 3 in 100% of patients. Other less prevalent locations of moderate-severe blood loss (BS grades 2 & 3) were cutaneous bleeding (83% of patients), and following procedures of dental extraction or surgery (50% and 83% of patients, respectively). Mild bleeding symptoms were reported at other sites (bleeding from minor wounds, oral cavity, gastrointestinal, postpartum, or hematomas) (mean BS of 1.03). The overall BS in BSS patients was 12.83.

Disease-causing homozygous mutations were found in 7 out of 8 patients -five lacking a history of known family consanguinity-, while in the remaining patient a mutation was identified in only one allele of *GP1BA* (Table 4). Six mutations were detected (3 in *GP9*, 2 in *GP1BA*, and 1 in *GP1BB*); half of them had been already described in the literature *(Wright SD et al. 1993, Simsek S et al. 1994, Noris P et al. 1997, Liang HP et al. 2005)* and the other 3 were new (one in *GP1BA*, one in *GP1BB*, and one in *GP9*). Of these novel mutations, 2 of them were

missense, Trp71Arg in exon 3 of *GP9*, and Pro64Ser in exon 2 of *GP1BB*. Of note, the p.Gln90_Leu92del mutation, which leads to the loss of 3 amino acids in one of the leucine rich-repeat sequences of GPIbα, was found in heterozygosis in one patient. We did not consider this patient as having the milder monoallelic autosomal dominant form of BSS *(Miller JL et al. 1992, Savoia A et al. 2001, Kunishima S et al. 2006, Vettore S et al. 2008)*, due to the absent GPIbα and GPIX expression by flow cytometry, and the low platelet count (<20 x $10^9 L^{-1}$). Additionally, this variant was also identified in the patient's mother, with no BSS phenotype at all. Hence, there might be other unknown genetic defects or different molecular mechanisms, such as transcription factors or postraductional defects *(Savoia A et al. 2011)* that could lead to BSS phenotype in this patient. It is worth mentioning that the variant p.Phe55Ser, previously described in a patient from the Netherlands *(Noris P et al. 1997)* was identified in 3 unrelated patients from the central region of Portugal, suggesting that this mutation could have an ancient origin in that geographical area.

To predict the effects of the 2 novel missense mutations, we performed *in silico* analysis of the new variants. The analysis of the variant p.Trp71Arg in GPIX reflected a change in the isoelectric point that could affect the interaction with GPIbβ, potentially influencing the correct folding and expression of the receptor on the platelet membrane. As for the p.Pro64Ser in GPIbβ, *in silico* studies did not show any relevant alteration of the protein structure but mutation analysis using different bioinformatics tools (Poly-Phen2, Mutation Taster) characterized this change as a damaging variant for the protein and a disease causing mutation (data not shown).

Table 4. Molecular diagnosis of Bernard Soulier syndrome patients.

Patients With Molecular Diagnosis	Gene	Inheritance	Mutation	Reference
6F, 2M, median age of 41 yrs; mean BS: 12.83				
BSS-1	*GP9*	Homozygous	p.Trp71Arg	new
BSS-2	*GP1BA*	Homozygous	p.Cys209Ser	*(Simsek S et al. 1994)*
BSS-3	*GP1BA*	Compound Heterozygous	p.Gln90_Leu92del	new
			-	-
BSS-4	*GP9*	Homozygous	p.Asn45Ser	*(Wright SD et al. 1993, Liang HP et al. 2005)*
BSS-5	*GP9*	Homozygous	p.Phe55Ser	*(Noris P et al. 1997)*
BSS-6	*GP9*	Homozygous	p.Phe55Ser	*(Noris P et al. 1997)*
BSS-7	*GP9*	Homozygous	p.Phe55Ser	*(Noris P et al. 1997)*
BSS-8	*GP1BB*	Homozygous	p.Pro90Ser	new

Abbreviation: BS, bleeding score. BSS, Bernard Soulier Syndrome.

3.2. Other IPDs

The molecular characterization of patients with the clinical suspicion of other IPDs resulted in the identification of 8 molecular alterations, 3 recently reported by us *(Sanchez-Guiu I et al. 2014, Sanchez-Guiu I et al. 2014)*. We did not confirm any of the 2 PvWD suspicions. Two homozygous mutations in *LYST* corroborated the diagnosis of CHS in 2 patients: p.Cys258Arg and p.Gly3725Arg *(Sanchez-Guiu I et al. 2014*a*)*, while HPS was confirmed in one patient (p.Glu204Stop) *(Sanchez-Guiu I et al. 2014*b*)*. Three monoallelic mutations, already described in the literature *(Seri M et al. 2000, Saposnik B et al. 2014)*, were detected in MYH9 patients: 2 patients had been referred to our center with the clinical suspicion of MYH9 (p.Arg1165Cys, p.Glu1841Lys), while the remaining individual had been clinically misdiagnosed as having BSS. In this patient with macrothrombocytopenia and no evident neutrophil inclusions on conventional May-Grünwald-Giemsa-stained blood, whole exome sequencing detected the p.Asp1424Glu variant in *MYH9 (Saposnik B et al. 2014)*. Molecular diagnosis of CAMT was attained in one patient by the identification of the homozygous p.Arg102Cys *(Germeshausen M et al. 2006)* mutation in *MPL*, while that of THC2 was achieved in an additional patient as the

monoallelic c.-128G>A variant *(Pippucci T et al. 2011)* in the 5'UTR region of the *ANKRD26* gene was detected. (Table 5). Patients presenting with non-syndromic normal or mildly reduced platelet counts, decreased aggregation responses to collagen, arachidonic acid, and/or TRAP, and impaired externalization of platelet activation markers in response to TRAP were lumped in the remarkably heterogeneous group of platelet secretion and signal transduction defects, and no further analysis were performed.

Table 5. Molecular diagnosis of other inherited platelet disorders.

Patients With Molecular Diagnosis	Gene	Inheritance	Mutation	Reference
Chediak-Higashi Syndrome (CHS) 1M, 1F, median age of 12 yrs				
CHS-1	*LYST/CHS1*	Homozygous	p.Gly3725Arg	*(Sanchez-Guiu I et al. 2014)*
CHS-2	*LYST/CHS1*	Homozygous	p.Cys258Arg	*(Sanchez-Guiu I et al. 2014)*
Hemansky-Pudlak Syndrome (HPS) 1 Male, 28 yrs				
HPS-1	*HPS1*	Homozygous	p.Glu204Stop	*(Sanchez-Guiu I et al. 2014)*
MYH9 disorders (MYH9) 2F, 1M, median age of 44 yrs				
MYH9-1	*MYH9*	Heterozygous	p.Arg1165Cys	*(Seri M et al. 2000)*
MYH9-2	*MYH9*	Heterozygous	p.Glu1841Lys	*(Seri M et al. 2000)*
MYH9-3	*MYH9*	Heterozygous	p.Asp1424Glu	*(Saposnik B et al. 2014)*
Congenital Amegakaryocytic Thrombocytopenia (CAMT) 1 Male, 20 yrs				
CAMT-1	*MPL*	Homozygous	p.Arg102Cys	*(Germeshausen M et al. 2006)*
Thrombocytopenia 2 (THC2): 1 Female, 33 yrs				
THC2-1	*ANKRD26*	Heterozygous	c.128G>A	*(Pippucci T et al. 2011)*

4. Discussion

In this study, we evaluated the largest cohort of BSS and GT patients in the Iberian Peninsula, following the aim of the project “Functional and Molecular Characterization of Patients with Inherited Platelet Disorders”. By reviewing the medical records, BS questionnaire, performing functional studies, and when possible, identifying the underlying genetic abnormality, amongst the 70 patients referred to us for a diagnostic confirmation of IPD, we concluded that 32.9% had been previously misdiagnosed, while 40% achieved a diagnosis at a molecular genetic level (Table 1).

IPDs are one of the most complex, poorly standardized and time consuming disorders to diagnose. A recent worldwide survey *(Gresele P et al. 2014)* confirms results from previous ones *(Jennings I et al. 2008, Cattaneo M et al. 2009)* showing large variations between laboratories in platelet function testing practice, and clearly demonstrates that the required diagnostic technologies are not always readily available to many facilities, and referral to specialized centers may be necessary for definitive diagnosis *(Nurden P et al. 2007)*. Even though sending samples to a distant laboratory might pose the problem of missing mild platelet defects, due to the time lag between blood extractions and testing, differences in platelet functional responses to strong agonists between patients and paired controls allowed us not only to functionally identify GT and BSS patients, but also to characterize individuals within the heterogeneous category of defects in platelet secretion and signal transduction.

In that sense, we restricted the study to patients with significant bleeding history. The selection of candidate patients justifies the differences in the prevalence of confirmed IPD between our study and the previous recent worldwide survey *(Gresele P et al. 2014)*, with a higher percentage of patients with GT (28.8% vs. 9.8%, respectively), with BSS (17.7% vs. 4.2%,

respectively), and the percentage of patients that achieve a diagnosis at a molecular level (40% vs. 8.7, respectively). A reflection of the need to improve the recognition of IPDs and the absence of referral centers is that 93% of the patients investigated at our institution were adults, and that even in GT and BSS, defects in which the diagnosis is rather straightforward, even for non-specialized laboratories, the percentage of patients that had been misdiagnosed was high. The long delay in reaching a definite diagnosis was not without consequences, since 8 subjects underwent undue splenectomy before our observation (2 with MYH9-related disease, 5 with BSS, 1 with CAMT).

The analysis of the 13 GT patients showed a consistent and homogeneous functional profile and bleeding complications leading to significant morbidity. Most patients had a type I phenotype, in accordance with previous reports *(George JN et al. 1990, Kannan M et al. 2009)*. The molecular characterization of patients revealed 16 mutations (half of them not reported previously) along both genes encoding for the $\alpha_{IIb}\beta_3$ receptor: 11 mutations were located in the *ITGA2B* gene within 8 patients (3 of them were compound heterozygous) and 5 in the *ITGB3* in 4 more patients. According to a regularly updated database (http://sinaicentral.mssm.edu/intranet/research/glanzmann), and to what has been previously stated *(Nurden AT et al. 2013)*, we observed more mutations affecting *ITGA2B* possibly because, although a smaller gene than *ITGB3*, it has 30 exons (and double number of splice sites) compared to 15 in *ITGB3*. In patient GT-12, who has unequivocally been diagnosed by functional studies as GT, no mutations were detected. It is known that no single screening technique warrants detection of all causative mutations; previous reports have identified mutations in 80% of GT patients, by using different techniques, such as denaturing gradient gel electrophoresis *(Vinciguerra C et al. 2001)*, or by conformation sensitive gel electrophoresis *(Kannan M et al. 2009)*. This suggests that defects in regulatory elements that adversely affect

the transcription of the *ITGA2B* and *ITGB3* genes or abnormalities in mechanisms that are responsible for post-translational modifications and trafficking of integrin subunits may also potentially account for some cases of GT.

The detected mutations were analyzed for penetrance into the individual family members; heterozygous mutations were seen in all of the available samples from the parents of GT, except in one case. Of note, patient GT-6 exhibited two *de novo* missense variants in the α_{IIb} subunit, together with a nonsense mutation of parental origin. Such missense mutations have been previously described in a patient also with triple heterozygosity in the α_{IIb} gene *(Nurden AT et al. 2004)*, indicating that both missense mutations, 8 aminoacids from each other, occur non-randomly but that variants are tightly linked. At this moment, the causative molecule or defect in the repair mechanism responsible for these *de novo* mutations are not known. While one of the missense variants (p.Ala958Thr) was considered to be a polymorphism in the previous report *(Nurden AT et al. 2004)*, using exome databases (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/)), this change was identified as a mutation (allele frequency < 0.01). Further studies are needed to clarify the linkage disequilibrium between both mutations in exon 29 of *ITGA2B*.

BSS was diagnosed in 8 individuals presenting with macrothrombocytopenia, decreased/absent levels of GPIbα and GPIX, and a bleeding tendency with variable severity among individuals. Similar to GT, all menstruating women had severe menorrhagia (grades 2 & 3), a finding that might justify the predominance of females (n=15) over males (n=6) in these two disorders, and suggesting that men with GT or BSS are being underdiagnosed. All cases but one (patient BSS-3) were homozygous for the same mutated allele inherited from parents, suggesting that these patients shared a common ancestor (5 out of 8 did not refer a history of

known family consanguinity). This is of particular relevance in the case of the variant p.Phe55Ser in GPIX, identified in 3 apparently unrelated patients from central Portugal.

It has been previously shown that missense mutations hit GPIbβ and GPIX more frequently than GPIbα, suggesting that folding and stability of these glycoproteins are more sensitive to aminoacid substitution than GPIbα *(Savoia A et al. 2014)*. Accordingly, of the 3 new mutations identified in our study, the two novel missense variants, p.Trp71Arg, and p.Pro64Ser affected GPIX, and GPIbβ, respectively. Structural modeling of these variants predicted deleterious effects.

Until 2011, original studies reported one or two patients with BSS, with the largest case series including 13 cases *(Savoia A et al. 2011)*. More recently, the BSS Consortium, constituted of research groups from 15 countries worldwide, has helped establish the most comprehensive database of BSS, and data from the 112 different variants characterized so far have been deposited in the Leiden Open Variation Database (LOVD) at http://www.lovd.nl/GP1BA, http://www.lovd.nl/GP1BB, and http://www.lovd.nl/GP9 for the *GP1BA*, *GP1BB*, and *GP9* genes, respectively *(Savoia A et al. 2014)*. Such database would be enriched, with the inclusion of the 3 novel mutations identified here.

As for other IPDs, it is known that the molecular diagnosis of HPS may not be that simple. Mutations in 9 genes (*HPS1, AP3B1, HPS3, HPS4, HPS5, HPS6, DTNBP1, BLOC1S3*, and *BLOC1S6*) can cause HPS in humans. By adopting a classical approach to reach a molecular diagnosis in the 2 patients with clinical suspicion of HPS, we selected *HPS1* as the first candidate gene to be responsible for the disease, as it is the most frequently affected *(Carmona-Rivera C et al. 2011)*. We identified a homozygous p.Glu204Stop mutation in exon 7 of this gene in one of the patients *(Sanchez-Guiu I et al. 2014*b*)*. In the second patient, further sequencing of *HPS4*

revealed no variations; we did not sequence the rest of less frequently affected genes. Two additional patients presented with syndromic forms, suggestive of CHS, and a definitive diagnosis was made based on molecular findings *(Sanchez-Guiu I et al. 2014*a*)*. One individual who had been previously misdiagnosed as having ITP and treated with steroids and splenectomized, exhibited a bone marrow aspirate and biopsy showing decreased cellularity and megakaryocyte counts, with 24% of the metaphases revealing monosomy 7. With the clinical suspicion of CAMT, sequencing of the *MPL* gene detected a homozygous point mutation, p.Arg102Cys previously described *(Germeshausen M et al. 2006)*; with a diagnosis of CAMT the patient underwent a matched unrelated bone marrow transplant. A woman with non-syndromic autosomal-dominant thrombocytopenia was categorized as having a THC2, due to the presence of a heterozygous c.-128 G>A ANKRD26 mutation. One patient, who was initially thought by the referring physician to be affected by BSS, was not confirmed by functional studies as having such disease (normal expression of GPIbα and GPIX, as well as platelet agglutination induced by ristocetin). In this patient, the use of exome sequencing, led us to identify a monoallelic mutation in the *MYH9* gene (p.Asp1424Glu). This observation supports the possibility that exome sequencing could be routinely used as a diagnostic tool in the near future, to help identify new genetic defects in patients who do not fall into known categories of IPDs, and to gain further insight into the molecular regulation of platelet production and function.

5. Conclusion

This project highlights the urgent need to significantly improve the diagnosis of IPDs at the level of the platelet pathway and by molecular analysis, considering the high percentage of

patients that are still being misdiagnosed, and those in whom, the molecular mechanisms causing these defects are still unknown. The requirement of highly specialized tests represents a particular problem in resource-limited countries, and collaborative international efforts should facilitate advances in patient diagnosis and care. Consensus standardized criteria for the diagnosis of these disorders are expected to overcome the present heterogeneity between facilities, and the creation of reference centers should be considered to help health care providers in the diagnosis of these disorders.

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Chapter II

Chediak-Higashi syndrome: description of two novel homozygous missense mutations causing divergent clinical phenotype and review of the literature

1. Introduction

Chediak-Higashi syndrome is a rare autosomal recessive disorder characterized by hypopigmentation, recurrent infections, microscopic findings of giant organelles in virtually all granulated cells, bleeding diathesis, and neurologic abnormalities. The occurrence of this disorder is rare, and less than 500 cases have been reported worldwide in the past 20 years *(Kaplan J et al. 2008)*. Pathologic mutations in the lysosomal trafficking regulator (*LYST/CHS1*) gene localized at 1q42.1-2 are responsible for this defect *(Barbosa MD et al. 1996, Nagle DL et al. 1996)*. More than two thirds of patients with CHS manifest with the severe "childhood" form of the disease and experience at least one life-threatening "accelerated phase", similar to hemophagocytic lymphohistiocytosis (HLH) that leads to repeated infections, bleeding, and organ failure that generally proves fatal unless treated by allogeneic bone marrow transplantation.[1] However, about 10-15% of patients follow a less severe clinical course of CHS, the "adolescent" and "adult" forms. These children present with mostly hypopigmentation and survive until adulthood without experiencing an "accelerated phase", but develop severe, debilitating neurologic manifestations in adolescence and early adulthood.

Allogeneic transplantation from an HLA-matched sibling or an unrelated donor or cord blood transplant is the treatment of choice to correct the immunologic and hematologic manifestations of early-onset CHS, although it does not affect the progressive neurologic deterioration or the oculocutaneous albinism *(Tardieu M et al. 2005)*. Transplantation appears to be most successful if performed prior to the accelerated phase *(Eapen M et al. 2007)*. Therefore, it would be of great interest to early differentiate patients that would present with the childhood form of the disease so as to premature enrollment into a transplantation protocol, from those that would exhibit clinical phenotypes of adolescent and adult CHS, to

preclude such a treatment.

It has been suggested a reasonably straightforward genotype-phenotype correlation of the disease. To date, 59 CHS1/LYST mutations have been described (Table 6), 28 substitutions (21 nonsense, 7 missense) *(Nagle DL et al. 1996, Barbosa MD et al. 1997, Certain S et al. 2000, Karim MA et al. 2002, Zarzour W et al. 2005, Westbroek W et al. 2007, Manoli I et al. 2010, Morrone K et al. 2010, Tanabe F et al. 2010, Jessen B et al. 2011, Kaya Z et al. 2011, Ogimi C et al. 2011, Bhambhani V et al. 2013)*, 18 deletions *(Nagle DL et al. 1996, Barbosa MD et al. 1997, Karim MA et al. 1997, Dufourcq-Lagelouse R et al. 1999, Certain S et al. 2000, Karim MA et al. 2002, Zarzour W et al. 2005, Westbroek W et al. 2007, Scherber E et al. 2009, Jessen B et al. 2011)*, 9 insertions *(Barbosa MD et al. 1996, Karim MA et al. 1997, Certain S et al. 2000, Karim MA et al. 2002, Scherber E et al. 2009, Tanabe F et al. 2010, Jessen B et al. 2011, Ogimi C et al. 2011)*, and 4 acceptor splice sites *(Jessen B et al. 2011)*. Among patients in whom molecular studies have thus far been reported with the severe childhood form of CHS, except for one case (patient 22, Table 6) only mutant alleles (nonsense or frameshift mutations) that would lead to premature termination and abolish expression of the full length CHS1/LYST polypeptide have been observed. With the exceptions of patients 22 and 42 (Table 6) who show compound heterozygosity for nonsense and missense mutations in the LYST/CHS1 gene *(Jessen B et al. 2011, Bhambhani V et al. 2013)*, missense mutant alleles have only been reported among patients with clinically mild forms of the disorder, either as homozygotes or compound heterozygotes with protein-null mutant alleles. These observations suggest that some patients with missense mutations may make a partially functional CHS1/LYST protein *(Karim MA et al. 2002)*.

Similar to other congenital platelet disorders, such as Wiskott Aldrich and congenital amegakaryocytic thrombocytopenia, in which hematopoietic stem cell transplantation could be

postponed in those patients whose missense mutations predict residual activity of the protein *(Imai K et al. 2004, Germeshausen M et al. 2006)*,genotype characterization could be a useful tool for predicting long-term prognosis for patients with CHS.

Here, we have characterized two patients on opposite ends of the CHS clinical spectrum, both bearing two novel homozygous missense CHS1/LYST mutations (p.G3725R, and p.C258R). We report that the p.G3725R mutation exhibits markedly reduced protein stability with no reduction in mRNA expression in cultured fibroblasts. Structural consequences including important changes in surface charge may underlie the pathogenic mechanism of this genetic change.

Table 6. LYST/CHS1 mutations, illness severity, and effect on CHS1/LYST protein of patients described in the literature.

Case	Genotype	Missense		Nonsense/Frameshift		Form of CHS	Reference
		mRNA	Protein	mRNA	Protein		
1	Homozygous			c.1467delG	E489fsX566	Childhood	*(Nagle DL et al. 1996)*
2	Homozygous			c.1899insA	K633fsX638	Childhood	*(Bhambhani V et al. 2013)*
3	Homozygous			c.9590delA	Y3197fsX3258	Childhood	*(Karim MA et al. 1997, Certain S et al. 2000, Bhambhani V et al. 2013)*
4	Homozygous			c.3085C>T	Q1029X	Childhood	*(Barbosa MD et al. 1997)*
5	Homozygous			c.2620delT	F874fsX898	Childhood	*(Certain S et al. 2000, Scherber E et al. 2009)*
6	Homozygous			c.10395delA	K3465fsX3467	Childhood	*(Karim MA et al. 2002)*
7	Homozygous			c.7060-7066del 7bp	L2354fsX2369	Childhood	*(Certain S et al. 2000)*
8	Homozygous			c.7555delT	Y2519fsX2528	Childhood	*(Certain S et al. 2000)*
9	Homozygous			c.9106-9161del 56bp	G3036fsX3051	Childhood	*(Certain S et al. 2000)*
10	Homozygous			c.6078C>A	Y2026X	Childhood	*(Karim MA et al. 2002)*
11	Homozygous			c.5004delA	G1668fsX1717	Childhood	*(Karim MA et al. 1997)*
12	Homozygous			c.5519delC	S1840fsX1842	Childhood	*(Karim MA et al. 1997)*
13	Homozygous			c.3310C>T	R1104X	Childhood	*(Certain S et al. 2000)*
14	Homozygous			c.11102G>T	E3668X	Childhood	*(Manoli I et al. 2010)*
15	Homozygous			c.5506C>T	R1836X	Childhood	*(Kaya Z et al. 2011)*
16	Homozygous			c.7060-1G>A	Acceptor splice site	Childhood	*(Jessen B et al. 2011)*
17	Homozygous			c.10551_10552del2	Y3517X	Childhood	*(Jessen B et al. 2011)*
18	Homozygous			c.5506C>T	R1836X	Childhood	*(Jessen B et al. 2011)*
19	Homozygous			c.2374_2375delGA	D792fsX797	Childhood	*(Jessen B et al. 2011)*
20	Homozygous			c.4508C>G	S1483X	Childhood	*(Jessen B et al. 2011)*
21	Homozygous			c.5506C>T	R1836X	Childhood	*(Jessen B et al. 2011)*
22	Compound heterozygous	c.2570C>G	S857C	c.9930delT	F3310fsX3346	Childhood	*(Jessen B et al. 2011)*
23	Compound heterozygous			c.1540C>T c.9893delT	R514X F3298fsX3304	Childhood	*(Zarzour W et al. 2005)*
24	Compound heterozygous			c.3622C>T c.11002G>T	Q1208X E3668X	Childhood	*(Morrone K et al. 2010)*

25	Compound heterozygous			c.10445insCA Not specified	V3483fsX3516 R2403X	Childhood	*(Ogimi C et al. 2011)*
26	Heterozygous			c.5317delA	R1773fsX1785	Childhood	*(Certain S et al. 2000)*
27	Heterozygous			c.9228ins 10bp	K3077fsX3080	Childhood	*(Certain S et al. 2000)*
28	Heterozygous			c.118insG	A40fsX63	Childhood	*(Barbosa MD et al. 1996)*
29	Heterozygous			c.3073+3074delA	N1025fsX1030	Childhood	*(Barbosa MD et al. 1997)*
30	Heterozygous			c.2454delA	K818fsX823	Childhood	*(Karim MA et al. 2002)*
31	Heterozygous			c.3434-3435insA	H1145fsX1153	Childhood	*(Karim MA et al. 2002)*
32	Heterozygous			c.4052C>G	S1351X	Childhood	*(Karim MA et al. 2002)*
33	Heterozygous			c.3944-3945insC	T1315fsX1331	Childhood	*(Tanabe F et al. 2010)*
34	Compound heterozygous			c.7060-1G>T c.11196-1G>A	Acceptor splice site Acceptor splice site	Childhood	*(Jessen B et al. 2011)*
35	Compound heterozygous			c.7982C>G c.8281A>T	S2661X R2761X	Unknown	*(Tanabe F et al. 2010)*
36	Homozygous					Unknown	*(Jessen B et al. 2011)*
37	Homozygous			c.4688G>A	R1563H	Adult	*(Karim MA et al. 2002)*
38	Homozygous	c.5996T>A	V1999D		Acceptor splice site	Adult	*(Karim MA et al. 2002)*
39	Compound heterozygous	c.10127A>G	N3376S	c.2413delG	E805fsX806	Adult	*(Westbroek W et al. 2007)*
40	Compound heterozygous	c.8428G>A	E2810K	c.4274delT	L1425fsX1426	Adolescent	*(Karim MA et al. 2002)*
41	Compound heterozygous	c.4361C>A	A1454D	c.5061T>A	Y1687X	Adolescent	*(Karim MA et al. 2002)*
42	Compound heterozygous	c.9925G>A	G3309S	c.1507C>T	R503X	Adult	*(Bhambhani V et al. 2013)*
43	Heterozygous			c.8583G>A	W2861X	Adult	*(Karim MA et al. 2002)*
44	Heterozygous			c.148C>T	R50X	Adult	*(Barbosa MD et al. 1997)*
45	Heterozygous			c.3944-3945insC	Q1847fsX1850	Adult	*(Tanabe F et al. 2010)*
46	Homozygous			c.3310C>T	R1104X	Adolescent	*(Nagle DL et al. 1996)*
47	Homozygous			c.575insT	L192FfsX6	Adult	*(Karim MA et al. 1997)*
48	Homozygous			c.575_576insT	L192fsX197	Adult	*(Jessen B et al. 2011)*
49	Homozygous			c.3310C>T	R1104X	Adult	*(Jessen B et al. 2011)*

CHS, Chediak-Higashi syndrome. GenBank: U67615.1

2. Materials and Methods

2.1. Case Material

The severe early-onset form of CHS was represented by patient 1. He was the only child from a Moroccan couple known to be consanguineous. History revealed a normal pregnancy and delivery. Until admission he had no history of serious recurrent infections nor excessive bleeding, and the infant was meeting developmental milestones appropriately for age. At 3 year of age, the infant presented with high fever, abdominal distension and pain. Examinations revealed pale skin color, dark-gray silvery hair and hepatosplenomegaly, but there was no clinically significant lymphadenopathy. The hemoglobin was 8.4 g/dL, the white blood cell count was 1.9 x 10^9/L, and platelets were 74 x 10^9/L. Peripheral blood smear showed giant granules in polymorphonuclear leukocytes (Figure 6A), and bone marrow aspirate evidenced numerous large azurophilic or eosinophilic cytoplasmic inclusion bodies in cells of myeloid lineage that reacted strongly to peroxidase stain (Figure 6C-D). Ultrastructural studies showed that the granules contained a dense matrix and occasionally "finger print" structures at the periphery of the monocytic cells; in some inclusions, fibrillar structures of myelinic figures could be seen (Figure 6E-F). Microscopic examination of the hair revealed evenly distributed, regular melanin granules, larger than those seen in normal hairs. Under polarized light microscopy, shafts exhibited a bright and polychromatic refringence appearance.

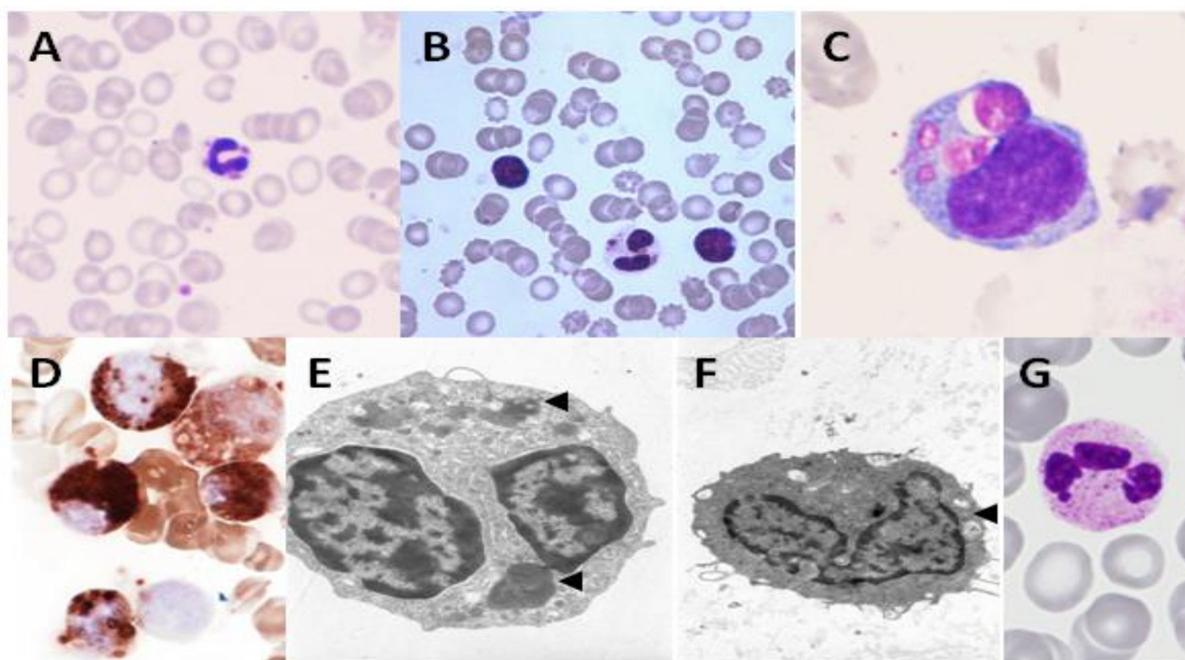

Figure 6. Microscopic evaluation of peripheral blood and bone marrow aspirate samples. Wright stain of peripheral smear, showing a polymorphonuclear leukocyte(s) with abundant giant intracytoplasmic granules from patient 1 (A) and patient 2 (B). Bone marrow myelocyte showing three large cytoplasmic granules and one huge vacuole containing residual hyaline material, and a transparent, watery content (C). Peroxidase staining of bone marrow cells. There are markedly positive granules and some ringed structures (D). Electron micrographs of bone marrow showing a cytoplasm filled with normal-sized organelles, as well as giant lysosomes and fibrillar structures in a myeloid cell (E), as well as onion-like figures inside monocytic cells (F). Patient 1 Wright-stained blood smear after transplantation (G). Figure C–F is from patient 1. All figures have 1000 x magnification, except for E and F (4000 x).

Platelet function studies performed when platelets were above 100 x 10^9/L with the platelet function analyzer (PFA-100) demonstrated increased ADP (124 seconds; reference range, 57-100 seconds) and epinephrine (158 seconds; reference range, 81-131 seconds) closure times. Platelet dysfunction was confirmed with platelet aggregometry studies that showed monophasic response after exposure to different concentrations of ADP (5 and 10 μM) and epinephrine (0.5 and 5 μM), and markedly impaired aggregation in response to other agonists (25 μM TRAP, 1.25 mg/mL ristocetin, 1.6 mM arachidonic acid, and 2 and 10 μg/mL collagen). There was an obvious decrease in the total uptake of ^{14}C 5-hydroxytryptamine (16% of control values). In addition, upon stimulation with 25 μM TRAP we observed an important reduction in the platelets flow cytometric externalization of fluorescein-labelled CD63 (4.5% positivity in patient vs 73.6% in control) and negative mepacrine test in the patient. These

findings are consistent with a storage pool deficiency with reduced dense bodies and consequent defects of secretion-dependent aggregation.

Based on the clinical presentation and hematological findings, a diagnosis of accelerated phase of the CHS was made. Chemo-immunotherapy according with the hemophagocytic lymphohistiocytosis (HLH)-2004 protocol including etoposide, dexamethasone, and cyclosporine was started *(Henter JI et al. 2007)*. The patient exhibited an excellent response, with the disappearance of the biological and clinical parameters of the massive lymphohistiocytic infiltration. Six months after diagnosis he underwent bone marrow transplantation from an unrelated donor. At present, 4 years after transplant, peripheral blood and bone marrow examination show absence of granule defects in neutrophils and in myelomonocytic series, the white blood cell count is in the normal range, and donor engraftment is 100%.

The milder adult late-onset of CHS was represented by patient 2. She is a 21-year old woman who presented at 6 years of age with an upper respiratory infection, in good general condition. Chediak-Higashi syndrome was diagnosed based on giant granules in peripheral white blood cells (Figure 6B). On examination she had pallor, silvery hair, and neither enlarged liver nor spleen were found. Platelet function studies exhibited platelet counts of 227 x 109/L. There was a mild decrease in the total uptake of 14C 5-hydroxytryptamine (53% of control values). In addition, upon stimulation with 25 μM TRAP we observed a slight reduction in the platelets flow cytometric externalization of fluorescein-labelled CD63 (29.8% positivity in patient vs 65.3% in control) and negative mepacrine test in the patient. These findings are consistent with a mild storage pool deficiency due to reduced dense bodies.

At present she is alive and well, without severe infections, and with normal blood counts. She is the first of three children, and while the second child is healthy, with normal

blood smear, the youngest brother was diagnosed at 3 months of age of CHS, based on peripheral blood smear, when he presented with a respiratory infection. He is now 5 years old, has dark-grey silvery hair, and his psychomotoric development is normal.

The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000, and samples were obtained after subjects provided informed consent.

2.2. Primary Fibroblast Culture

Primary dermal fibroblasts from a skin biopsy of patient 1, and from a normal control, were cultured. Written informed consent was obtained from the parents of the propositus, and from the control. The samples were aseptically collected, and washed with phosphate buffered saline (PBS) containing 100 μg/mL penicilin/streptomycin, 4 μg/mL gentamycin, and antimicotic (100 U/ml amphotericin). After removing the epidermis, the pure dermis was cut into small pieces and once attached, kept in the upside-down position for 12 hours, and then inverted, in the presence of culture medium (DMEM-F12 with 10% FBS containing 4 μg/mL gentamicin, 100 μg/mL penicillin-streptomycin, 2.5 μg/mL Amphotericin, 1% non-essential aminoacids, and 1% ITS liquid media supplement). The cultures were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO_2, and fresh growth medium was added to the cells every 3 to 7 days. After 14 days, the cultured fibroblasts were harvested by trypsinization and culture expanded into new T25 culture flasks with expansion degree of 1:4. When the subcultures reached 80-90% confluence, serial passaging (up to 5) was done by trypsinization.

2.3. RNA Extraction, cDNA Synthesis, and LYST/CHS1 Amplification and Sequencing

Blood was obtained by venipuncture and processed for total RNA from peripheral blood

by using the PureLink Total RNA Purification Kit (Invitrogen, Paisley, UK), according to the manufacturer´s recommendations. One hundred nanograms of total RNA were used for cDNA synthesis by the Superscript First Strand Synthesis System for RT-PCR (Invitrogen), following the manufacturer´s protocol.

Samples from known heterozygous or homozygous family members, and from normal donors were used as controls.

The entire coding region (13.5 kb) of the *LYST/CHS1* gene was amplified by PCR using 2 µl of cDNA in 9 overlapping reactions (mean size, 1.4 kb) and submitted to later nested PCR. The PCR products were sequenced in the ABI 3130xl genetic analyzer (Applied Biosystems, Carlsbad, CA), as previously reported *(Certain S et al. 2000)*.

2.4. mRNA Quantification

Total RNA was extracted from blood of patients 1 and 2, from known heterozygous or homozygous family members, and from normal donors using the Trizol reagent (Life Technologies Corporation, Carlsbad, CA) and the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA was also isolated from cultured fibroblast from patient 1 and from a normal control. RNA samples were stored at -80°C until used in the experiments. mRNA quantifications were carried out as previously described *(Teruel R et al. 2011)*.

Transcript relative quantification of *LYST/CHS1* was performed by qRT-PCR using TaqMan® Gene Expression Assay (hs00915897m1; Life Technologies Corporation, Carlsbad, CA). Expression of β-actin (Hs99999903_m1; Life Technologies Corporation, Carlsbad, CA) was employed as endogenous reference control. The PCR reactions were performed using an LC480 Real Time PCR system (Roche Applied Science, Barcelona, Spain). Expression analysis was

performed in triplicate for each sample. The fold difference for each sample was obtained using the $2^{\Delta Ct}$ method. Ct is the Threshold Cycle and ΔCt = Ct sample gene - Ct Actβ.

2.5. Western Blot

Cultured fibroblasts from Patient 1 and from a normal control were washed with PBS and homogenized with ice-cold PBS supplemented with 0.5 % Triton X-100, 0.5 % NP-40, protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St Louis, MO). The homogenates were centrifuged at 12,000 x g for 20 minutes at 4º C and the supernatants were stored at -80° C until being used. BCA assays (ThermoFisher, Waltham, MA) were performed to determine protein concentration of the lysates. One hundred µg of proteins were electrophoresed on 5% acrylamide gels under reducing conditions, and the proteins were wet transferred to polyvinylidene difluoride membrane. The membrane was incubated using a 1:200 dilution of mouse-anti CHS1/LYST antibody (Abnova Gmbh, Heidelberg, Germany). Detection was performed using adequate horseradish peroxidase conjugate secondary antibodies (GE Healthcare, Waukesha, WI) and the use of an ECL kit (GE Healthcare, Waukesha, WI). Densitometric analysis was performed with Quantity One 1-D analysis software (Bio-Rad Laboratories, Hercules, CA).

2.6. Immunofluorecence

Human fibroblasts from control and patient 1 were fixed in 4% formaldehyde in PBS buffer (22 ºC, 15 min). After fixation, cells were washed with PBS, permeabilized and blocked with 2% BSA/0.1% Triton X-100 in PBS (30 min). All subsequent incubations and washes contained 0.5% BSA/0.1% Triton X-100 in PBS buffer. LAMP-3 (a lysosomal marker) was

localized by immunofluorescence microscopy using a mouse anti LAMP-3 antibody labelled with phycoerythrin (BD Bioscences, Madrid, Spain) at 1:20 (12h, 4ºC). Images were captured on a Leica 6000B microscope using its associated software (Leica Microsystems, Barcelona, Spain).

2.7. Homology Modeling

In order to investigate if *LYST/CHS1* mutations found on patients 1 and 2 had structural consequences on the protein, structural models for p.G3725R and p.C258R of CHS1/LYST were generated. Tertiary structure of these CHS1/LYST were predicted by a homology modeling approach using MODWEB program, (http://modbase.compbio.ucsf.edu/ModWeb20-html/modweb.html) *(Eswar N et al. 2003)*. The CHS1/LYST p.G3725R mutation found in patient 1 is located in the sixth WD domain at the C-terminal part of the protein, while the p.C258R mutation of patient 2 is located in the ARM/HEAT repeat domain, at N-terminal. Homology modeling of native and mutated CHS1/LYST proteins (in C-terminal, residues: 3449-3781) was performed using human WDR5 crystal, chain A (PDB code 2gnqA) *(Schuetz A et al. 2006)*. Homology modeling of native and mutated CHS1/LYST proteins (in N-terminal, residues: 1-525) was performed using crystal structure of human importin 13 bound to RanGTP (PDB code 2X19B) as template *(Kippert F et al. 2009)*. Importin13 is a β importin, included in the class IMB of the 3 cluster of HEAT repeats.

The resultant CHS1/LYST sequence was submitted to the fully automated server using default values.

Three dimensional diagrams were visualized on Swiss-PdbViewer 4.01 *(Guex N et al. 1997)*. Electrostatic potential was studied using Poisson-Boltzmann computation method implemented in Swiss-PdbViewer 4.01.

3. Results

3.1. Mutation analysis

Patient 1. The patients' parents were first-cousins. Full length RT-PCR products for the 9 *LYST/CHS1* overlapping fragments were obtained from the cDNA of the patient and from his parents. Patient was found to be homozygous for a novel missense mutation c.11362 G>A, p.G3725R (GenBank: U67615.1) (Figure 7A). His parents were both found to be heterozygous for this mutation (Figures 2B-C). The detected point mutation located in exon 51 targets a highly conserved WD40 domain at the C-terminal part of the CHS1/LYST protein (Figure 8).

Patient 2. Genetic mutation analysis of the entire coding region of *LYST/CHS1* was performed in the patient, her youngest brother, and in her parents. Patient was found to be homozygous for a novel missense mutation c.961 T>C, p.C258R (GenBank: U67615.1) (Figure 7D). Her parents were both found to be heterozygous for this mutation (Figures 7E-F), while the youngest brother also presented the mutation in homozygosity (Figure 7G).The detected point mutation is located in exon 5 and targets ARM/HEAT repeat at the N-terminal part of the CHS1/LYST protein.

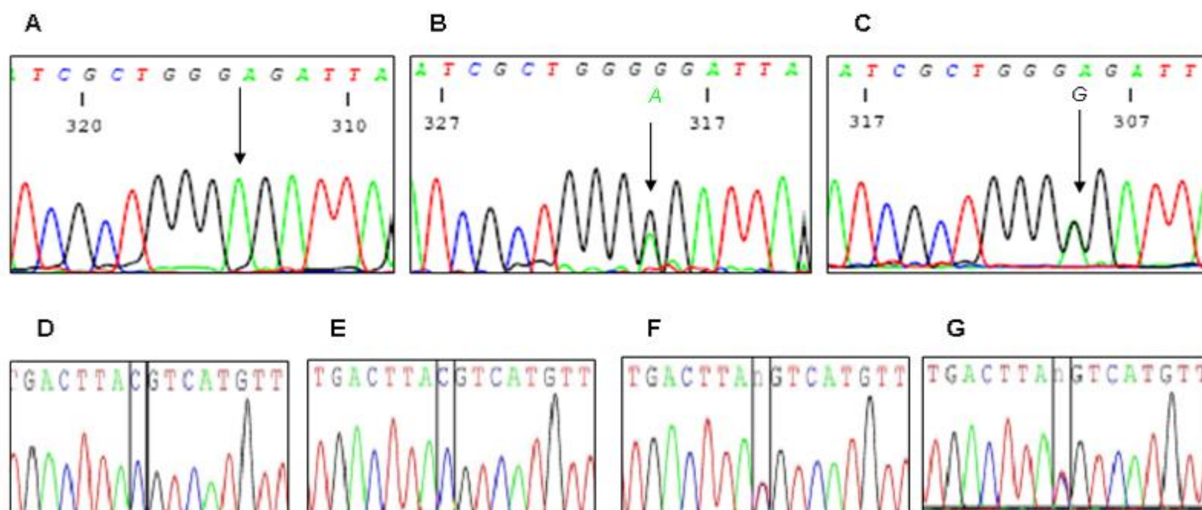

Figure 7. Sequence patterns showing the patient's 1 homozygous LYST/CHS1 mutation in exon 51, c. 11362G>A (p.G3725R) (A), and the heterozygous mutation both in mother (B) and father (C). Sequence patterns showing the patient's 2 homozygous LYST/CHS1 mutation in exon 5, c.961 T>C (p.C258R) (D) and in her brother (E), and the heterozygous mutation both in mother (F) and father (G).

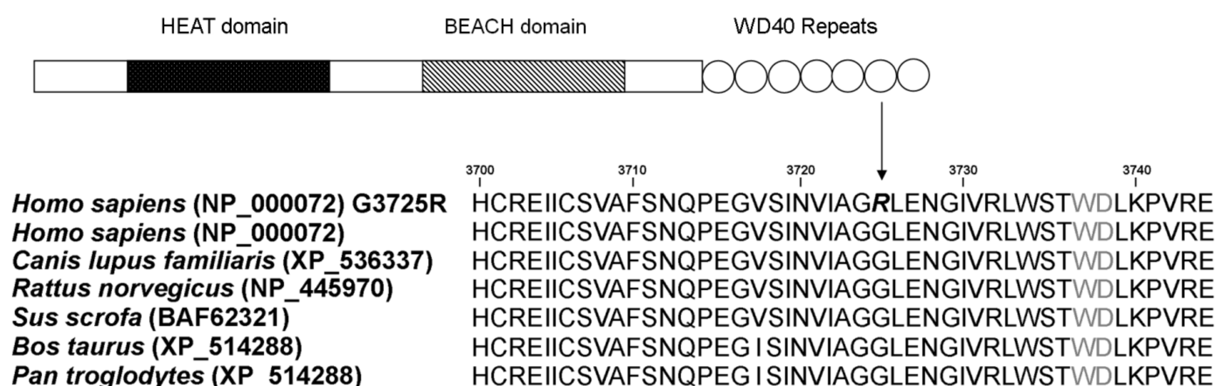

Figure 8. Schematic representation of CHS1/LYST protein structure with the HEAT/ARM domain, BEACH domain, and WD repeats. Depicted is the evolutionary conservation of human amino acid residue 3725 of the CHS1/LYST. Residues 3700 to 3744 of the sixth WD repeat of the CHS1/ LYST protein are shown. The exchange residue (Arg3725) is shown in bold italic character. The consensus WD motif is shown in grey.

3.2. LYST/CHS1 mRNA quantification

The expression level of *LYST/CHS1* mRNA both in patient 1 and 2, were similar to control levels in peripheral whole blood cells (Table 7). Similarly, no substantial differences in *LYST/CHS1* mRNA were observed in whole blood from heterozygous and homozygous family members of patients, compared to human healthy subjects. While relative expression of *LYST/CHS1* mRNA in fibroblasts were consistently lower than those in peripheral blood, that expression was not substantially different between patient 1 and that of his matched healthy control.

Table 7. Quantification of the relative amounts of the LYST/CHS1 transcript ($2^{-\Delta Ct}$).

	Patient 1	Patient 2
	Whole Blood ($2^{-\Delta Ct}$×100)	
Propositus	2,80	3,72
Father	3,12	3,61
Mother	3,87	4,57
Brother	NA	2,53
Mean of 3 controls	2,01	3,23
	Cultured Fibroblasts ($2^{-\Delta Ct}$×10000)	
Propositus	1,21	NA
Control	1,64	NA

The ΔCt value is determined by substracting the β-actin (endogenous reference) Ct-value from the Ct value of the target investigated: ΔCt = Ct (*LYST/CHS1*) - Ct (β-actin). The relative concentration of the target (amount of target), $2^{-\Delta Ct}$, is calculated for the average ΔCt values.

3.3. Protein expression

To determine whether there are differences in the levels of CHS1/LYST in patient 1 carrying the p.G3725R mutation, we performed Western blotting with an anti-CHS1/LYST antibody in fibroblasts. By densitometric analysis of the Western Blot, we showed that the level of CHS1/LYST was markedly decreased (19.2% ± 13.6%) in the amount of CHS1/LYST protein

(~430 KDa) in fibroblasts of patient 1 as compared with a control subject (Figure 9).

3.4. Lysosome morphology

To study whether the p.G3725R CHS1/LYST protein mislocalizes in fibroblasts, immunofluorescence in fibroblasts stained with LAMP-3 was performed. In the control subject, normal sized lysosomes distributed in the perinuclear and peripheral region were observed, while fibroblasts from patient 1 exhibited enlarged lysosomes in the perinuclear area, according to previous reports[10,12] (Figure 10).

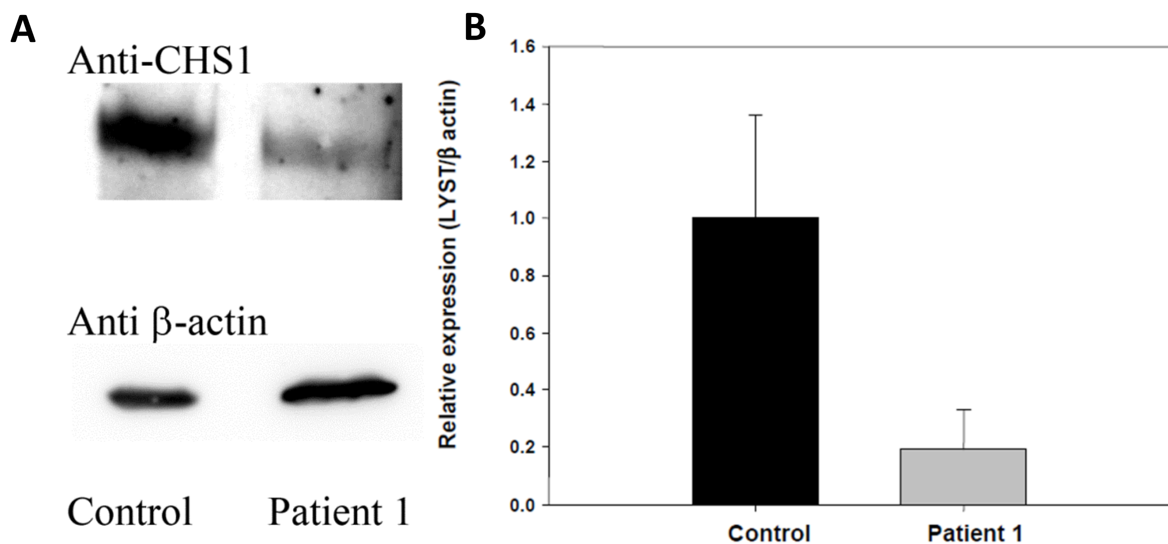

Figure 9. (A) Western blot analysis of protein lysates obtained from primary skin fibroblasts from patient 1 and from a control using an antibody against the CHS1/LYST protein. Also shown is a control blot probed for actin. (B) Quantification of CHS1/LYST levels normalized to actin is shown. Data shown represent the average of two experiments. Error bars correspond to one standard deviation.

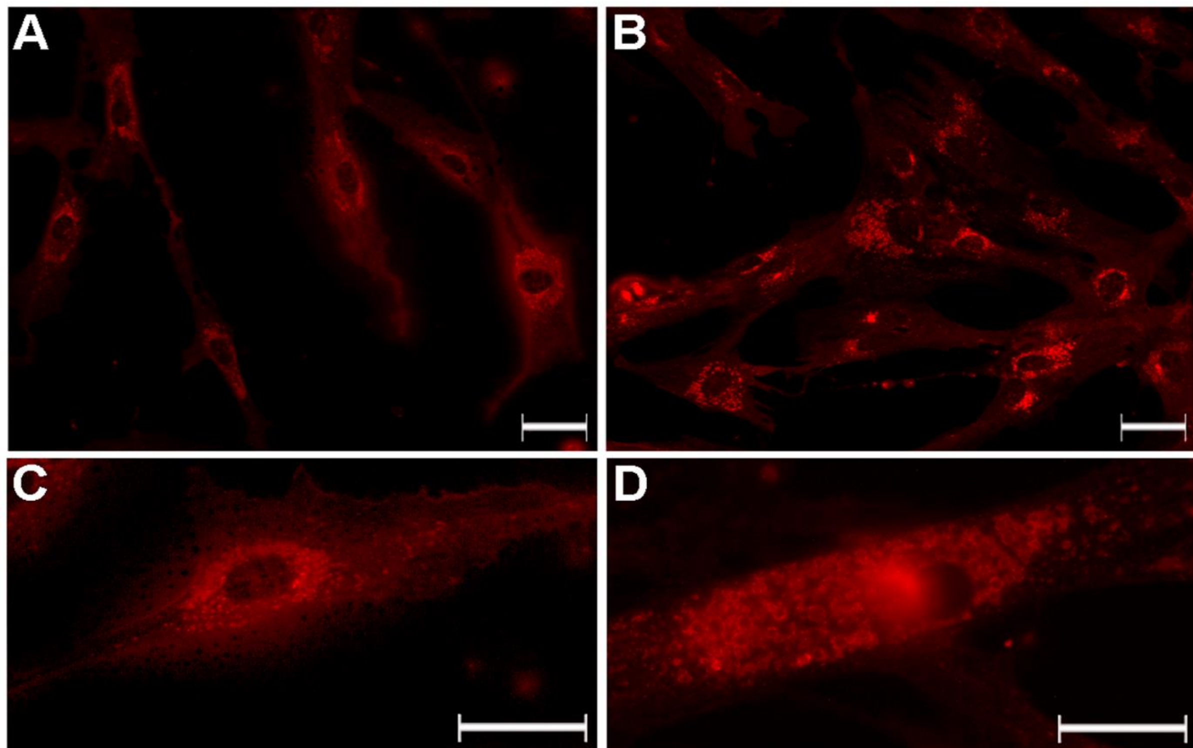

Figure 10. LAMP-3 staining in fibroblasts from a control (A) and from patient 1 (B). Patient's fibroblasts show enlarged lysosomes in the perinuclear area. Enlarged view in control (C), and patient (D). Scale bars, 20 μm

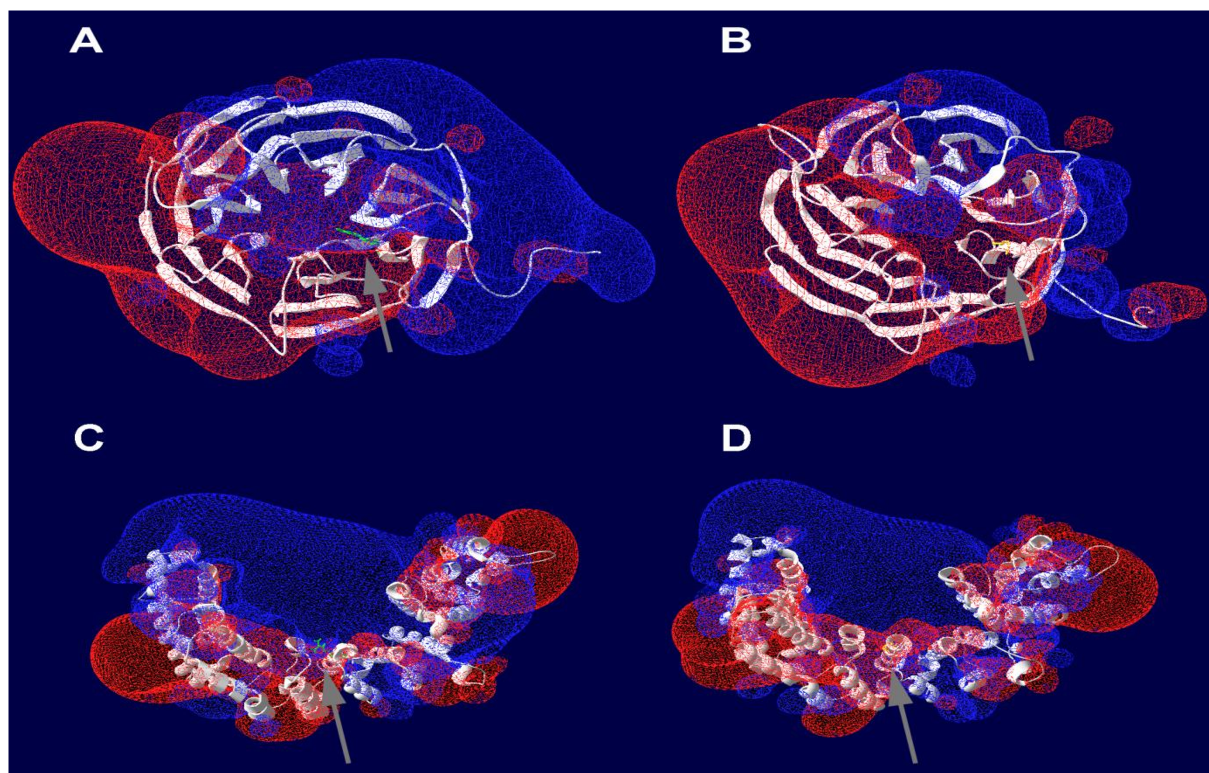

Figure 11. Electrostatic potential of CHS1/LYST protein in C-terminal (A and B) and N-terminal (C and D), evaluated by Poisson–Boltzmann computation method. Charge distribution is represented as a gradient in which positive potential is drawn in blue and negative in red. Amino acids involved in mutation are indicated by a grey arrow. (A)

CHS1/ LYST with Arg mutation displayed in green color. (B) CHS1/LYST native protein with Gly displayed in yellow color. (C) CHS1/LYST with Arg mutation displayed in green color. (D) CHS1/LYST native protein with Cys displayed in yellow color.

3.5. Molecular modeling and electrostatic potential

When analyzing the structural consequences of the p.G3725R mutation in patient 1, as the 3D structure of the seven-bladed WD β propeller of CHS1/LYST protein has not been determined, protein structure prediction and molecular modeling was performed with a solved structure with the highest homology used as template in the analysis of structural effects caused by the mutation. The replacement of glycine by the basic arginine leads to a shift of the electrostatic potential from the acid to the basic. Figure 11 A and B shows the computer model of the seven WD-40 domains of the CHS1/LYST protein. As shown, additionally to its influence on the electrostatic potential, the replacement of glycine by arginine may lead to significant conformational changes. Therefore, both structural changes, and specially, the drastical effect on electrostatic potential may explain the clinical consequences of this mutation (Figures 6 A-B).

As for patient 2, cysteine at position 258 of CHS1/LYST is not predicted to be involved in disulfide bonds. p.C258R mutation in the CHS1/LYST protein is located in the ARM/HEAT repeat domain, at N-terminal. This domain, rich in α-helix, may mediate membrane interactions. The cysteine/arginine interchange did not significantly affect the structure of the CHS1/LYST protein, and had a slight effect on the electrostatic potential (Figures 11C and D). The small consequences of this change on the protein might be related with the milder form of the disease, likely encoding CHS1/LYST polypeptides with partial function *(Karim MA et al. 2002)*.

4. Discussion

For both of the patients studied, the parents were heterozygous for the specific mutation; thus, each patient was homozygous by descent for a different pathologic mutation. Patient 1 carries a missense mutation at exon 51, of the LYST/CHS1 gene; this could be expected to allow for partial synthesis of the CHS1/LYST protein and result in a mild phenotype. However, initial presentation of this patient was unusual, since he presented with "childhood" severe clinical and biological CHS phenotype. Patient 2 also exhibited homozygous missense mutations, and in contrast to patient 1 and according to what has been previously established, presented with a milder form of the disease, allowing survival into adulthood.

It has been suggested an apparent genotype-phenotype correlation among the various clinical forms of the disorder *(Karim MA et al. 2002, Westbroek W et al. 2007, Kaplan J et al. 2008)*. Thus, early reports associated frameshift, nonsense, and splice site mutations resulting in an absent CHS1/LYST protein with severe childhood CHS, whereas milder adolescent or adult forms of CHS would present with at least one missense mutation probably encoding a partially functioning protein *(Karim MA et al. 2002)*. However, this linkage was not seen in other studies *(Certain S et al. 2000, Zarzour W et al. 2005, Jessen B et al. 2011)*, and few exceptions are known. Nonetheless, the association of mild clinical course with missense mutations strongly suggests the importance of CHS expression as determinant of disease severity. To define the unexpected effect of the missense mutation in the severe form of presentation in patient 1, we addressed three questions: whether CHS is expressed in the patient´s cells, the level of CHS expressed, and the molecular structure of the variant protein.

The CHS1/LYST protein is a highly conserved large cytosolic protein of approximately 430kDa. The N-terminal extreme presents several ARM/HEAT α-helix repeats, followed by

BEACH domain and a C-terminal domain of seven WD40 repeats (Figure 7). HEAT repeats are 40-50 amino acid segments containing conserved residues (proline, aspartic acid and arginine) with several flanking hydrophobic residues *(Andrade MA et al. 2001, Kaplan J et al. 2008)*; HEAT and BEACH are suggested to have a function in membrane interactions and organelle protein trafficking *(Andrade MA et al. 2001, Kaplan J et al. 2008)*, while the WD domain is generally supposed to be important for interference with other protein-binding partners *(Kaplan J et al. 2008)*. Recently, an important role of protein surface charge distribution for functional interactions in the members of the seven-bladed β-propeller WD-repeat family has been suggested *(Valeyev NV et al. 2008)*. A previous study analyzing the functional effects of different portions of BEACH proteins has shown that while the C-terminal WD domain is important for protein stability, in contrast, N-terminal deletions yield abundant protein expression *(Wu WI et al. 2004)*.

The described G to A transition at nucleotide position 11362 in exon 51 in patient 1 targets the highly conserved sixth WD domain at the C-terminal part of the CHS1/LYST protein (Figure 8), and represents the most distally located mutation in the LYST/CHS1 gene reported to date. Four patients have been described carrying mutations in the WD domain of the protein *(Manoli I et al. 2010, Morrone K et al. 2010, Jessen B et al. 2011)*; in all four cases, infants presented with classic CHS, and genetic studies revealed premature stop codons. In one case for the first time it was verified the absent expression of CHS1/LYST on cells *(Manoli I et al. 2010)*.

To gain further insights into the effects of the p.G3725R mutation, we studied dense granules on platelets, LYST/CHS1 mRNA, and levels of the protein variant. Dense granule studies of platelets of the patient evidenced marked storage-pool deficiency, according to the clinical presentation. This finding is interesting because while platelets from patients presenting with

"childhood" CHS have been reported to be markedly deficient or lack dense bodies *(White JG 2003, Nurden P et al. 2008, Masliah-Planchon J et al. 2013)*, dense bodies could be present in platelets of patients with late-onset CHS *(Westbroek W et al. 2007)*. This observation is also supported by the fact that platelets of patients with the milder form of the disease exhibit a lesser impairment of platelet function than patients presenting with the severe early-onset disease *(Buchanan GR et al. 1976)*, as seen in platelet functional studies of patient 2.

We found that both missense mutations did not reduce LYST/CHS1 mRNA levels, in whole blood, nor in the case of the p.G3725R mutation, in cultured fibroblasts (Table 7). However, a clear and specific decrease in the amount of CHS1/LYST protein in cultured fibroblasts from patient 1 as compared with control samples was verified. This reduction in the translated product suggests instability of CHS1/LYST at the protein level as a direct consequence of the amino acid substitution. Moreover, the severe phenotype of the patient raises the possibility of a toxic partially translated product with dominant negative effect.

It is known that electrostatic potential changes affect the specificity of protein-ligand or protein-protein interactions *(Honig B et al. 1995)*, and that those interactions play a dominant role in determining protein stability *(Borjesson U et al. 2003, Schwehm JM et al. 2003, Strickler SS et al. 2006)*. Computer modelling of the p.C258R mutation detected on patient 2 provided evidence that it might be tolerated by the fold, and does not seem to significantly disturb the electrostatic interactions between the α-helices. However, the exchange of glycine to the positively charged hydrophilic amino acid arginine at the position 3725 of the CHS1/LYST protein involves a change in the structure of the protein, and most dramatically, on its electrostatic potential, which may have a crucial effect on ligand binding, protein folding, and stability. Genetic conservation of amino acids at the sixth WD-40 domain of CHS1/LYST (Figure 8) may indicate the relevance of these residues on protein structure and/or function. Clearly

the position and nature of the mutations can influence the level of protein expression and the severity of the disease, and caution is required in correlating disease severity with mutations.

In conclusion, this is the first report of a severe early-onset CHS with a homozygous missense mutation. Clinical picture, and laboratory studies, confirmed presentation with a severe phenotype in the accelerated phase, contrary to the accepted premise of null mutant LYST/CHS1 alleles in individuals with the more severe CHS phenotype. These features are probably a direct consequence of the predicted changes in electrostatic surface potential, which might be related to high instability of the mutant protein and to the severity of the disease. In Chediak-Higashi, as well as in other disorders, attempts to link genotype and clinical phenotype require knowledge of the actual molecular effect of the mutation.

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Chapter III

Hermansky-Pudlak Syndrome: Overview of clinical and molecular features and case report of a new HPS-1 variant

Microsatellite markers as a rapid approach for autozygosity mapping in Hermansky-Pudlak syndrome: Identification of the second HPS7 mutation in a patient presenting late in life

Hermansky-Pudlak Syndrome: Overview of clinical and molecular features and case report of a new HPS-1 variant

1. Introduction

Inherited platelet function disorders (IPFDs) encompass a heterogeneous group of haemorrhagic diseases caused by congenital defects of platelets function affecting various elements of the platelet physiology (membrane receptors, intraplatelet signaling proteins, granules), and leading to different clinical manifestations *(Bolton-Maggs PH et al. 2006, Salles, II et al. 2008, Nurden AT et al. 2014)*. Platelets have three types of secretory granules that differ in their number, content and function. Each single platelet has between 50 and 80 alpha (α) granules, containing adhesion molecules like P-selectin, cytokines, coagulation and fibrinolysis factors, complement molecules and growth factors. Dense (δ) granules, much less frequent (only 3-8 per platelet) contain small molecules like calcium, nucleotides (ADP, ATP), serotonin and pyrophosphate. Platelets have also lysosomes containing different proteolytic enzymes and glycosidases *(Thon JN et al. 2012)*.

Congenital defects regarding the number and/or content of platelet granules include a range of disorders with variable reduction in the number and content of such granules (Table 8). In this review we will focus on the Hermansky-Pudlak syndrome (HPS), which is one of the most severe congenital disorders of δ-platelet granules, and report a patient with a novel homozygous mutation in *HPS1* causing this disorder.

Table 8. Congenital defects in platelet granules.

AFFECTED GRANULES	NAME	CLINICAL AND LABORATORY PHENOTYPE	INHERITANCE	GENES
α and δ	α y δ storage pool disease	Normal or discretely diminished platelets count. Normal platelet morphology. δ and or α granules defect by electron microscopy. LTA ↓. Granular protein release impairment measured by FC. Mild bleeding usually in the context of high haemorrhagic risk situations.	AR/AD	-
α	Grey platelet Syndrome (GPS)	Moderate thrombocytopenia (30-100 x109/L). Large and grey platelets. LTA N or ↓. Some patients show a selective defect in GPVI and activation by collagen. Defect in α-granular proteins in biochemical assays (β-TG, PDGF, etc.) Selective absence of α-granules in thin-section electron microscopy. Clinical: Early bone marrow fibrosis; mild haemorrhagic episodes, usually associated with high risk situations (dental procedures under antiaggregant treatment, surgery, delivery, trauma, etc.), occasional splenomegaly.	AR/AD	*NBEAL2*
	Quebec Syndrome (QS)	Moderate thrombocytopenia (≈100 $x10^9/L$), normal morphology. LTA N or ↓. Impaired procoagulant platelet activity. Enhanced fibrinolytic activity. Defect in α-granular proteins showed in FC (P-selectin, factor V) Clinical: cutaneous-mucose and post-surgery haemorrhagic episodes. Clinical response to fibrinolytic inhibitors. Absence of clinical response to platelet transfusion.	AD	*PLAU*
δ	Hermansky-Pudlak Syndrome (HPS)	Normal platelet count and morphology Selective defect of δ-granules by electron microscopy LTA N or ↓. Decrease in radiolabeled hydroxytryptamine and mepacrine uptake and reduced release of CD63 by FC. Clinical: oculocutaneous albinism, lysosomal accumulation of ceroid lipofuscin, pulmonary fibrosis, granulomatous colitis.	AR	HPS1-HPS9
	Chediak-Higashi Syndrome (CHS)	Normal platelet count and morphology Selective defect of δ-granules by electron microscopy LTA N or ↓. Decrease in radiolabeled hydroxytryptamine and mepacrine uptake and reduced release of CD63 by FC. Clinical: oculocutaneous albinism, large peroxidase positive lysosomal granules in neutrophils and other non-haematopoietic cells; impaired NK and cytotoxic T lymphocyte function. In most patients hemophagocytic lymphohistiocytosis is lethal unless allogeneic transplantation is performed.	AR	LYST
	Griscelli Syndrome (GS)	Normal platelet count and morphology Selective defect of δ-granules by electron microscopy Clinical: Albinism, silver hair, neurological defects, lymphohistiocytosis. Diminished NK cell and T-lymphocyte cytotoxic function.	AR	*RAB27, MYO5A,MLPH*
	Asyndromic deficiency	Normal platelet count. Reduced second aggregation wave. Absence of δ-granules by electron microscopy	AD/AR	-

LTA: Light Transmission Aggregometry (weak agonists and low concentrations: including ADP, epinephrine and collagen); LTA N, LTA normal; LTA ↓, reduced aggregation or absence of second wave. FC, Flow Cytometry; AR, Autosomic Recessive; AD, Autosomic Dominant; β-TG, β-Thromboglobulin; PDGF, Platelet-Derived Growth Factor

2. Hermansky-Pudlak Syndrome

2.1. General Concepts

The HPS, so called in honor of the two Czechoslovak pathologists who first described it *(Hermansky F et al. 1959)*, encompasses a group of clinically and biologically heterogeneous disorders with sensible differences in their molecular base. It is an inherited disease due to the congenital alteration of lysosome-related subcellular organelles, like delta granules in platelets or melanosomes in melanocytes.

Patients with HPS are also characterized by hemorrhagic diathesis with variable degree of severity depending on platelet alteration, and oculocutaneous albinism (tyrosinase positive) with nystagmus, and visual acuity loss. Many patients show other severe clinical manifestations depending on the HPS subtype (i.e. pulmonary fibrosis, granulomatous colitis, neutropenia, immunodeficiency and neurological symptoms) *(Gunay-Aygun M et al. 2004, Carmona-Rivera C et al. 2011, Masliah-Planchon J et al. 2013, Samuels JD 2013)*.

As it happens in the majority of IPFDs, the incidence of HPS is not accurately known and less than 1000 cases have been described all around the world *(Bolton-Maggs PH et al. 2006)*. Noteworthy HPS is particularly prevalent in Puerto Rico, especially in the north of the country, where 1/1800 people are affected. Two founding-effect mutations are responsible of this high HPS rate in Puerto Rico, a 16-bp duplication in exon 15 of the *HPS1* gene and a 3.9Kb deletion in *HPS3*. These mutations are not especially prevalent in other countries *(Carmona-Rivera C et al. 2011, Masliah-Planchon J et al. 2013)*. To the best of our knowledge, only two Spanish HPS patients have been reported, and only the one previously described by our group had a molecular diagnosis *(del Pozo Pozo AI et al. 2002, Gonzalez-Conejero R et al. 2003)*.

2.2. Physiopathology: Proteins and Genes Involved

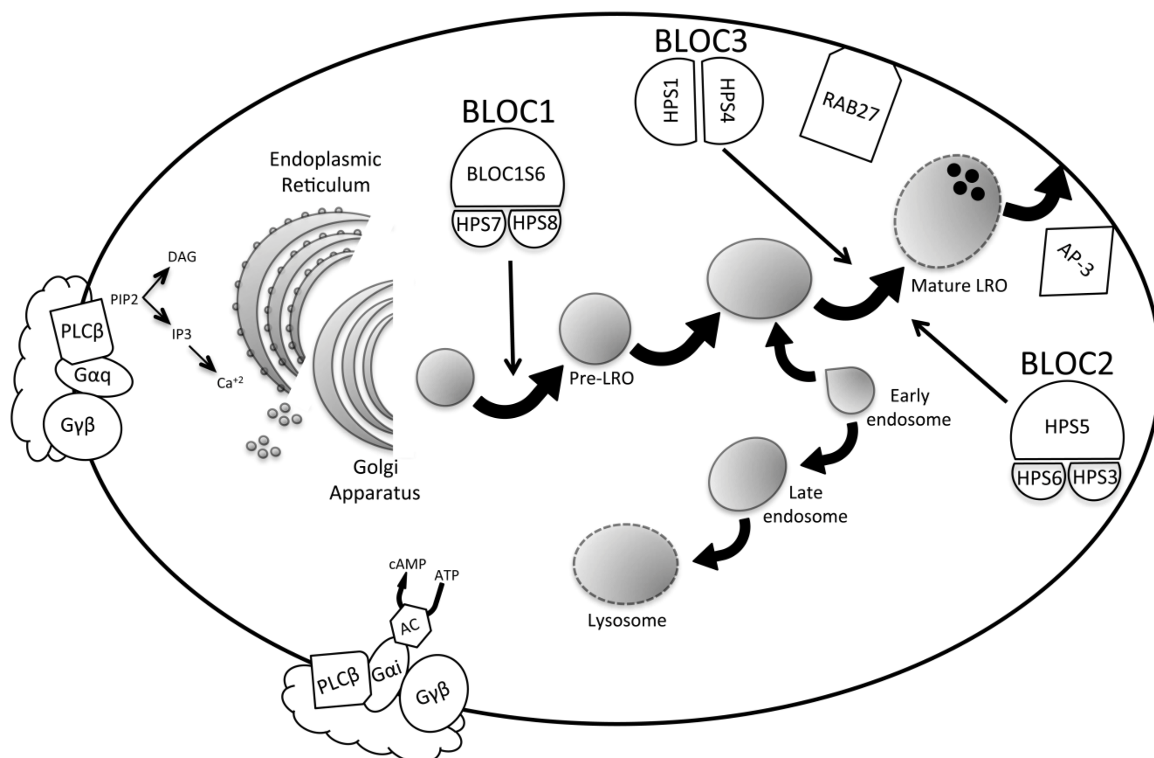
Congenital δ-granule disorders, including HPS, arise from genetic disorders involving formation and/or intracellular traffic of the lysosome-related organelles (LRO). LRO, a family to which platelet δ-granules belong, are a heterogeneous group of membranous vesicles sharing with lysosomes their synthesis pathways, certain membrane components and their acid pH. However, LRO differ from lysosomes in their morphology, composition and function. Different types of LRO are selectively present in cells like melanocytes, platelets, T-lymphocytes, neutrophils and pulmonary epithelial cells (Table 9), contributing importantly to their specialized function *(Huizing M et al. 2008, Wei AH et al. 2013)*.

Table 9. Main of intracellular lysosome related organelles (LRO), function and pathological consequences of their defects.

LRO	FUNCTION	CELL	PATHOLOGY
Melanosomes	Biosynthesis and storage of intracellular melanin. Transference of melanin to keratinocytes	Melanocytes, iris cells and retina cells	Oculocutaneous hypopigmentation
δ Granules	Storage molecules involved in the coagulation cascade	Platelets and megakaryocytes	Haemorrhagic diathesis
Cytyolytic granules	Intracellular degradation of macromolecules	T-cytotoxic lymphocytes and NK-cells	Immunodeficiency. Viral infections
Azurophil granule	Storage of lytic enzymes for bacterial destruction.Involved in pathological processes including inflammation.	Neutrophils and eosinophils	Neutropenia, immunodeficiency, bacterial infections
Basophil granules	Storage of histamine, serotonin, heparin, IL-4 and lysosomal proteases. Regulation of inflammation	Basophils and mast cells	Immunodeficiency, allergies
Lamellar bodies	Storing and secretion of surfactants for pulmonary function	Type II pneumocytes	Pulmonary fibrosis
Major histocompatibility complex class II compartments	Processing and incorporating antigens to cell membranes	B lymphocytes, macrophages, dendritic cells and other antigen presenting cells.	Immunodeficiency
Neuromelanin granules	Neuromelanin storage	Brain stem catecholaminergic neurons	Unknown
Ruffled Borders	Storage, activation and secretion acid hydrolases, used for bone resorption and remodeling	Osteoclasts	Osteoporosis
Weibel-Palade bodies	Storege and regulation of the secretion of haemostatic and proinflammatory factors (von Willebrand factor, P-selectin)	Endothelial cells	Hemorrhagic diathesis
Synaptic vesicles	Neutrotransmitters storage	Neurons	Abnormal behaviour

During the last decades, we have witnessed many remarkable advances in the knowledge of the basics of formation, function and molecular pathology of LRO, including HPS. Today, it is known that different subtypes of HPS (HPS-1 to HPS-9) arise from quite numerous mutations located in up to 9 different genes: *HPS1*, *AP3B1*, *HPS3*, *HPS4*, *HPS5*, *DTNBP1*, *BLOC1S3* and *BlOC1S6 (Gunay-Aygun M et al. 2004, Huizing M et al. 2008, Carmona-Rivera C et al. 2011, Masliah-Planchon J et al. 2013, Wei AH et al. 2013)* (Table 10). Except *AP3B1*, the rest of these genes encode proteins with an unknown function that are part of Biogenesis of Lysosome-related Organelles Complexes (BLOC), which are very ubiquitous structures located both anchored to cellular membrane and in a soluble cytoplasmic form (Figure 12) *(Wei AH et al. 2013)*.

Figure 12.Protein complexes involved in biogenesis and shuttle of Lisosome Related Organelles.



BLOC-1 regulates the traffic of vesicles in the endosomal system through its union to actin filaments of the cytoskeleton and participates in the early endosomal membrane and LROs fusion through its union to SNARE proteins. BLOC-1 interacts with other complexes like AP3 and BLOC-2 *(Huizing M et al. 2008)*. Mutations in BLOC-1 proteins are very infrequent, resulting in HPS-7, HPS-8 and HPS-9 subtypes that represent mild forms of HPS with limited clinical manifestations. So far, only 6 molecular alterations in subunits of this complex have been reported, two nonsense mutations in *BLOC1S6* causing HPS-9 *(Cullinane AR et al. 2011, Badolato R et al. 2012)*, one frameshift change and a simple deletion in *BLOC1S3* leading to HPS-8 *(Morgan NV et al. 2006, Cullinane AR et al. 2012)*, and 2 nonsense mutations in *DTNBP1* in two patients with HPS-7 *(Li W et al. 2003, Lowe GC et al. 2013)*.

BLOC-2 complex is a heterotrimer consisting of HPS3, HPS5 and HPS6 (Figure 12), although the involvement of other not yet identified proteins is not ruled out *(Huizing M et al. 2008)*. BLOC-2 interacts with BLOC-1 in the early endosomes and binds microtubules, clathrins and SNARE proteins to move throughout the cytoplasm. BLOC-2 complex is involved in transport of LRO components (e.g. TYR and TYRP1 enzymes in melanosomes from early endosomes to final LROs). BLOC-2 seems to be also involved in the secretion of lysosomes and other related granules, as suggested by the low lysosome secretion in mice platelets with HPS-5 and HPS-6 *(Huizing M et al. 2008)*. Patients with molecular alterations affecting BLOC-2 (*HPS3, HPS5* and *HPS6* genes) have a mild HPS phenotype (HPS-3, HPS-5, HPS-6) with moderate bleeding, variable hypopigmentation and sporadic granulomatous colitis, but do not show pulmonary fibrosis *(Gunay-Aygun M et al. 2004, Huizing M et al. 2008, Carmona-Rivera C et al. 2011, Masliah-Planchon J et al. 2013)*. To our knowledge, 9 molecular alterations have been reported in *HPS3 (Anikster Y et al. 2001, Huizing M et al. 2001, Boissy RE et al. 2005, Thielen N et al. 2010)*, 11 in *HPS5 (Zhang Q et al. 2003, Huizing M et al. 2004, Korswagen LA et al. 2008,*

Carmona-Rivera C et al. 2011) and 9 in *HPS6 (Zhang Q et al. 2003, Schreyer-Shafir N et al. 2006, Huizing M et al. 2009)*. Most of them are frameshift or nonsense mutations affecting the function of BLOC-2. As mentioned above, there is a 3.9 Kb deletion in *HPS3* with founder effect in the central area of Puerto Rico, where HPS-3 affects 1:4000 native people *(Anikster Y et al. 2001)*.

BLOC-3 complex is a heterodimer formed by HPS1 and HPS4, which shares no homology with other known proteins. The N- and C-termini of HPS1 interacts with the N-terminal and middle region of HPS4 to form the complex *(Carmona-Rivera C et al. 2013)*. Similar to other BLOCs, the precise function of BLOC-3 is not well known, but it is believed to be involved in lysosome and late endosome biogenesis and transport *(Huizing M et al. 2008)*. Mutations in HPS1 and HPS4 affecting BLOC-3 are the most frequent and the ones that give rise to the more severe forms of HPS, i.e. HPS-1 and HPS-4. In addition to other typical complications of this syndrome as hypopigmentation and hemorrhagic diathesis, these patients commonly suffer from granulomatous colitis (>30% cases) and pulmonary fibrosis (>80% cases in HPS-1 subtype). The reason for lung affectation is unclear, but it probably reflexes the relevant role of BLOC-3 in the biogenesis and function of lamellar bodies of pneumocytes *(Huizing M et al. 2008, Masliah-Planchon J et al. 2013, Wei AH et al. 2013)*. The clinical phenotype of HPS-4 is very similar to HPS-1 one, although usually less severe.

The *HPS1* gen, located in 10q24.2 is the most frequently mutated: 31 mutations have been described, including 2 amino acid change single mutations (p.L239P and p.L668P) and a majority of frameshift changes and nonsense mutations *(Oh J et al. 1996, Oh J et al. 1998, Shotelersuk V et al. 1998, Spritz RA et al. 1999, Anikster Y et al. 2001, Hermos CR et al. 2002, Gonzalez-Conejero R et al. 2003, Suzuki T et al. 2004, Ito S et al. 2005, Iwakawa J et al. 2005, Natsuga K et al. 2005, Merideth MA et al. 2009, Vincent LM et al. 2009, Wei A et al. 2009,*

Sandrock K et al. 2010, Sandrock K et al. 2010, Carmona-Rivera C et al. 2011, Carmona-Rivera C et al. 2011, Wei A et al. 2011). The aforementioned 16-pb duplication in exon 15 of *HPS1* with founder effect in northwest of Puerto Rico causes an extraordinary high prevalence or HPS-1 in this island. This HPS subtype is also common in Japanese, Chinese, Caucasian and non-Puerto Rican people, and it has been reported in few European cases *(Huizing M et al. 2008, Carmona-Rivera C et al. 2011, Masliah-Planchon J et al. 2013, Wei AH et al. 2013)*. Regarding the HPS-4 subtype, up to 13 mutations have been described in the *HPS4* gen, mostly nonsense and frameshift *(Suzuki T et al. 2002, Anderson PD et al. 2003, Bachli EB et al. 2004, Carmona-Rivera C et al. 2011, Jones ML et al. 2012, Gipson DS et al. 2013)*.

Unlike other genes causing HPS, *AP3B1* codifies the β3A subunit, namely AP3B1, of the adapter complex 3 (AP-3). This complex, synthesized in the Golgi apparatus, consists of four subunits that are differently combined in a form of AP-3 distributed very ubiquitously and a second AP-3 form with specific cerebral location. Both are believed to act in the recognition and selection of specific proteins such as LAMP or TYR that are necessary to the formation of new vesicles and for their transport throughout endosome/lysosome pathways *(Dell'Angelica EC 2009)*.

Molecular defects in *AP3B1* give rise to HPS-2, which is the only HPS subtype presenting with lymphohistiocytosis and immunodeficiency, and with a lower grade of albinism, hypopigmentation and bleeding. Immunodeficiency in HPS-2 patients has been related with the role of AP-3 in the formation and transport of lytic T lymphocyte granules *(Huizing M et al. 2008, Dell'Angelica EC 2009, Masliah-Planchon J et al. 2013)*. Up to 12 different mutations in *AP3B1* have been identified in HPS-2 patients, mainly frameshift and nonsense mutations *(Dell'Angelica EC et al. 1999, Huizing M et al. 2002, Clark RH et al. 2003, Enders A et al. 2006, Fontana S et al. 2006, Wenham M et al. 2010, Kurnik K et al. 2013)*.

Despite the high number of patients with different HPS subtypes and the wide amount of mutations identified, there is not a clear evidence of the link between these mutations and the severity of the clinical manifestations in each subtype of patients. It is important to mention that the genes described above might not be the only ones involved in HPS. Indeed, since the 70's murine models of HPS have been developed involving up to 15 candidate genes, and in some cases, the equivalent human genes are still unknown (Table 10). Research in these murine models have shed light on the biogenesis and function of LROs, and are promising to allow for better clinical characterization of these patients, including novel aspects like drug susceptibility and resistance to atherosclerosis *(Li W et al. 2004, Masliah-Planchon J et al. 2013, Wei AH et al. 2013)*.

Table 10. List of responsible genes for different types of HPS (in human and in mouse model) and mutations.

Human subtype HPS	Protein Complex	Gene	NCBI RefSeq	Chromosome location	Number of mutations	Murine Model
HPS-1	BLOC-3	*HPS1*	NM_000195	10q24.2	32	Pale ear
HPS-2	AP-3	*AP3B1*	NM_003664	5q14.1	12	Pearl
HPS-3	BLOC-2	*HPS3*	NM_032383	3q24	9	Cocoa
HPS-4	BLOC-3	*HPS4*	NM_022081	22q12.1	13	Light ear
HPS-5	BLOC-2	*HPS5*	NM_181507	11p15.1	11	Ruby eye-2
HPS-6	BLOC-2	*HPS6*	NM_024747	10q24.32	9	Ruby eye
HPS-7	BLOC-1	*DTNBP1*	NM_032122	6p22.3	2	Sandy
HPS-8	BLOC-1	*BLOC1S3*	NM_212550	19q13.32	2	Reduced pigmentation
HPS-9	BLOC-1	*BLOC1S6*	NM_012388	15q21.1	2	Pallid
-	BLOC-1	*BLOC1S5*	NM_001199323	6p24.3	-	Muted
-	BLOC-1	*BLOC1S4*	NM_018366	4p16.1	-	Cappuccino
-	BLOC-1	*BLOC1S1*	NM_001487	19p13.11	-	Kxd1-KO
-	AP-3	*AP3D1*	NM_001261826	19p13.3	-	Mocha
-	HOPS	*VPS33A*	NM_022916	12q24.31	-	Buff
-	Rab geranylgeranyl transferase alpha	*RABGGTA*	NM_004581	14q11.2	-	Gunmetal

2.3. Diagnostic approach to HPS patients: molecular and platelet characterization

While the clinical identification of many patients with HPS is facilitated by the typical symptomatology accompanying the bleeding diathesis, the laboratory demonstration of a platelet dysfunction related to a δ-granules deficiency, and the identification of the underlying molecular alteration can be difficult to obtain. In some, but not all, HPS patients, the semi-automatic PFA-100 test can show prolonged closures times *(Samuel CA et al. 2013)*, and light transmission aggregometry (LTA) is usually diminished and lack the second aggregation wave with weak platelet agonists such as ADP or epinephrine. However, neither PFA-100 nor platelet LTA are specific tests, so normal results do not exclude the diagnosis of HPS *(Favaloro EJ 2001, Barrowcliffe TW et al. 2006, Masliah-Planchon J et al. 2013)*.

Therefore, some additional tests are especially relevant to specifically evaluate the quantitative or functional defect of δ-granules, such as quantification of nucleotides content by lumiaggregometry or luminescence, the passive uptake of mepacrine and the assessment of CD63 expression by flow cytometry, or radioactive assays to determine the uptake/release of ^{14}C-5-hydroxytryptamine *(Hayward CP et al. 2006)*. Visual demonstration of δ-granules by electron microscopy (EM) remains the *gold standard* in HPS diagnosis *(Masliah-Planchon J et al. 2013)*. This can be performed by a simple whole mount assay with little manipulation of samples that allow to distinguish δ-granules as black dots due to their high density, or by a more complex EM analysis of platelet ultrastructure (thin sections) giving valuable information about the number and possible alterations in the morphology of these granules *(White JG 2003)*.

The molecular diagnosis may not be that simple; as previously stated mutations in 9 genes (*HPS1, AP3B1, HPS3, HPS4, HPS5, HPS6, DTNBP1, BLOC1S3 and BLOC1S6*) cause HPS in

humans. The large number of HPS culprit genes (>118 exons) and the lack of genotype-phenotype correlation, hamper the molecular diagnosis of HPS. For many years, the standard approach for the molecular diagnosis of HPS has been based on a HPS candidate gene analysis, considering *HPS1* the most frequently affected gene and also and potential clinical characteristics of patients such as greater severity, presuming a diagnosis to HPS-1 or HPS-4, or a immunodeficiency, which suggests HPS-2. This approach, arbitrary in some aspects, might not always be successful.

Additionally, there may be disease-causing genes not yet identified, this disorder could be often underdiagnosed and patients not be molecularly characterized. Current high-throughput DNA specific technologies provide a good alternative to address the molecular diagnosis of these patients. Several techniques like microsatellite genetic screening or next generation sequencing are being implemented in this setting *(Badolato R et al. 2012, Cullinane AR et al. 2012, Lowe GC et al. 2013)*.

2.4. Management of HPS patients

Similar to other IPFDs, the clinical management and treatment of patients with HPS must be done in specialized centers, individualized according to the severity of its clinical manifestations *(Barrowcliffe TW et al. 2006, Seligsohn U 2012)*. To date, no curative treatment exists and the use of aggressive alternatives like hematopoietic cell transplantation is restricted to very severe cases and must be preceded by an exhaustive risk-benefit evaluation*(Seligsohn U 2012)*. Patients should be advised to pay meticulous attention to oral and dental care, and to avoid drugs known as interfering with platelet function (such as aspirin, other non-steroidal anti-inflammatory agents, or serotonin reuptake inhibitors) and intramuscular injections. Mild

bleeding episodes might be effectively treated with antifibrinolytic agents. Desmopressin is not always useful in this context and it has the additional disadvantage of increasing the vasoconstriction due to high serotonin levels present in some patients with δ-granules deficiency. There is also limited experience in the use of recombinant factor VII in HPS. Platelet transfusion must be restricted to major bleeding episodes or previous to major surgery procedures, and in these cases the platelet products must be ideally HLA compatible to minimize the risk of alloimmunization *(Seligsohn U 2012, Masliah-Planchon J et al. 2013)*.

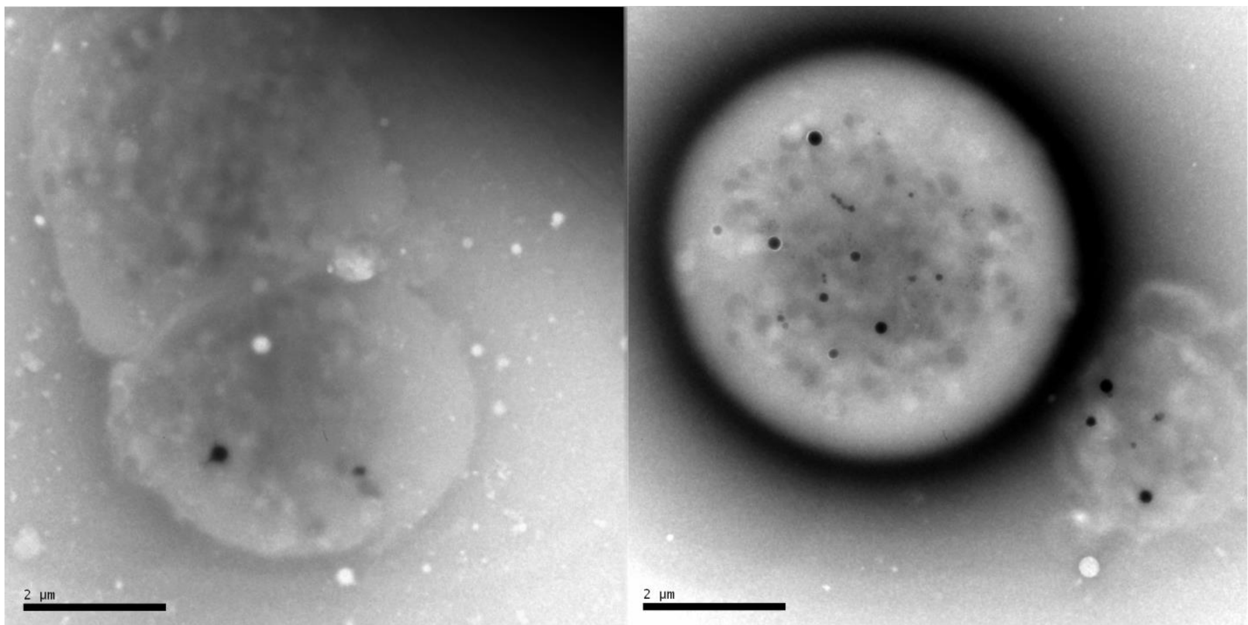
3. Case material

A 28 year old male with a family history of parental consanguinity was referred to our center with a clinical suspicion of HPS. He is the second of three children, and while the first brother is healthy, the youngest sister was diagnosed with juvenile idiopathic scoliosis. History revealed megaureter due to ureter ostium stenosis at birth. The patient suffered from excessive bleeding following urological surgery, as well as other hemorrhagic episodes, including epistaxis requiring cauterization, and excessive bleeding from small wounds. Examinations revealed oculocutaneous albinism, and nystagmus.

The hemoglobin and the white blood cell count were in range, and platelets were 159 x 10^9/L. Platelet function studies with the platelet function analyzer (PFA-100) demonstrated increased ADP (111 seconds; reference range, 57-100 seconds) and epinephrine (>300 seconds; reference range, 81-131 seconds) closure times. Platelet dysfunction was confirmed with platelet aggregometry studies that showed monophasic response after exposure to ADP (5 mM), and markedly impaired aggregation in response to epinephrine (5 mM), and 2 mg/mL collagen. There was an obvious decrease in the total uptake of ^{14}C-5-hydroxytryptamine (54% of

control values). In addition, upon stimulation with 25 mM TRAP we observed an important reduction in the platelets flow cytometric externalization of fluorescein-labelled CD63 (15.7% positivity in patient vs 73.6% in control) and negative mepacrine test in the patient. These findings are consistent with a storage pool deficiency with reduced dense bodies and consequent defects of secretion-dependent aggregation. Whole mount electron microscopy revealed no δ-granules in the patient´s platelets (Figure 13).

Figure 13. Whole mount of patient's platelets (A) and healthy control's (B).



Based on the clinical presentation and hematological findings consistent with HPS, we aimed to molecularly characterize this patient. We first considered studying *HPS1*, as he did not exhibit neutropenia or immunodeficiency, characteristic of HPS-2, and that he did present bleeding manifestations that are not typical of HPS-9. The entire coding region (20 exons and flanking regions) of *HPS1*, the most frequent gene being affected in HPS, were sequenced by Sanger method. Patient was found to be homozygous for a novel nonsense mutation c.844 G>T,

p.Glu204Stop located in exon 7. His parents were both found to be heterozygous for this mutation.

At present he is alive and well, except for mild bleeding, has not developed granulomatous enteropathic disease or pulmonary fibrosis, and his psychomotoric development is normal.

To our knowledge, this is the second case of molecular characterization of a HPS patient in Spain, with the previous patient also being described by our group *(Gonzalez-Conejero R et al. 2003)*. In that case, the patient was found to be a compound heterozygous, with the maternal allele inherited containing the insC974 mutation and a *de novo* mutation in exon 5 of the paternal allele also affecting *HPS1*, and he suffered from severe mental retardation, which is not characteristic of this disorder *(Gonzalez-Conejero R et al. 2003)*.

Despite, there is no curative approach for this disease, increased awareness and early identification of HPS patients may be useful for their clinical management. With a high degree of clinical suspicion, the study of this disease, its causative genes and the phenotype associated is of enormous interest to both cell biology and medicine.

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Microsatellite markers as a rapid approach for autozygosity mapping in Hermansky-Pudlak syndrome: Identification of the second HPS7 mutation in a patient presenting late in life

Hermansky-Pudlak syndrome (HPS) is an autosomal recessive disorder characterized by oculocutaneous hypopigmentation and a bleeding diathesis caused by a lack of dense granules in platelets. HPS is genetically heterogeneous with variable skin, hair, and iris hypopigmentation, and visual impairment. In addition, some forms of HPS give rise to granulomatous colitis and pulmonary fibrosis *(Davies BH et al. 1976)*. HPS has been associated with mutations in 9 human genes: HPS1 (mouse model pale ear), AP3B1/HPS2 (pearl), HPS3 (cocoa), HPS4 (light ear), HPS5 (ruby-eye 2), HPS6 (ruby-eye), HPS7/dysbindin (sandy), HPS8/ reduced pigmentation and HPS9/pallid *(Dell'Angelica EC 2004, Wei ML 2006)*. In addition, genes have been identified for a further 6 mouse models of HPS (mocha, gunmetal, ashen, muted, buff, and subtle gray) *(Dell'Angelica EC 2004)*. In general, HPS is a rare disorder, but HPS1 has a high prevalence in northwest Puerto Rico (1/1800) due to a founder mutation *(Witkop CJ et al. 1990)*.

The large numbers of potential HPS culprit genes (>118 coding exons), together with minimal guidance offered by the genotype-phenotype correlations between HPS genes, provides a significant challenge for the molecular diagnosis of these disorders. In cases with suspected consanguinity, autozygosity mapping can be used as a high speed tool to prioritize mutation screening for a specific HPS gene *(Morgan NV et al. 2006)*. Further, this can be facilitated using microsatellite markers flanking the HPS genes to exclude genes in the case of heterozygous markers/haplotypes and prioritize direct sequencing of specific genes where autozygosity is noted. We illustrate the proof of principle of this approach in the identification

of the second case of a mutation in the HPS7 (encoding Dysbindin) gene in a patient with a bleeding history and hypopigmentation. A 77 year old Caucasian female was referred to the bleeding disorder clinic with a lifelong bleeding tendency (Figure 14A). There was no history of a bleeding disorder in this participant's half siblings, parents or children. Her parents were first cousins. She had pale skin and hair and had reduced visual acuity and nystagmus throughout life. She also had spontaneous epistaxes as a teenager which lasted for several hours and required medical attention, and several episodes of prolonged bleeding from minor injuries that required surgical hemostasis. She bled for several days after dental extractions in her twenties and thirties and required packing of the tooth sockets to control the bleeding. She had menorrhagia from menarche which eventually necessitated a hysterectomy at the age of 37. She experienced heavy post-partum bleeding after all of her three vaginal deliveries. Surgical procedures in this patient, including surgery for an ectopic pregnancy in the 1960s, abdominal hysterectomy and salpingo-oophrectomy in 1971 and excision of a lipoma from her back in 2002, were followed by prolonged bleeding requiring blood product transfusion. She had two episodes of severe per-rectal bleeding. The first one occurred in 1979 and was attributed to a rectal polyp which was surgically removed. She bled significantly after this procedure and required a platelet transfusion. A further episode occurred in 2009 which was attributed to Crohn's disease. During this episode the patient required red cell and platelet transfusions and tranexamic acid and was being considered for a right hemicolectomy at the time of her hematology referral. Colonic biopsies showed florid granulomatous inflammation with no co-existing infection (Figure 14B). Acid fast bacilli were absent. She was reviewed by a respiratory physician in 2010 and had no evidence of any respiratory disease, with normal lung function tests (FEV1 100% predicted, FVC 95% predicted, TLC 80% predicted and KCO 105% predicted, all within normal ranges). A chest X ray showed no active lung disease, and a CT thorax with

contrast showed no convincing features of fibrosis.

The patient gave informed consent and was recruited to the GAPP (Genotyping and Phenotyping of Platelets, NIHR ID 9858, Regional Ethics Committee reference 06/ MRE07/36) study. Platelet function testing was performed on platelet rich plasma (PRP) by lumiaggregometry using Chronolume® to assess secretion. The response to intermediate concentrations of PAR-1 peptide (30 µM) and collagen (1 µg/ml) was reduced relative to the control on the day and also a panel of over 70 healthy volunteers. Normal aggregation was observed at higher concentrations of these agonists (not shown). A lack of dense granule secretion with the entire panel of agonists tested *(Dawood BB et al. 2012)*was noted, as illustrated for PAR-1 and PAR-4 peptides in Figure 14C and D. The lack of platelet ATP secretion was consistent with an absence of platelet dense granules, and in combination with the patient's clinical features were diagnostic of HPS. Such platelet function testing has previously been shown to be successful in diagnosing other HPS patients with complete lack of secretion from platelet dense granules *(Morgan NV et al. 2006, Dawood BB et al. 2012)*.

DNA was extracted from peripheral blood and genetic studies were undertaken. As the patient's parents were related by blood, we were able to apply autozygosity linkage mapping by genotyping several microsatellite markers flanking all of the known human HPS genes. This was carried out to prioritize a limited number of HPS genes for direct sequencing. Strikingly the only HPS locus that displayed autozygosity for both flanking markers and over an extended region of genetic distance was the HPS7 locus. Therefore the most likely candidate HPS gene was HPS7/Dysbindin on chromosome 6p22.3. The 10 coding exons of DTNBP1 (encoding dysbindin) including exon-intron boundaries were PCR-amplified and sequenced. Sequencing of DTNBP1 revealed a homozygous nonsense mutation in exon 4 (c.177 G>A; p.Trp59Stop) confirming a diagnosis of HPS type 7 (Fig 14E).

Dysbindin is important for normal platelet-dense granule and melanosome biogenesis and is mutated in the sandy (sdy) mouse *(Swank RT et al. 1991)*. Dysbindin is a component of the biogenesis of lysosome-related organelles complex 1 (BLOC-1) which regulates trafficking to lysosome related organelles. The BLOC-1 complex is ubiquitously expressed and along with Dysbindin contains seven predicted coiled-coil-forming proteins (pallidin, muted, cappuccino, snapin, BLOC1S1, BLOC1S2 and BLOC1S3) *(Li W et al. 2003)*, all of which are associated with Hermansky-Pudlak syndrome in mice.

The only previously reported HPS7 mutation in humans was a homozygous nonsense mutation (p.Q103X) found in a 48 year old Portuguese woman with oculocutaneous hypopigmentation, ease of bruising and a bleeding tendency. She had mild shortness of breath on exertion and reduced lung compliance but otherwise normal pulmonary function *(Li W et al. 2003)*. Although the patient described here has been diagnosed with Crohn's disease, it is likely that this represents granulomatous colitis given the histological findings reported on colonic biopsy. She has no evidence of pulmonary fibrosis. Colitis has been reported in HPS1, HPS3 and HPS4 and pulmonary fibrosis in HPS1, HPS4 and HPS2 *(Hermos CR et al. 2002, Anderson PD et al. 2003, Gochuico BR et al. 2012)*.

In conclusion, we report a novel mutation in the HPS7 (Dysbindin) gene causing a premature stop codon which was rapidly identified following autozygosity mapping using microsatellite markers. This high speed technique provides a rapid approach to identify candidates HPS genes for Sanger sequencing in order to identify a disease causing mutation. An alternative method to identify the genetic defect in this patient would have been to utilize second generation sequencing strategies such as whole exome sequencing or custom built arrays, however this would have been far more costly and time consuming given the consanguinity in this family which we exploited to narrow down the culprit gene. Despite

having had numerous hospital visits and lifelong excessive bleeding, the cause of this patient's bleeding was not elucidated until she was in her eighth decade. Mild inherited platelet disorders should therefore be considered in patients presenting with excessive bleeding later in life.

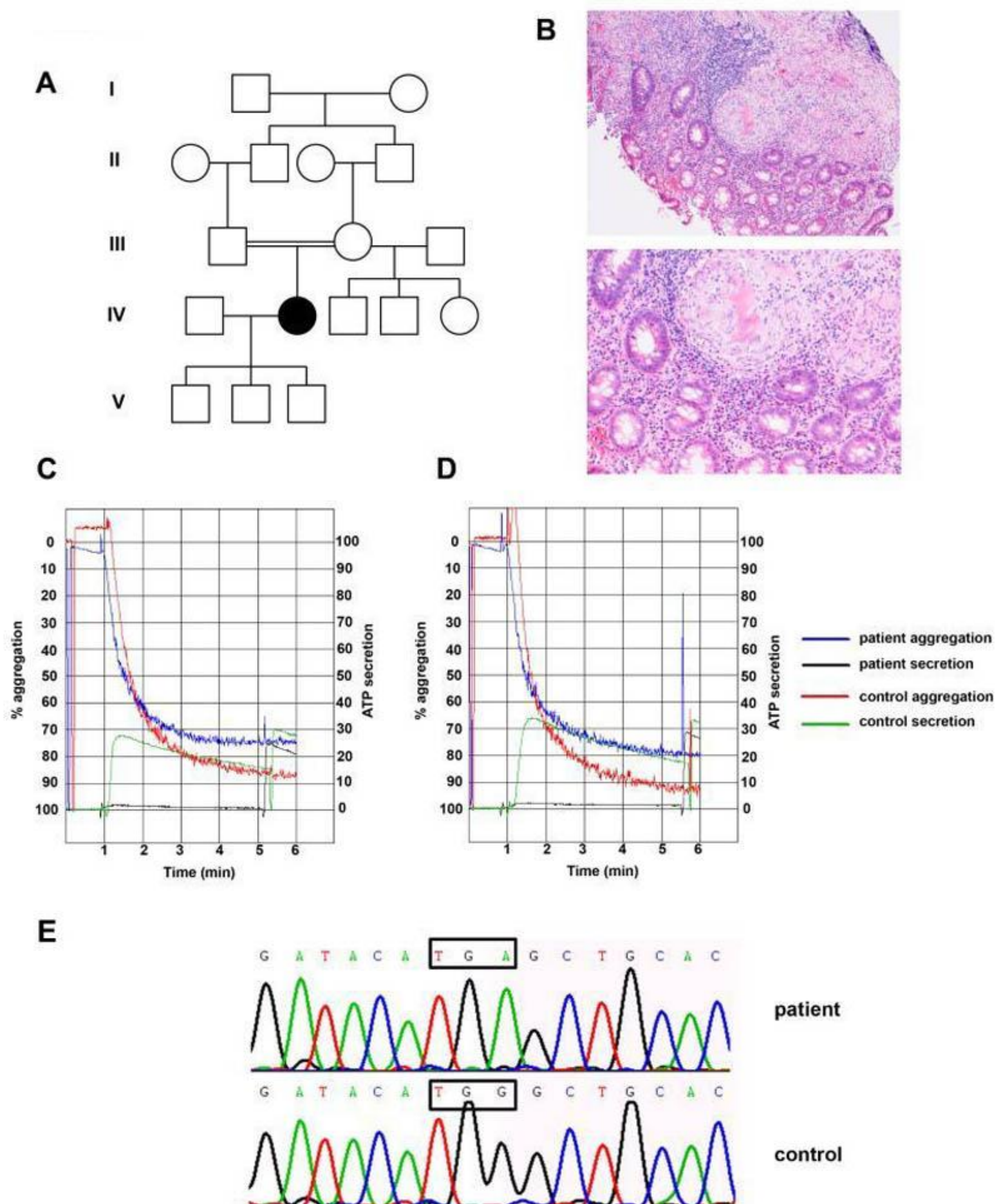

Figure 14. (A) Pedigree of a consanguineous family with Hermansky-Pudlak syndrome. The affected individual is represented by a solid symbol. (B) Images of a colonic biopsy from the patient in low power (upper panel) and high power (lower panel). Both images show inflammatory infiltrates and granulomata with numerous giant cells, alongside normal bowel mucinous glands. No caseous necrosis is seen, and special stains showed no evidence of micro-organisms (including mycobacteria).(C and D) Absence of secretion (black trace) to high doses of PAR-1 peptide 100µM (C) and PAR-4 peptide 500µM (D) in this patient. Left sided Y axis depicts percentage aggregation, and right sided Y axis represents platelet ATP secretion assessed using Chronolume®. 1.6nmol of ATP standard were added to each cuvette in order to calculate absolute secretion and secretion normalized to platelet count in PRP for both patient and control. (E) Identification of a homozygous single base substitution (c.177 G>A) in Dysbindin leading to a premature stop codon (p.Trp59Stop). Sanger sequencing showing wild-type and mutant *DTNBP1* sequence traces. The position of the mutation is indicated by the boxed regions.

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Chapter IV

An atypical IgM class platelet cold agglutinin induces GPVI-dependent aggregation of human platelets

1. Introduction

The platelet cold agglutinins (PCA) are rare and poorly characterized phenomenons *(Watkins SP, Jr. et al. 1970)*, most frequently recognized as incidental pseudothrombocytopenia. PCAs are usually detected due to *in vitro* platelet clumping in ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood leading to spuriously low platelet counts in automated cell counters and occur in up to 0.15 % of all blood counts performed *(Vicari A et al. 1988, Garcia Suarez J et al. 1991, Bizzaro N 1995, Muniz-Diaz E et al. 1995, Zandecki M et al. 2007, Froom P et al. 2011)*. Clumping is generally mediated by autoantibodies of IgM and IgG classes that recognize cryptic epitopes exposed within platelet surface proteins after Ca^{2+} chelation and low temperature *(Onder O et al. 1980, Pegels JG et al. 1982, von dem Borne AE et al. 1986)*. More rarely, IgM-mediated pseudothrombocytopenia occurs at 37ºC *(Bizzaro N 1995)* or in the presence of other anticoagulants such as citrate or heparin *(van Vliet HH et al. 1986, von dem Borne AE et al. 1986, Cunningham VL et al. 1992, Kuijpers RW et al. 1992, Schimmer A et al. 1998, Akbayram S et al. 2011)*. Pseudothrombocytopenia is seen in otherwise healthy subjects independent of age, gender, or specific acquired situations. It does not necessarily increase the risk of autoimmune diseases, hemorrhagic or thrombotic processes, and in some subjects pseudothrombocytopenia extends for decades without pathological effects *(Bizzaro N 1995)*. However, coincidental pseudothrombocytopenia has been described in patients with various diseases including autoimmune and inflammatory diseases, tumors, metabolic syndromes and infections *(Hoyt RH et al. 1989, Irisawa A et al. 1992, Takeuchi T et al. 1993, Mori M et al. 2000, Hsieh AT et al. 2003, Lambertucci JR et al. 2011)*, following surgery *(Wenzel F et al. 2011)*, stem cell transplantation *(Gillis S et al. 2003)*, or treatment with drugs *(Casonato A et al. 1990, Sane DC et al. 2000, Yoshikawa H 2003, Beyan C*

et al. 2010, Albersen A et al. 2013). Autoantibodies are generally acquired in patients with pseudothrombocytopenia, and often alleviated with disease resolution or cessation of therapy *(Bizzaro N 1995)*.

Generally, pseudothrombocytopenia does not require clinical monitoring or treatment. However, failure to recognise pseudothrombocytopenia can lead to unnecessary monitoring, inappropriate platelet transfusions and other treatments *(Onder O et al. 1980, Solanki DL et al. 1983, Payne BA et al. 1984, Nilsson T et al. 1986, Carrillo-Esper R et al. 2004, Lau LG et al. 2004, Sharma A et al. 2011)*. A higher risk of major bleeding in patients with pseudothrombocytopenia undergoing cardiac surgery under hypothermic conditions, means that timely recognition of this condition is critical in these patients *(Bizzaro N 1999, Wilkes NJ et al. 2000, Arat-Ozkan A et al. 2004, Nair SK et al. 2007, Kocum TH et al. 2008)*.

Here, we report an unusual mechanism of action of a singular IgM type PCA, identified in a patient with chronic moderate thrombocytopenia and apparent EDTA-independent pseudothrombocytopenia, which induced activation and aggregation of autologous and allogeneic platelets via engagement of platelet GPVI collagen receptor and activation of Src kinases and spleen tyrosine kinase (Syk) signaling pathways.

2. Subjects and Methods

A 37-year-old woman (the proposita) with lifelong mild bleeding diathesis and chronic moderate thrombocytopenia (≈100 $x10^9$ platelets L^{-1}) was referred to our facility with clinical suspicion of Glanzmann's Thrombasthenia (GT) due to prolonged closure times (CT>300 s) in platelet function analyser-100 (PFA-100®) using citrated whole blood and collagen/epinephrine (CEPI) and collagen/ADP (CADP) cartridges, and abnormal aggregation responses to all agonists

by Multiplate® testing. Complete blood count performed using a hematological analyzer XS-1000i (Sysmex Europe GmbH, Norderstedt, Germany) revealed normal parameters except for significant thrombocytopenia (5 x 10^9 platelets L^{-1}) in citrated blood and mild thrombocytopenia in EDTA-anticoagulated blood (100 x 10^9 platelets L^{-1}) suggestive of uncommon EDTA-independent pseudothrombocytopenia. There were no alterations in kidney and liver function. Levels of C3, C4, C reactive protein and immunoglobulins were normal, and antinuclear, cardiolipin, and antiphospholipid antibodies were undetectable.

Blood samples were also obtained from healthy volunteers and from patients with well-described GT, GPVI deficiency or afibrinogenemia. All subjects gave written informed consent to participate in this study, which complied with the Helsinki Declaration and was formally approved by the Ethics Committee of Hospital Universitario Reina Sofía.

2.1. Reagents

Phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) against platelet granule proteins P-selectin (CD62) and granulophysin (CD63), and PE-conjugated anti-human IgG and fluorescein isothiocyanate (FITC)-conjugated anti-human IgM were from Becton Dickinson (San Jose, CA). LJ-P3 anti-GPIbα and LJ-CP8 anti-αIIbβ3 complex mAbs were provided by Dr. ZM Ruggeri (Scripps Research Institute, La Jolla, CA), anti-active $\alpha_{IIb}\beta_3$ single chain (sc) antibody was from Professor K Peter (Baker IDI, Melbourne, Australia) *(Schwarz M et al. 2006)* and anti-VWF mAb NMC-4 from Dr. L De Marco (Centro di Riferimento Oncologico, Aviano, Italy). Anti-GPVI mAb 1G5 was produced as described *(Al-Tamimi M et al. 2009)*, and anti-FcγRIIa function-blocking IV.3 mAb was a gift from Dr. RH Aster (Blood Center of Southeastern Wisconsin, Milwaukee, WI). 5-OH-^{14}C-labelled tryptamine (^{14}C-serotonin) was from GE Healthcare

(Barcelona, Spain). A recombinant ectodomain of GPVI fused with the Fc portion of IgG was kindly provided by Dr. M Moroi (University of Cambridge, UK) *(Miura Y et al. 2002)*. Platelet agonists, platelet inhibitors, and other reagents were commercially available.

2.2. Blood Sampling and Preparation of Platelet and Plasma Samples

Blood samples were collected from the proposita, other patients and healthy volunteers, by routine venipuncture in tubes containing standard concentrations of EDTA, sodium citrate, acid citrate dextrose (ACD) or lithium heparin as anticoagulants, or in empty tubes for preparation of serum. Platelet-rich-plasma (PRP) and platelet-poor plasma (PPP) were separated from blood samples by stepwise centrifugations at 140 x *g* for 10 min and then 1200 x *g* for 20 min at room temperature (RT). Serum was obtained from non-anticoagulated blood by incubation at 37ºC for 30 min followed by centrifugation (1200 x *g*, 20 min) and heat-inactivation (56ºC for 30 min). PPP and serum aliquots were stored frozen at -80ºC until used in platelet aggregation/activation assays, or analyzed for soluble GPVI (sGPVI) levels by enzyme-linked immunosorbent assay (ELISA) *(Al-Tamimi M et al. 2009)*. In some studies, platelets from platelet concentrates (PCs) *(Plaza EM et al. 2014)*, or washed platelets resuspended in Tyrode´s buffer *(Guerrero JA et al. 2005)* were used.

2.3. Platelet Function Assessment

PFA-100® tests (CADP and CEPI cartridges) were performed using citrated-blood samples from the proposita or healthy donors according to manufacturer's instructions (Siemens Healthcare, Barcelona, Spain).

2.4. Sensitization of Allogeneic Platelets with Plasma or Serum from the Proposita

Whole blood or PRP were incubated with patient or control plasma or serum for 1 h at RT, unless otherwise stated. In some experiments 5 mM EDTA, LJ-CP8 antibody (20 μg/mL), other antibodies, or selected platelet inhibitors, were included to evaluate their effect on platelet aggregation/activation induced by patient plasma/serum. In platelet activation assays, anti-CD62*PE or anti-CD63*PE were added in the mixtures, or platelets labelled with ^{14}C-serotonin were used.

2.5. Lactic Dehydrogenase (LDH) and TXB_2 Determination, ^{14}C-Serotonin Release Assays

LDH levels in plasma or in the supernatant of platelet-plasma mixtures were determined using a COBAS C701 biochemical analyzer (Roche, Basel, Switzerland). TXB2 levels, as an index of TXA2 generation, was measured by enzyme immunoassay (GE Healthcare, Barcelona, Spain). PRP from patients or healthy subjects, or PC samples were mixed with 14C-serotonin and washed *(Holmsen H et al. 1989)*, then incubated with plasma, serum or other agonists, and the levels of 14C-serotonin released into the supernatant were determined *(Guerrero JA et al. 2005)*.

2.6. Flow Cytometric Assessment of Platelet Activation and GP Expression

Platelet populations were identified by flow cytometry using a FACscalibur instrument and CellQuest software (Becton Dickinson) on the basis of forward and side scatter properties, and binding of antibodies against unique platelet markers *(Plaza EM et al. 2012)*. Levels of GPIbα, GPIX, αIIbβ3, α2 and GPVI, were assessed by binding of specific antibodies (10 μg/ml) against CD42b, CD42a, CD61, CD41, CD49b or GPVI, respectively. Platelet activation was

evaluated by P-selectin and granulophysin expression on the platelet surface.

2.7. Analysis of Antiplatelet Antibody

Both platelet associated Ig (PAIg) and antiplatelet plasma antibodies were evaluated in patient samples. For the PAIg test, washed platelets (6 x 10^6 per 50 μL) from the proposita or from a healthy donor (background binding) were incubated at RT for 30 min with saturating levels of PE-conjugated anti-human IgG or FITC-conjugated anti-human IgM. To evaluate antiplatelet antibodies in patient plasma, control washed platelets (1.5 x 10^7 in 50 μL) were mixed with 50 μL heat inactivated serum from the patient or a healthy control for 30 min at RT, washed, and incubated for 30 min at RT with saturating levels of PE-conjugated anti-human IgG or FITC-conjugated anti-human IgM. Bound fluorescence activity was quantified by flow cytometry. To permit quantitative comparison of frequency distributions of the sample and a reference range, data were analysed using the Kolmogorov-Smirnov statistical test. Additionally, the proposita's serum was tested for antibodies directed against HLA-1 class antigen or human platelet antigens (HPA) on platelet GPs Ib/IX, Ia/IIa (HPA 5a and 5b), or αIIbβ3 (HPA-1a/1b, HPA-3a/3b, HPA-4a/4b), using the immunoassay PAK® 12 (GTI Diagnostics, Waukeha, WI, USA).

2.8. Depletion of IgM and IgG from Serum

Serum samples from the patient were depleted of IgM and IgG by successive elutions through a mannose binding protein (MBP) agarose column (Thermo Scientific Pierce IgM Purification Kit, Rockford, IL) and a HiTrap Protein G column using an Äkta Purifier (GE Healthcare, Barcelona, Spain). Depleted sera contained less than 5% of original IgG and about 20% of the original IgM level as assessed by nephelometry using an Immage 800 device

(Beckman Coulter Inc., Brea, CA) and by western blot (not shown).

2.9. Platelet Ultrastructural Analysis by Electron Microscopy

Samples from PCs were incubated with patient plasma, in the absence or in the presence of LJ-CP8 or platelet agonists, fixed in 1.25% glutaraldehyde, washed and post fixed in 1% osmic acid containing 1.5% potassium ferrocyanide, dehydrated using graded alcohols and propylene oxide and embedded in Epon as described *(Navarro-Nunez L et al. 2011)*. Embedded samples were sectioned, stained, and visualized using a Philips Tecnai 12 transmission electron microscope and a Megaview III camera (FEI, Hillsboro, OR).

3. Results

3.1. Evidence of Spontaneous Platelet Clumping and Platelet Activation

Across a 5-year period we monitored the platelet count in a 37-42 year old female with a history of bleeding. In all evaluations, she demonstrated mild thrombocytopenia in EDTA-anticoagulated blood (100.1 ± 4.9 x 10^9 platelets L^{-1}; mean platelet volume 10.2 ± 3.3 fL; n=11), and severe thrombocytopenia in parallel blood samples collected in citrate (8.4 ± 2.1 x 10^9 platelets L^{-1}; n=11), ACD or heparin. Similar samples from a healthy donor showed platelet counts within normal ranges in all anticoagulants (Figure 15A). The reduction in the patient platelet count in citrated blood occurred rapidly at RT and less efficiently at 37°C (Figure 15B), and was inhibited by functional blockade of $\alpha_{IIb}\beta_3$ but not GPIbα nor VWF (Figure 15C). Analysis of blood smears from citrated blood from the patient maintained at RT revealed platelet clumps (not shown). These data suggested that patient blood contained a temperature-dependent

clumping factor that was sensitive to EDTA but insensitive to other anticoagulants.

PFA-100® testing of blood from the proposita produced abnormal closures times (CT) for both CEPI and CADP cartridges when performed using citrated blood maintained at RT for 1 h after phlebotomy (259s and 203s, respectively; normal range 76-131s and 57-100s, respectively). CT were shorter when tests were performed in 37°C-warmed citrate-blood immediately after drawing (CEPI CT=185s, and CADP CT=121s).

As platelet receptor levels may influence CTs, and to assess whether patient platelet activation coincided with the observed platelet clumping, we examined levels of platelet proteins and activation markers by flow cytometry. Patient platelets displayed levels of αIIbβ3 complex (Fig. 15D) and GPIX and α2 integrin (data not shown) that were similar to levels observed on PRP from a healthy donor. However, patient platelets were significantly deficient in GPVI (~50%) and GPIbα (30-45%) expression (Figure 15D), receptors that are known to be metalloproteolytically shed from platelets upon platelet activation. Patient platelets also showed abnormally high levels of P-selectin (60 ± 23%, n=6) and CD63 (49 ± 23 %, n=4), compared with a healthy donor (P-selectin positive platelets 12 ± 8 %, n=6; CD63 positive platelets 2 ± 2 %, n=4). EDTA plasma samples from the proposita demonstrated significantly elevated levels of TXA2 compared with parallel samples from healthy subjects (100.5 ± 49.5 vs. 0.7 ± 0.4 g-6L-1, n=2). Together these data imply an on-going activation of patient platelets in vivo.

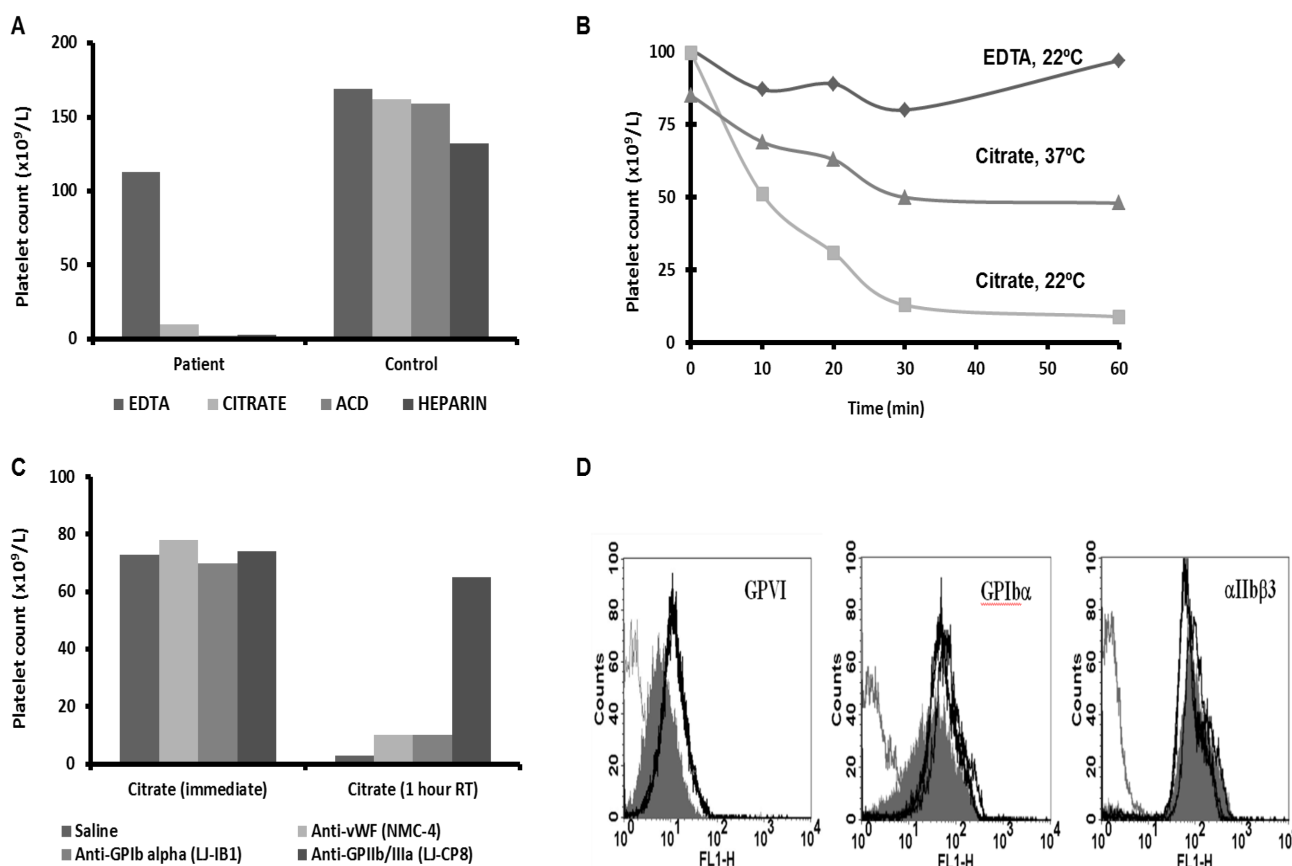


Figure 15. Patient platelet counts under different conditions and expression of platelet membrane glycoproteins. (A) Platelet counts were measured in blood collected from the patient or a healthy donor (control) on the same day in different anticoagulants. (B) Blood collected in citrate and maintained at 22°C or 37°C or in EDTA at 22°C was monitored for platelet count at various times over 1 h. (C) Blood collected in citrate from the patient was treated with 20 μg/ml of function blocking antibodies against αIIbβ3 (LJ-CP8), GPIbα (LJ-Ib1) or VWF (NMC-4) and blood counts were performed immediately or after 1 h incubation at RT. Plots A-C correspond to a representative experiment. (D) Levels of GPVI, GPIbα or αIIbβ3 assessed by flow cytometry in platelets from the patient (grey filled histograms) or three healthy donors (dark line, unfilled histograms) analysed on parallel. Plots also show nonspecific binding of secondary antibody (grey line, unfilled histogram).

3.2. Identification of an IgM anti-platelet autoantibody

We investigated whether the clumping factor was an antiplatelet autoantibody. Antiplatelet IgG antibodies could not be detected by direct or indirect flow cytometry assays. However, both PAIgM and IgM antiplatelet antibody in patient serum were identified (Figure 16) and frequency distribution curves were confirmed to be significantly different from reference curves using the Kolmogorov-Smirnov statistical test. Screening of patient serum

using the immunoassay PAK® 12 failed to detect antibodies against common autoantigens (HLA-1, GPIb/IX, $\alpha 2\beta 1$ or $\alpha IIb\beta 3$). Taken together these data indicate that the patient has an antiplatelet IgM that binds to an unidentified platelet antigen in a manner that activates platelets in citrated blood.

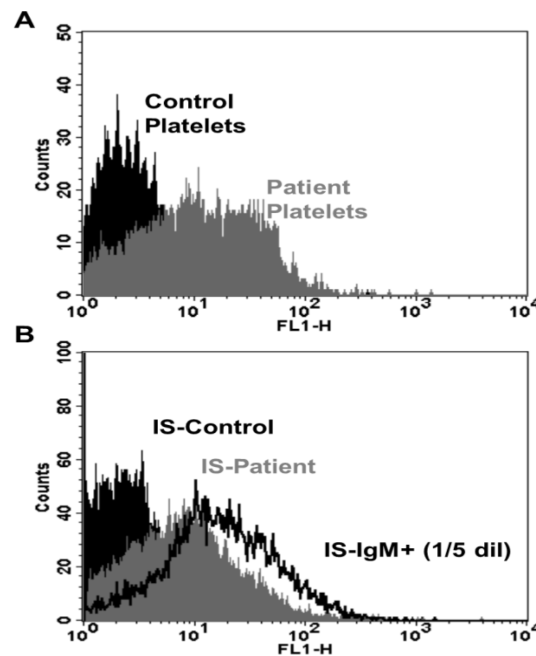


Figure 16. Anti-platelet IgM detected on patient platelets and in serum from the patient. (A) Washed platelets from a healthy donor (control) or the patient were mixed with FITC-conjugated anti-human IgM and analysed by flow cytometry. (B) Washed platelets from a healthy donor were sensitized with inactivated serum (IS) isolated from an isogroup donor (IS-control), or IS from the patient (IS-Patient), or IS from a patient with a confirmed anti-platelet IgM (IS-IgM) that had been diluted 5-fold. Bound antibody was detected by incubation with FITC-labelled anti-human IgM and analysed by flow cytometry.

3.3. An IgM within patient plasma and serum reduces platelet count and activates suspensions of allogeneic platelets

To determine whether the platelet aggregation activity was limited to patient platelets, patient plasma or serum samples were mixed at RT for 1 h with citrated whole blood, PRP or washed platelets from healthy donors or PCs, and platelet count was measured in an automated analyser. Patient citrated plasma or serum routinely induced a significant decline in platelet count (Figure 17A) that was not caused by lysis of platelets as LDH levels in the

supernatants remained stable in all incubations (data not shown). The platelet count for washed platelets, PRP from a patient with congenital afibrinogenemia, formalin-fixed platelets, or platelets that were pretreated with LJ-CP8 to block $\alpha_{IIb}\beta_3$ function did not fall after incubation with patient serum indicating that functional platelets, $\alpha_{IIb}\beta_3$ and fibrinogen were all essential for aggregation. PRP from either GT patients (n = 4) or from a patient with severe platelet GPVI deficiency (as determined by flow cytometry and western blot [data not shown]), also did not show a decrease in their platelet count in response to proposita plasma or serum (Figure 17A), confirming that the plasma- or serum-mediated drop of the platelet count was due to platelet aggregation, implying that GPVI may be the antigenic target of the patient IgM autoantibody.

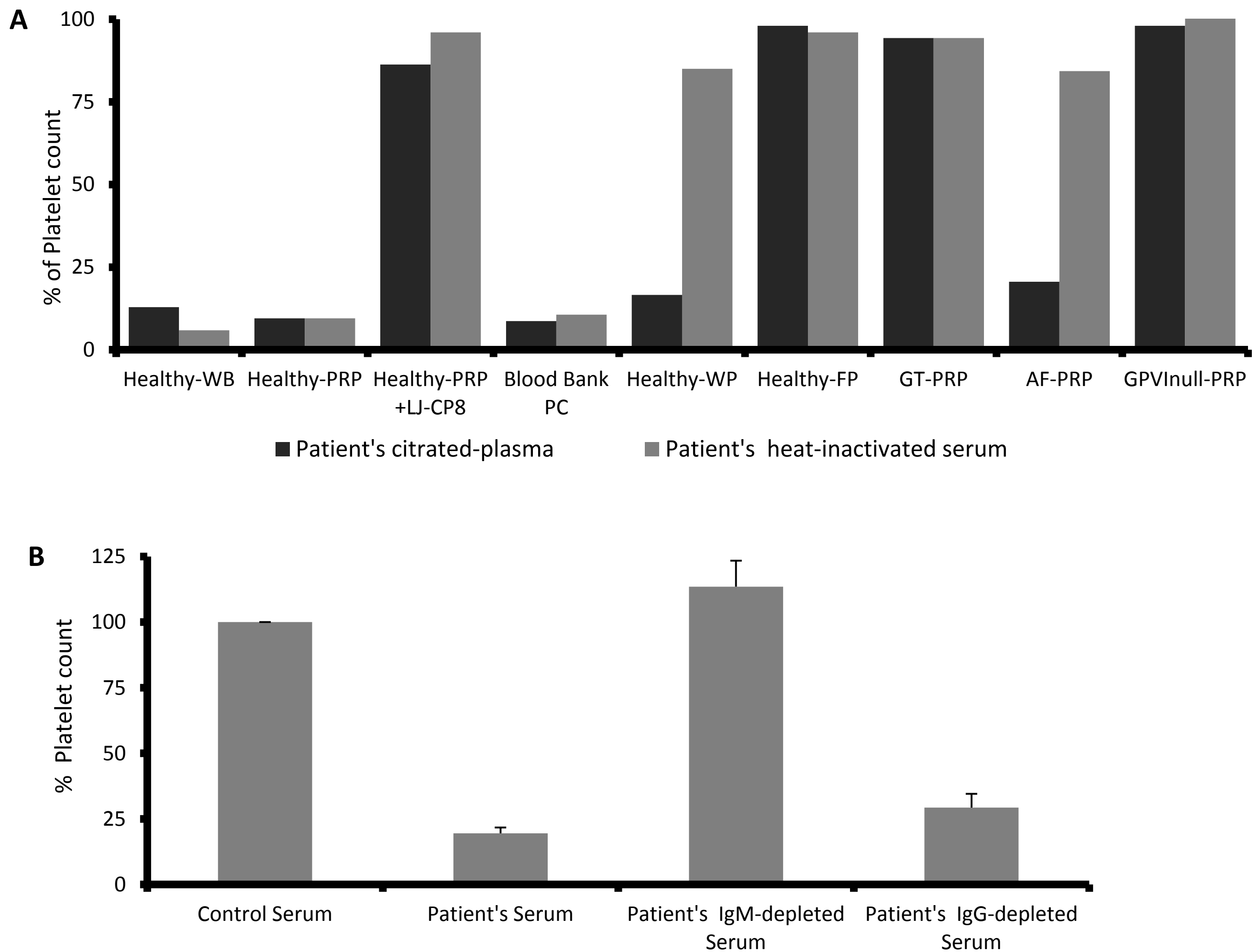


Figure 17. Effect of patient plasma or serum and Ig-depleted serum in allogeneic platelets. Platelet samples were incubated for 1 h at RT in the presence of citrated-plasma or heat-inactivated serum from the proposita (A), or with the proposita IgM-depleted serum or with ABO matched plasma or serum from a healthy volunteer (B). Platelet counts were determined using an automated hematological counter, and expressed as percentage of platelet counts in samples incubated in parallel with control plasma or serum. (A) Representative data from at least two experiments using different donors, except for PRP samples from afibrinogenemia (AF-PRP) and a GPVI-deficient patient (GPVInull-PRP) which were from single patients. (B) Depletion of IgM, but not of IgG, from patient serum minimised serum-induced aggregation of allogeneic platelets. Abbreviations: WB: citrated whole blood; PRP: citrated platelet-rich plasma; PC: platelet concentrate (35% plasma-65% platelet additive solution); WP: washed platelets; FP: washed platelets formalin-fixed; GT: Glanzmann Thrombasthenia; AF: congenital afibrinogenemia; GPVInull: patient with no detectable platelet expression of GPVI.

Table 11 summarises the consequences of incubation of platelets from healthy donors, GT patients or congenital afibrinogenemia with patient or control plasma at RT in the presence of LJ-CP8 to prevent platelet aggregation. Treatment with patient plasma resulted in a significant percentage of platelets expressing P-selectin and CD63, similar to levels achieved by

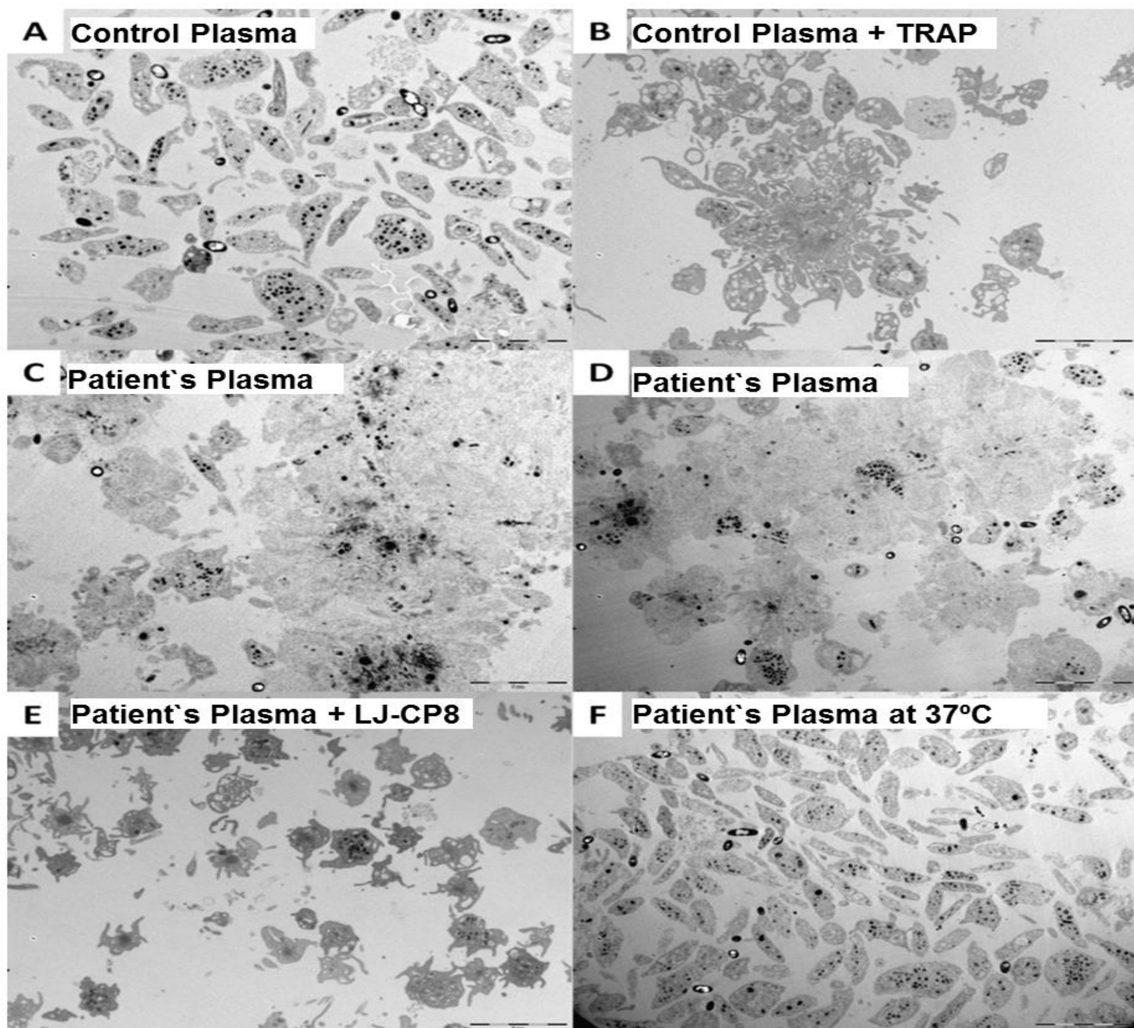
platelet agonists such as 25 μM thrombin receptor-associated peptide (TRAP) (data not shown). This effect was not seen when the experiment was performed at 37°C or when PRP from a GPVI-deficient patient was used. Incubation with the proposita’s plasma induced degranulation as measured by ^{14}C-serotonin release from pre-labelled healthy donor or GT platelets, and generation of TXA_2 production. Visually, platelet activation and aggregation was apparent by thin section electron microscopy in incubations carried out at RT but not at 37ºC (Figure 18A-F) and platelet shape change was still evident when platelet aggregation and/or fibrinogen binding to $\alpha_{IIb}\beta_3$ was disrupted either by inclusion of LJ-CP8 or using PRP from an afibrinogenemia patient or a GT patient.

Table 11. Platelet activation induced by exposure to plasma or heat-inactivated serum from patient.

	% CD62+ platelets		**% CD63+ platelets**		**% ^{14}C –Serotonin release**		**TXA_2 (ng/mL)**	
Plasma /Serum / **PRP**	**Control**	**Patient**	**Control**	**Patient**	**Control**	**Patient**	**Control**	**Patient**
Healthy Control at RT	15.1 ± 13.8	63.6 ± 9.8	1.6 ± 0.5	46.3 ± 4.5	5.8 ± 4.0	93.6 ± 13.4	16.9 ± 3.9	396.9 ± 278.8
Healthy Control at 37ºC	7.4	7.9	-	-		0	-	28.0
Glanzmann’s Thrombasthenia	17.8 ± 10.5	63.6 ± 21.3	2.9 ± 0.2	61.2±1.5	4.5	81.1	-	
Congenital Afibrinogenemia	2.9	93.1	-	-	-	-	-	
GPVI deficiency	2.91	8.95	-	-	-	-	-	

PRP from healthy volunteers (n=3), patients with Glanzmann’s Thrombasthenia (n=1-4), congenital afibrinogenemia (n=1) or GPVI deficiency (n=1) were incubated for 1 h at RT with citrated-plasma or heat-inactivated serum from the proposita or with control plasma from a healthy donor. Levels of surface P-selectin (CD62) and CD63 are shown as % positive ± standard deviation (SD). Platelet dense granule release was assessed by ^{14}C-serotonin release in response to control or patient plasma and expressed as percentage of the total ^{14}C-serotonin stored ± SD. Supernatants isolated from PRP-plasma mixtures were assessed for TXA_2 level as described in Subjects and Methods.

To assess whether the antiplatelet IgM present in the patient blood was responsible for platelet activation and aggregation of allogeneic platelets, patient serum was depleted of IgM or IgG as described above. Figure 17B and Figure 18G show that removal of IgG did not affect the capacity of the patient's serum to promote platelet count decline and upregulation of P-selectin on allogeneic platelets; however serum IgM depletion drastically impaired platelet activation and aggregation effects.



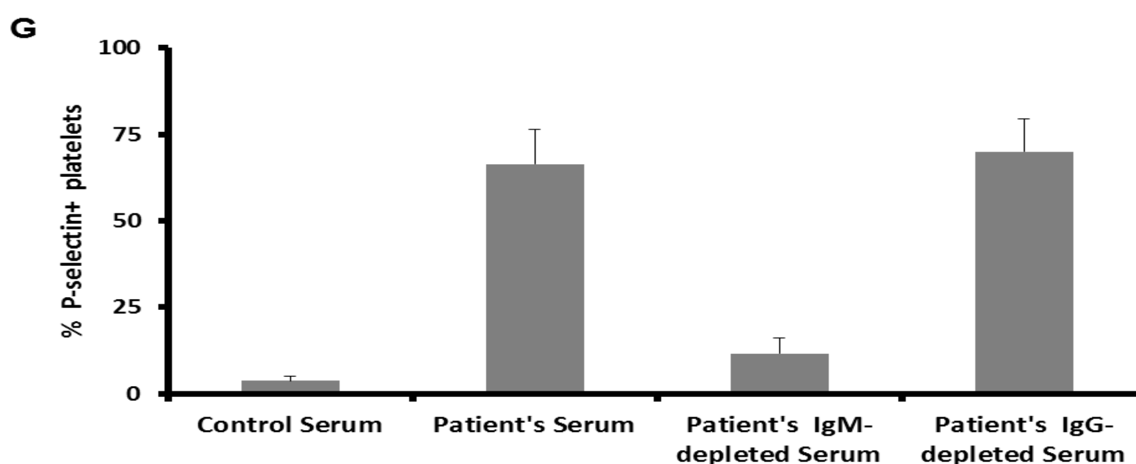


Figure 18. Electron Microscopy evaluation and assessment of P-selectin of donor platelets after treatment with patient plasma or IgM depleted serum. (A-F) Aliquots of washed platelets from a healthy donor (10^8 platelets) were incubated for 1 h at 22ºC or 37ºC with 25 μM TRAP, citrated control plasma or plasma from the proposita ± anti- αIIbβ3 antibody LJ-CP8 as indicated. Platelets were processed for analysis by electron microscopy as described in methods. All images are magnified 2850x. (G) Depletion of IgM, but not of IgG, from patient serum minimised serum-induced activation of allogeneic platelets. P-selectin expression was evaluated by flow cytometry. Values are mean ± SD from two experiments using different donor platelet samples.

3.4. Inhibitors of platelet activation and aggregation pathways block patient IgM activity

The signalling pathways downstream of GPVI and FcγRIIA involving Src and Syk activities *(Bergmeier W et al. 2013)*, promote platelet activation and induce ADP and TXA_2 release, which then act back on platelets. Binding of antiplatelet autoantibodies to FcγRIIA may also trigger platelet activation *(Brandt KJ et al. 2013)*. To investigate whether this signalling is influenced by the IgM activity, selective platelet inhibitors were included in the analysis of platelet activation and aggregation induced by the plasma/serum of the proposita.

Figure 19 shows that preincubation of PCs with a concentration of aspirin sufficient to block platelet aggregation induced by arachidonic acid, did not affect the decline in platelet count or increase in P-selectin expression promoted by patient serum. In contrast, treatment with cangrelor or apyrase impaired platelet aggregate formation by 40-50% indicating contributing roles for ADP release and $P2Y_{12}$ engagement. Aspirin (inhibits TXA_2 synthesis),

cangrelor (blocks the $P2Y_{12}$ ADP receptor) or apyrase (a scavenger of ADP) treatment had no effect on P-selectin upregulation induced by patient IgM. Pretreatment of PCs with dasatinib or PRT318 to block Src and Syk signalling events, respectively, completely ablated platelet aggregation and P-selectin upregulation induced by exposure to patient IgM. As FcγRIIA also activates Src and Syk, the contribution of FcγRIIA to these activation steps was assessed by pretreating PCs with function-blocking anti-FcγRIIA monoclonal antibody IV.3, a specific inhibitor of FcγRIIA. Saturating amounts of IV.3, previously shown to block FcγRIIA *(Iturbe T et al. 2011)*, failed to block aggregation or P-selectin expression induced by the patient IgM, indicating no contribution of FcγRIIA engagement to platelet activation. Taken together, our data indicate that the patient IgM triggers activation and aggregation of healthy donor platelets, and this effect is mediated by GPVI engagement and triggering of platelet signalling events.

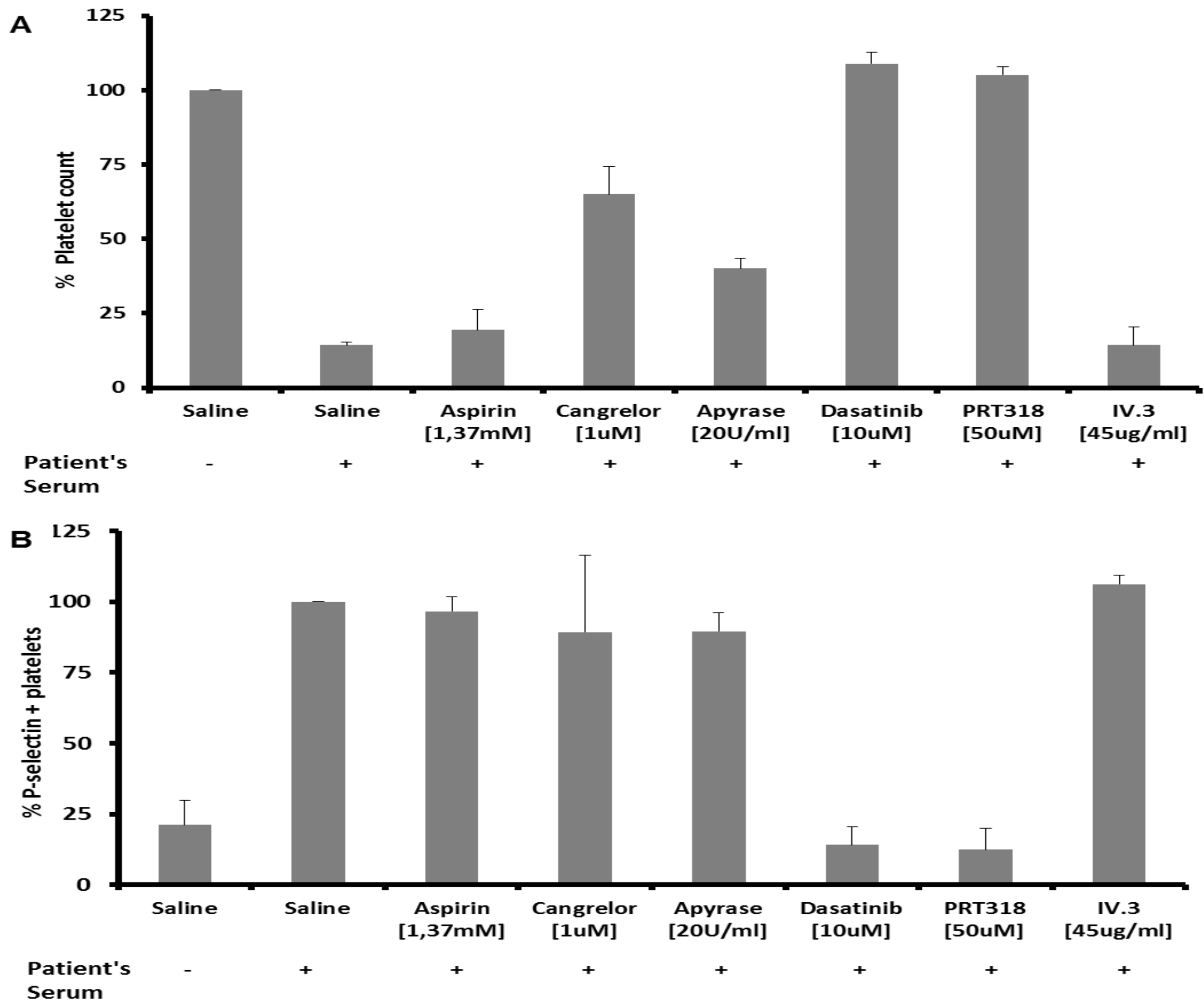


Figure 19. Inhibitors of platelet activation and signalling pathways block platelet activation and aggregation induced by the patient IgM. PCs were incubated at RT for 1 h with heat-inactivated serum from the proposita in the absence or presence of inhibitors of platelet signaling pathways. Platelet counts (A) and P-selectin levels (B) were determined as described in Methods. Platelets treated with saline alone were used as negative controls. Data is representative of at least two assays with different platelet preparations.

3.5. Levels of sGPVI in patient plasma

One consequence of GPVI engagement is the rapid metalloproteolytic shedding of this receptor from platelets. This loss of receptor occurs in response to engagement by physiological and experimental ligands, upon generation of active Factor X as occurs in serum samples *(Al-Tamimi M et al. 2011)*, or after exposure of platelets to an anti-GPVI autoantibody isolated from a patient with immunothrombocytopenia (ITP) *(Gardiner EE et al. 2008)*. Receptor shedding

from human platelets is mediated by A Disintegrin And Metalloproteinase (ADAM) 10 and results in the release of a 55-kDa extracellular fragment of GPVI which can be measured by ELISA. As levels of platelet GPVI were already shown to be approximately 50% of levels on platelets from a healthy donor (Figure 15D), levels of sGPVI were measured in blood samples from the patient or a healthy donor. Data in Table 2 shows that the levels of sGPVI in healthy donor citrate samples were in agreement with published values (19.7 ± 8.1 ng/mL, n=159) *(Al-Tamimi M et al. 2011)*. Levels of sGPVI were elevated in patient samples regardless of the anticoagulant used and this difference increased when levels were corrected for platelet count (data not shown). As expected, sGPVI levels were increased in serum samples, compared to anticoagulated samples from both control and patient, consistent with elevated sGPVI shedding occurring in response to generation of active Factor X in samples collected in serum and indicating that the GPVI shedding mechanism was intact in patient platelets *(Al-Tamimi M et al. 2011)*. If sGPVI levels in serum represent complete loss of GPVI from platelets (i.e. the total amount of GPVI that could be released) levels of sGPVI in citrate- or EDTA-anticoagulated plasma from the patient represented approximately one third of total GPVI compared with ~10% in a healthy donor (Table 12).

Table 12. : Levels of sGPVI in patient plasma are elevated compared with a healthy donor.

	sGPVI (ng/mL ± SD**)**		
Collection tube	**Healthy donor**	**Patient**	**P-value**
Citrate	10.7 ± 1.6 (8.4)	35.2 ± 0.8 (37)	0.0030
EDTA	13.6 ± 0.4 (10.7)	33.71 ± 2.7 (34.7)	0.0090
Heparin	19.6 ± 0.05 (15.4)	59.1 ± 1.7 (62)	0.0009
Serum	127.6 ± 7.2 (100)	95.1 ± 1.55 (100)	0.0250

Plasma was isolated from the patient or a healthy donor as described, and levels of sGPVI were evaluated by ELISA. Data are expressed as the average of duplicate samples (ng/mL) ± standard deviation (SD). Values in parentheses represent the % of GPVI present as sGPVI with levels of sGPVI in serum set to 100%. P values reflect levels of significant differences between healthy donor and patient using multiple t tests.

3.6. Activation of donor platelets induced by patient plasma was inhibited by a recombinant GPVI-Fc fusion protein

To investigate whether blockade of engagement of GPVI was sufficient to prevent platelet activation by the patient IgM, citrated PRP from a healthy subject was incubated with autologous citrated plasma containing 10 μg/mL of 1G5, a GPVI binding and platelet-activating antibody, or patient citrated plasma in the absence or presence of 20 μg/mL recombinant GPVI-Fc fusion protein. Figure 20 shows that treatment with 1G5 or patient IgM induced both P-selectin upregulation and binding of a fluorescently labelled single chain antibody that recognises active αIIbβ3. Platelet activation was blocked by inclusion of the GPVI-Fc fusion protein implying that engagement of GPVI by 1G5 or the patient autoantibody and subsequent activation of donor platelets was disrupted by inclusion of the fusion protein.

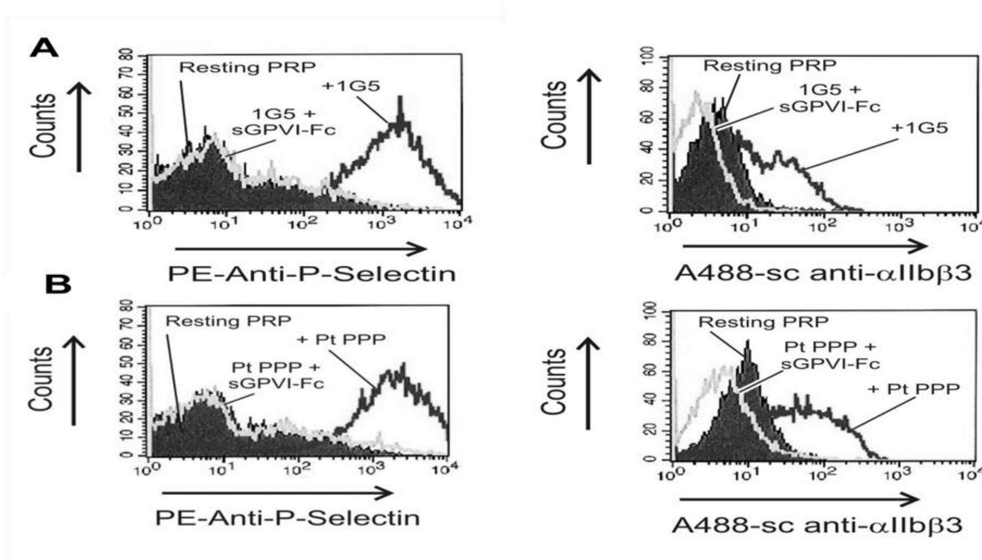


Figure 20. Activation of donor platelets by patient IgM is blocked by a soluble recombinant fragment of GPVI. Citrated PRP from a healthy subject was incubated with autologous citrated plasma containing 10 μg/mL 1G5 (A) or patient citrated plasma (B) in the absence or presence of 20 μg/mL recombinant GPVI-Fc fusion protein. Platelet activation induced by 1G5 or patient plasma was assessed by measurement of P-selectin upregulation (left panels) or binding of a single chain antibody that recognises active $\alpha_{IIb}\beta_3$ (right panels) by flow cytometry.

4. Discussion

Pseudothrombocytopenia is not a catastrophic clinical condition, however awareness of this possibility in the haematology clinic is vital to avoid inappropriate treatments *(Kumar TB et al. 2014, Nagler M et al. 2014)*. Mechanisms underlying pseudothrombocytopenia remain poorly understood. We have investigated a rare EDTA-independent pseudothrombocytopenia in a patient with chronic moderate thrombocytopenia, a mild bleeding diathesis, and abnormal platelet function testing. We detected a rapid spontaneous decline of her platelet count in citrate- or heparin-anticoagulated blood at RT, but not in EDTA-anticoagulated blood. An IgM antiplatelet antibody was detected on patient platelets and in patient plasma and serum. In contrast to most reported cases of pseudothrombocytopenia, where the PCA is directed against $\alpha_{IIb}\beta_3$ *(van Vliet HH et al. 1986, Kuijpers RW et al. 1992, Casonato A et al. 1994)*, our data indicate that the IgM bound platelets via GPVI.

To the best of our knowledge, pseudothrombocytopenia has not previously been associated with platelet dysfunction or increased platelet activation *(Ryo R et al. 1994, Schimmer A et al. 1998)* and it is thought to be independent of physiological platelet aggregation, as sera from EDTA- pseudothrombocytopenia can induce platelet clumping in blood from patients with afibrinogenemia *(Casonato A et al. 1994)*. In contrast, the singular PCA reported here was shown conclusively to promote patient or donor platelet activation and αIIbβ3-mediated platelet aggregation, rather than simple platelet clumping. Thus, platelets from a GT patient or a patient with afibrinogenemia failed to aggregate in response to patient IgM and functional blockade of $\alpha_{IIb}\beta_3$ prevented aggregation of healthy donor platelets. Activation was associated with increased levels of P-selectin and CD63 after blood drawing, and generation of significant amounts of TXA_2. These activation steps required functional platelets

but not functional $\alpha_{IIb}\beta_3$.

We provide clear evidence indicating that GPVI was the target of this PCA. First, activation of donor platelets by the patient IgM could be blocked by inclusion of inhibitors of Src or Syk kinases. Human platelets contain three immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptors that signal strongly through Src and Syk. These are the C-type lectin-like receptor-2 (CLEC-2) which binds podoplanin, the FcγRIIA receptor which binds the Fc portion of immune complexes, and GPVI which binds collagen and laminin *(Bergmeier W et al. 2013)*. Inhibitors of ADP-mediated platelet activation resulted in minor interference of platelet aggregation, consistent with an auxiliary role for $P2Y_{12}$ activity in GPVI-mediated platelet aggregation. Second, IgM-induced platelet activation could be totally blocked by inclusion of a recombinant GPVI extracellular domain fragment of GPVI. This fragment also blocked anti-GPVI mAb 1G5-triggered binding and activation of platelets, a process known to be mediated by engagement of FcγRIIA by the Fc portion of 1G5 *(Al-Tamimi M et al. 2009)*. IgM antibodies bind poorly to Fc receptors *(Klimovich VB 2011)*, and we ruled out a role for FcγRIIA in mediating platelet activation as functional blocking of this receptor by mAb IV.3 did not ablate platelet activation/aggregation induced by the patient IgM. There was no detectable platelet activation in the presence of recombinant GPVI fragment implying that CLEC-2 did not play a major role in IgM-induced platelet activation. Third, platelets isolated from a patient with selective impairment in GPVI-mediated aggregation, with no response to collagen, convulxin or collagen-related peptide, and undetectable levels of GPVI by flow cytometry, were not affected by the patient IgM. Taken together, we conclude that GPVI mediates platelet activation and subsequent aggregation induced by the unusual PCA detected in our patient.

It remains to be determined whether the presence of an anti-GPVI autoantibody in the patient plasma was responsible for the observed moderate thrombocytopenia and mild

bleeding diathesis. Platelet function assessments in the proposita by standard tests in citrated blood or PRP (PFA-100®, Multiplate, or aggregometry in PRP) lead to spurious abnormal results due to the spontaneous platelet clumping. We detected bound anti-platelet antibody on the surface of patient platelets and diminished levels of GPVI and GPIbα. Patient plasma contained elevated levels of sGPVI compared to a healthy donor with a normal platelet count, indicating that GPVI may be engaged in vivo by the IgM to an extent sufficient to trigger a low level of GPVI shedding and GPVI dysfunction in patient blood in vivo. Shedding of GPVI ectodomain by ADAM family metalloproteinases, occurs following platelet activation by GPVI agonists such as collagen, convulxin or collagen related peptide and has been reported as a consequence of an anti-GPVI autoantibody in a patient with ITP *(Gardiner EE et al. 2008)*. Autoantibodies can induce GPVI depletion by different pathways including internalization and metalloproteinase-mediated shedding, either dependent or independent of activation *(Arthur JF et al. 2007, Al-Tamimi M et al. 2012, Andrews RK et al. 2014)* and sGPVI is increased in diseases where there is ongoing platelet activation such as disseminated intravascular coagulopathy, stable angina pectoris and atrial fibrillation *(Al-Tamimi M et al. 2012)*.

Although temperature-dependence of IgM agglutinins binding to target epitopes is a known phenomenon *(Petz LD 2008)*, it is not clear why platelet activation by this agglutinin occurs at RT and less efficiently at 37ºC. Platelet activation at physiological temperature by this IgM may be weak, but at lower temperature the balance between inhibitory phosphatases and kinase activity may favour kinase activation and thus platelet responses.

In summary, we provide the first description of an IgM autoantibody causing temperature-dependent platelet activation and aggregation of human platelets in a patient with lifelong thrombocytopenia and mild bleeding tendency. Our data indicate that this atypical PCA activates platelets via GPVI. The persistence of the PCA activity in this patient across a 5-

year follow up period suggests this PCA has a causative role in her chronic thrombocytopenia and bleeding.

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Conclusions

1. Under the scope of this Thesis we have promoted a collaborative project for the recruitment and functional and molecular diagnosis of Spanish and Portuguese patients with clinical suspicion of inherited platelet disorders. This project has permitted the accurate diagnosis of a high number of patients that will benefit from more specific medical management.
2. This Thesis comprises the study of 70 patients with clinical suspicion of an inherited platelet disorder, and represents the largest cohort of these type of patients ever investigated in the Iberian Peninsula.
3. Our study demonstrates that while a strategy for sending blood samples to a distant facility may not be ideal and not free of technical limitations, is valid for functional identification of patients with severe phenotypes, such as those with GT and BSS, as there are big differences in platelet functional responses to strong agonists between patients with severe diseases and paired controls. In the case of mild platelet defects, such as defects in platelet secretion and signal transduction, this strategy may still be valid, provided that the conditions of blood sampling and mailing are carefully controlled.
4. Amongst samples from the patients that had been referred to our laboratory, the clinical suspicion of a particular inherited disorder was confirmed by specialized platelet studies in 67% of the cases, while in the remaining 33% the clinical suspicion was not ratified. Thus, our study demonstrates that correct diagnosis of inherited platelet disorders may not be as straightforward, even in severe disorders such as GT and BSS, and supports the benefit of specialized platelet laboratories for the diagnostic confirmation in these patients. Misdiagnosis of inherited platelet disorders was not without consequences, since a relatively high number of patients underwent undue splenectomy.

5. A high percentage of patients (40%) achieved a diagnosis at a molecular level, 28.8% with GT, and 17.7% with BSS. The molecular characterization of thirteen patients with GT revealed sixteen mutations along both genes encoding for the $\alpha_{IIb}\beta_3$ receptor, half of them not reported previously. In the case of BSS, six different mutations were identified in eight individuals, and three of these mutations were firstly described in this study. These data further extent the wide molecular variability of these disorders.
6. We identified two new variants of CHS in this study. Noteworthy, we describe for the first time a case of a severe infant-onset CHS due to a homozygous missense mutation, with features that are probably a direct consequence of the high instability of the mutant protein. This indicates that in this disease, attempts to link genotype and clinical phenotype require knowledge of the actual molecular effect of the mutation.
7. This study describes the second case of molecular characterization of a HPS patient in Spain, adopting a classical approach to reach a molecular diagnosis of *HPS1*. A novel mutation in the *HPS7* gene was also identified in an additional HPS patient following autozygosity mapping using microsatellite markers, emphasizing the potentiality of this high speed technique to identify candidate genes for Sanger sequencing in the study of disease causing mutations.
8. The analysis of other individual cases with clinical suspicion of inherited platelet disorder, led us to attain a molecular diagnosis of CAMT in one patient, THC2 in an additional patient, and MYH9-RD in three additional individuals.
9. In the study of patient with lifelong chronic thrombocytopenia and mild bleeding tendency without signs of any other disorder, we have provided the first evidence of an IgM autoantibody causing temperature-dependent activation and aggregation of autologous and allogeneic platelets, through interaction with the platelet collagen

receptor GPVI. The platelet cold activity in this patient persisted over a 5-year follow-up period, supporting a causative role in her chronic thrombocytopenia and bleeding.

In summary, this Thesis highlights the need to improve the diagnosis of inherited platelet disorders at the level of the functional and by molecular analysis. Standardized criteria for diagnosis of inherited platelet disorders would overcome the present heterogeneity between facilities, and the creation of reference centers should be considered to help healthcare providers in the diagnosis of these disorders.

Appendix I

The results presented in this Thesis have been disseminated to the scientific community through the following articles and communications to national and international conferences:

1. Scientific articles

1.1. Published

1. Gillian C. Lowe*, Isabel Sánchez-Guiu*, Oliver Chapman, José Rivera, Marie Lordkipanidzé, Natalia Dovlatova, Jonathan Wilde, Steve P. Watson, and Neil V. Morgan. *Microsatellite markers as a rapid approach for autozygosity mapping in Hermansky-Pudlak syndrome: Identification of the second HPS7 mutation in a patient presenting late in life*. Thromb Haemost. 2013 April 8;109(4): 766–768.
2. Isabel Sánchez-Guiu, Ana I Antón, Nuria García-Barberá, José Navarro-Fernández, Constantino Martínez, Jose L Fuster, Jose M Couselo, Francisco J Ortuño, Vicente Vicente, Jose Rivera and Maria L Lozano. *Chediak-Higashi syndrome: description of two novel homozygous missense mutations causing divergent clinical phenotype and review of the literature*. Eur J Haematol. 2014 Jan;92(1):49-58.
3. Sánchez-Guiu I, Torregrosa JM, Velasco F, Antón AI, Lozano ML, Vicente V [1], Rivera J. *Hermansky-Pudlak Syndrome: Overview of clinical and molecular features and case report of a new HPS-1 variant*. Hamostaseologie. 2014 Aug 13;34(4).

1.2. Submitted for publication

1. Isabel Sánchez-Guiu, Ana I Antón, José Padilla, Francisco Velasco, José F Lucia, Miguel Lozano, Ana Rosa Cid, Teresa Sevivas, Mª Fernanda Lopez-Fernandez, Vicente Vicente,

Consuelo González-Manchón, José Rivera and María L Lozano. *Functional and Molecular Characterization of Inherited Platelet Disorders in the Iberian Peninsula: Results from a Collaborative Study.*

2. Sánchez Guiu MI, Martínez-Martinez I, Martínez C, Navarro-Fernandez J, Garcia-Candel F, Ferrer-Marín F, Vicente V, Watson SP, Andrews RK, Gardiner EE, Lozano ML, Rivera J. *An atypical IgM class platelet cold agglutinin induces GPVI-dependent aggregation of human platelets*.

2. Communication to Congress

1. Sánchez-Guiu I, Antón AI, Cid AR*, Lozano M&, Lucia JF#, Martínez C, Corral J, Roldán V, Vicente V, Rivera J, Lozano ML. *Identificación y caracterización de tres nuevas anomalías moleculares responsables del Síndrome de Bernard-Soulier*. LII REUNIÓN NACIONAL SEHH-XXVI CONGRESO NACIONAL SETH. 2010 Las Palmas, Spain.
2. Sánchez-Guiu I, Antón AI, Cid AR*, Candel F&, Lucia JF#, Martínez C, Corral, J, Roldán V, Vicente V, Lozano ML, Rivera J. *Caracterización funcional y molecular de siete nuevos casos de Trombastenia de Glanzmann. Identificación de dos nuevas mutaciones en la integrina $\beta 3$*. LII REUNIÓN NACIONAL SEHH-XXVI CONGRESO NACIONAL SETH. 2010 Las Palmas, Spain.
3. Antón AI, Sánchez-Guiu I, Navarro-Fernández J, *Fuster JL, González MH, Ortuño FJ, Vicente V, Rivera J, Lozano ML. *Primer caso de mutación missense en un caso de Chediak-Higashi infantil con presentación en fase acelerada: cae el dogma de correlación fenotipo-genotipo*. LII REUNIÓN NACIONAL SEHH-XXVI CONGRESO NACIONAL SETH. 2010 Las Palmas, Spain.

4. J. Rivera, M.L. Lozano, I. Sánchez-Guiu, A.I. Antón, A.R. Cid, J.F. Lucía, F. Candel, M. Lozano, R.M. Fisac, F. Velasco, M.F. López, T. Sevivas, K. Arribalzaga, I.M. Ancín, J.L. Fuster, J.M. Couselo, N. Bermejo, G. Iruin, J.L. Rodríguez Martorell, C. González-Manchón, C. Martínez, J. Corral, I. Martínez-Martínez, J. Navarro Fernández, V. Roldán, V. Vicente. *Caracterización fenotípica y molecular de trombopatías congénitas de España y Portugal: resultados de cinco años de trabajo en un centro de referencia*. LIV REUNIÓN NACIONAL SEHH-XXVIII CONGRESO NACIONAL SETH. 2012 Salamanca, Spain

5. Sánchez-Guiu I, Antón AI, Padilla J, González-Manchón C, Vicente V, Lozano ML, Rivera J. *Five year report of a multicenter project for characterization of inherited platelet disorders (IPD) in the Iberian Peninsula. Diagnosis of 23 severe IPD and identification of 14 new genetic variants.* XXIV CONGRESS OF THE INTERNATIONAL SOCIETY OF THROMBOSIS AND HAEMOSTASIS. 2013 Amsterdam, Holland.

6. Isabel Sánchez Guiu, Gillian C Lowe, Francisco Velasco, Marie Lordkipanidzé, Natalia Dovlatova, Jonathan Wilde, Steve P Watson, María Luisa Lozano, Neil V Morgan, José Rivera. *Characterization of the second patient with Hermansky-Pudlak Syndrome type-7 (HPS7), and a novel HPS1 mutation. Value of an autozygosity mapping approach to prioritize mutation screening in HPS.* XXIV CONGRESS OF THE INTERNATIONAL SOCIETY OF THROMBOSIS AND HAEMOSTASIS. 2013 Amsterdam, Holland.

7. Sánchez Guiu MI, Martínez-Martinez I, Plaza E, Navarro-Fernandez J, Martínez C, Ferrer F, Roldán V, Vicente V, Lozano ML, Rivera J. *First identification and biological characterization of an IgM type platelet cold agglutinin causing temperature-dependent activation, secretion and aggregation of human platelets.* XXIV CONGRESS OF THE INTERNATIONAL SOCIETY OF THROMBOSIS AND HAEMOSTASIS. 2013

Amsterdam, Holland.

8. Sánchez Guiu MI, Martínez-Martínez I, Plaza E, Navarro-Fernández J, Martínez C, Ferrer-Marín F, Roldán V, Vicente V, Lozano ML, Rivera J. *Activación y agregación plaquetaria inducida por IgM: Caracterización de un nuevo mecanismo no descrito previamente.* LV REUNIÓN NACIONAL SEHH-XXVIII CONGRESO NACIONAL SETH. 2013 Sevilla, Spain.
9. Soler Sánchez G, Sánchez Guiu MI, Antón A, López MF, Lucia JF, Vicente V, Lozano ML, Corral J, Rivera J. *La secuenciación del exoma permite la identificación de alteraciones moleculares responsables de macrotrompocitopenias clínica y fenotípicamente inespecíficas.* LV REUNIÓN NACIONAL SEHH-XXVIII CONGRESO NACIONAL SETH. 2013 Sevilla, Spain.
10. Sanchez Guiu Isabel, Sánchez-Guiu MI, Martínez-Martínez I, Plaza E, Navarro-Fernández J, Martínez C, García-Candel F, Roldán V, Ferrer F, Vicente V, Lozano ML, Watson SP, Gardiner EE, Rivera J. *Identificación de la GPVI como diana de interacción de una aglutinina fría de tipo IgM: Mecanismo de activación plaquetaria.* LVI REUNIÓN NACIONAL SEHH-XXVIII CONGRESO NACIONAL SETH. 2014 Madrid, Spain.
11. Sanchez Guiu Isabel, Sánchez-Guiu MI; Muiña B; Periago A; Jaimes D; Soler MH; Vicente V; Lozano ML; Rivera J. *Enfermedad asociada a MYH9: descripción de dos mutaciones con fenotípico clínico no característico.* LVI REUNIÓN NACIONAL SEHH-XXVIII CONGRESO NACIONAL SETH. 2014 Madrid, Spain.

Resumen en Castellano

1. Introducción

1.1. Función Hemostática de las plaquetas:

Las plaquetas desempeñan un papel crítico en el mantenimiento de la integridad vascular y la prevención del sangrado. En condiciones fisiológicas normales, las plaquetas, gracias a su pequeño tamaño, circulan en estrecho contacto con el endotelio de la pared del vaso sanguíneo. Inicialmente tras una lesión, las plaquetas se adhieren a la pared del subendotelio dañado mediante la interacción del complejo GPIb/IX/V con el Factor von Willebrand (FvW) inmovilizado sobre las fibras de colágeno, la unión directa de las fibras de colágeno con sus receptores (GPVI y $\alpha 2\beta 1$ integrina) y la unión del FvW soluble con el receptor $\alpha_{IIb}\beta_3$, creando así una monocapa de plaquetas sobre la superficie dañada. Las interacciones de estos receptores con sus correspondientes ligandos inducen eventos de señalización intraplaquetaria de dentro hacia fuera (*inside-out*) y del exterior al interior (*outside-in*) que conducen a eventos de activación tales como el cambio de forma, la movilización de calcio intracelular, la generación de tromboxano (TxA_2) y la liberación del contenido granular (gránulos alfa, α, y densos, δ).

En la fase de extensión, los agonistas solubles como ADP, TxA_2, epinefrina y trombina, liberados/sintetizados por las plaquetas adheridas a la zona dañada del subendotelio, actúan de forma autocrina y paracrina en las plaquetas de la zona, uniéndose a sus receptores de membrana específicos, lo que desencadena una nueva oleada de señales intraplaquetarias que refuerzan y promueven la activación de las plaquetas y su incorporación al trombo en formación, en el proceso que conocemos como agregación plaquetaria. A medida que el agregado crece se inicia un proceso de

estabilización del mismo sobre la zona lesionada. Esta estabilización se lleva a cabo gracias al contacto estrecho entre las plaquetas dentro del trombo que favorece la formación de puentes directos o indirectos entre moléculas de plaquetas adyacentes, y la mayor concentración de agonistas plaquetarios y de sustancias bioactivas en el seno del trombo.

1.2. Alteraciones plaquetarias congénitas:

Esta función hemostática de las plaquetas puede verse comprometida por defectos adquiridos o congénitos que afectan ya sea la producción/destrucción de las plaquetas, la expresión, o la funcionalidad de cualquiera de los elementos involucrados en la fisiología plaquetaria, provocando diátesis hemorrágica en pacientes con esas alteraciones.

Los trastornos plaquetarios congénitos los agrupamos en trombocitopenias hereditarias, en las que los defectos genéticos provocan una disminución anormal del número de plaquetas en la sangre; y las trombopatías o trastornos hereditarios de la función plaquetaria. Distintas trombocitopenias hereditarias llevan asociada una trombopatía o viceversa.

En el primer grupo, trombocitopenias hereditarias, se incluyen las trombocitopenias sindrómicas, en el que la afectación plaquetaria forma parte de un conjunto de alteraciones presentes en los pacientes afectados. Las más destacadas son el Síndrome de Wiskott-Aldrich, la trombocitopenia con ausencia de radio, la enfermedad asociada a MYH9, el Síndrome DiGeorge y el Síndrome Paris-Trousseau o Jacobsen. Las trombocitopenias no sindrómicas más características son la

trombocitopenia ligada al cromosoma X, la trombocitopenia del tipo 2, la trombocitopenia amegacariocítica congénita, el trastorno plaquetario familiar con malignidad mieloide asociada y la trombocitopenia asociada a mutaciones en *GATA-1.*

Por su parte, las trombopatías se pueden clasificar según el elemento de la fisiología plaquetaria que este afectado. Así, un primer grupo de trombopatías son los defectos en los distintos receptores de membrana plaquetarios, que suelen afectar sobre todo a la fase de adhesión plaquetaria y formación inicial del trombo en la zona de la lesión vascular. Aquí, los más graves y mejor conocidos son el Síndrome Bernard-Soulier (SBS) y la Trombastenia de Glanzmann (TG). El SBS se debe a cambios moleculares patológicos en los genes *GPIBA, GPIBB,* y *GP9* que codifican las proteínas del complejo GPIb/IX/V, el principal receptor plaquetario del FvW. Mutaciones raras en *GPIBA* también dan lugar a un receptor GPIb/IX/V de afinidad supranormal por el FvW y son causa del trastorno conocido como enfermedad de von Willebrand de tipo plaquetaria. Las alteraciones en los genes *ITGA2B* e *ITGB3* causan fallos de expresión y/o funcionalidad de la integrina $\alpha_{IIb}\beta_3$ el principal receptor plaquetario de fibrinógeno, y provocan TG. En la actualidad, se han descrito un número relativamente alto de pacientes con SBS y TG (cerca del millar), mientras que son mucho más raros (menos de 25) los pacientes descritos con alteraciones genéticas de otros receptores plaquetarios como P2Y12, uno de los receptores de ADP, TP, receptor del TxA2, GPIa/IIa y GPVI, receptores de colágeno, o ADRA2A receptor de epinefrina. No se han identificado pacientes con mutaciones en los receptores de trombina (PAR-1 y PAR-4).

Un segundo grupo de trombopatías son los defectos hereditarios en la formación o el contenido de gránulos plaquetarios, que suelen comprometer las fases de extensión y estabilización del trombo. Entre las alteraciones que afectan a los gránulos α

destacan el Síndrome de Plaqueta Gris y el Síndrome de Quebec, y entre las que afectan relevantemente a la formación o transporte de los gránulos δ se encuentran el Síndrome de Hermansky-Pudlak (HPS), el Síndrome de Chediak-Higashi (CHS) y el Síndrome de Griscelli.

Otros defectos congénitos que pueden alterar la función plaquetaria son los que afectan a proteínas con papel relevante en la transmisión de señales intraplaquetarias que inician y amplifican la activación de las plaquetas. En este grupo se encuadran deficiencias congénitas de enzimas efectoras (nucleótido ciclasas, fosfolipasas, ciclooxigenasas, quinasas), alteraciones en las proteínas G (Gαq, Gαi, Gαs), defectos en factores de transcripción y anomalías en proteínas del citoesqueleto como talin o Kindlin-3.

Por último, entra la miscelánea de otras trombopatías hereditarias destaca en Síndrome de Scott causado por mutaciones en genes que codifican las proteínas clave en la externalización de fosfolípidos negativos en la membrana plaquetaria durante la activación. Este proceso es crítico para la formación de los complejos de los tenasa y protrombina sobre las plaquetas y su actividad procoagulante.

1.3. Epidemiología, manifestaciones clínicas y diagnóstico de las alteraciones plaquetarias congénitas

Las alteraciones congénitas de las plaquetas constituyen un grupo raro y heterogéneo de enfermedades. Pese a su rareza, su prevalencia real se desconoce pero estudios recientes sugieren que, en conjunto, las alteraciones plaquetarias pueden ser tan frecuentes como la enfermedad de Von Willebrand, que es la diátesis hemorrágica más

prevalente (0.1-1% de la población). Los síntomas clínicos relacionados con el sangrado son muy variables; debido a que la mayoría de pacientes presentan una tendencia hemorrágica leve o moderada, muchos no son diagnosticados durante la infancia. Sin embargo, tras traumatismos o cirugías puede tener lugar un sangrado extenso e inesperado. Su diagnóstico precoz es deseable para prevenir el sangrado asociado a situaciones de alto riesgo hemorrágico –como intervenciones dentales, cirugías o partos– y evitar tratamientos médicos y farmacológicos inapropiados. Además del sangrado, distintos tipos de alteraciones plaquetarias presentan también otras manifestaciones clínicas relevantes, cuadros sindrómicos que afectan a distintos órganos y tejidos (alteraciones auditivas, renales, cardiacas, cognitivas, músculo-esqueléticas, fibrosis, inmunodeficiencia, etc.).

El diagnóstico de los trastornos plaquetarios congénitos requiere una investigación clínica exhaustiva, un estudio de laboratorio con pruebas generales de bioquímica y coagulación, hemograma y frotis, con especial atención a la cifra de plaquetas y su morfología. El análisis del funcionalismo de las plaquetas es importante y se realiza con distintas pruebas como la agregación plaquetaria clásica, lumiagregometría, citometría de flujo para medir las glicoproteínas de la superficie y activación plaquetaria, inmunoensayos y otros análisis bioquímicos de sustancias liberadas o de proteínas por métodos electroforéticos. Para el estudio de pacientes con alteraciones morfológicas de gránulos plaquetarios, la microscopía electrónica suele ser una herramienta muy útil.

Además de las pruebas de función plaquetaria, que son las más usadas en los laboratorios clínicos, un diagnóstico completo e inequívoco de las enfermedades de las plaquetas se alcanza con la identificación del defecto genético en cada paciente. La detección de la alteración molecular es importante para el mejor manejo médico los

pacientes afectados, evitado tratamientos inadecuados, para proporcionar consejo genético, y para satisfacer la demanda cada vez mayor de información de los pacientes sobre sus enfermedades.

Cuando el estudio funcional permite identificar un trastorno plaquetario congénito concreto de base genética conocida, por ejemplo un SBS o una TG, el análisis molecular es más simple y se dirige a la amplificación por PCR del gen(es) candidatos y su secuenciación mediante el método clásico de Sanger, comparando la secuencia obtenida con las de referencia en bases de datos públicas y fiables (http://www.ensembl.org/index.html). Sin embargo, en muchos casos los estudios de función plaquetaria ofrecen patrones inespecíficos, compatibles con alteraciones en un gran número de proteínas estructurales, receptores y otras proteínas implicadas en la señalización de las vías plaquetaria. El diagnóstico molecular de estos trastornos congénitos supone un reto importante y se necesitan nuevas aproximaciones moleculares, cada vez más accesibles. Entre estos nuevos enfoques están los análisis de ligamiento, que identifican la región cromosómica en la que reside el gen de interés, y la secuenciación de siguiente o nueva generación (*Next Generation Sequencing*), la cual permite la secuenciación masiva en paralelo de fragmentos de ADN a partir de una sola muestra. Mediante esta última técnica podemos secuenciar el exoma o genoma completo. Gracias a estas aproximaciones moleculares, en los últimos años se han podido identificar los genes asociados a enfermedades plaquetarias como el Síndrome de Plaqueta Gris o la trombocitopenia con ausencia de radio.

2. Objetivos de la tesis doctoral

Considerando que, la caracterización completa de los trastornos plaquetarios hereditarios se ve obstaculizada por la variabilidad y la baja especificidad de las pruebas comunes de función plaquetaria, la complejidad y la limitada accesibilidad del diagnóstico molecular, y, en España, por la falta de centros de referencia altamente especializados para el estudio de estos enfermedades, los objetivos de esta tesis han sido:

1. Facilitar el acceso al diagnóstico funcional y molecular especializado de trastornos hereditarios de las plaquetas, para la comunidad médica de la Península Ibérica, promoviendo y apoyando la formación de un proyecto de colaboración para el reclutamiento y estudio de pacientes con un diagnóstico previo o con sospecha clínica de cualquier trastorno plaquetario congénito que afecte al número y tamaño plaquetario como a la función plaquetaria.
2. Realizar una nueva evaluación cuantitativa de diátesis hemorrágica en los pacientes reclutados por medio de una escala común de sangrado.
3. Completar un análisis detallado y estandarizado de la función plaquetaria en las muestras de los pacientes reclutados, con el objetivo de confirmar o descartar un diagnóstico funcional.
4. Lograr un diagnóstico molecular en los individuos con un diagnóstico confirmado en el laboratorio.
5. Establecer las posibles relaciones entre fenotipo clínico y/o de laboratorio y el genotipo, en diferentes tipos de trastornos plaquetarios hereditarios.

3. Resultados

3.1. Capítulo I: Caracterización Funcional y Molecular de Trastornos Plaquetarios Congénitos en la Península Ibérica: Resultados de un Estudio Colaborativo

El diagnóstico de pacientes con trastornos plaquetarios congénitos es complejo y laborioso, lo que resulta en un gran número de pacientes diagnosticados y clasificados incorrectamente. Con el fin de evaluar estos trastornos plaquetarios congénitos en individuos con sospecha clínica, y para proporcionar una herramienta de diagnóstico para centros que no tienen acceso a estudios específicos de plaquetas, establecimos el proyecto "Caracterización Funcional y Molecular de Pacientes con Trombopatías Congénitas" en el ámbito de la Sociedad Española de Trombosis y Hemostasia.

Los sujetos de estudio fueron pacientes con sospecha clínica de trastorno plaquetario congénito y voluntarios sanos estudiados en paralelo. Los estudios funcionales incluyeron ensayos de agregación plaquetaria, citometría de flujo, análisis Western-blot de glicoproteínas plaquetarias, y estudios de la retracción del coágulo (en los casos con sospecha de TG). El análisis molecular se realizó principalmente, mediante ampliación de regiones codificantes de los genes de interés mediante la reacción en cadena de la polimerasa y posterior secuenciación con el método de Sanger.

De los 70 casos con sospecha de trastornos plaquetarios congénitos remitidos para estudio a nuestro centro, se caracterizaron funcional y molecularmente 12 pacientes con TG, 8 pacientes con SBS, y 8 con otras formas de desórdenes plaquetarios. (Tabla 1). En estos pacientes se identificaron 12 mutaciones que no habían sido descritas previamente en la literatura. El estudio sistemático de los pacientes reveló que casi un tercio de los pacientes había sido previamente mal diagnosticado.

Nuestro estudio proporciona una visión global de las limitaciones actuales y el acceso al diagnóstico de pacientes con sospecha de un trastorno plaquetario congénito, identifica y confirma nuevas variantes genéticas que causan estos trastornos, y destaca la necesidad de crear centros de referencia que puedan ayudar en el reconocimiento y correcto diagnóstico de estos pacientes.

3.2. Capítulo II: Síndrome de Chediak-Higashi: descripción de dos nuevas mutaciones missense en homocigosis causando fenotipo clínico divergente y revisión de la literatura

Síndrome de Chediak-Higashi (SCH) es una enfermedad autosómica recesiva rara, resultado de mutaciones en el gen *LYST/CHS1*, que codifica para una proteína de 429 kDa, CHS1/LYST, que regula el tráfico de vesículas y determina el tamaño de los lisosomas y otros orgánulos celulares. Hasta la fecha, se han descrito 59 mutaciones diferentes, y se ha sugerido una correlación fenotipo-genotipo.

En esta Tesis se caracterizan dos pacientes con nuevas mutaciones de cambio de aminoácido (*missense*) situados en extremos opuestos del espectro clínico de SCH. El paciente 1 tiene una mutación *missense* en homocigosis (c.11362 G> A, p.Gly3725Arg) en el gen *LYST/CHS1,* y representa el primer caso conocido de SCH en el que una mutación de cambio de aminoácido se asocia a una sintomatología grave, síndrome mileoproliferativo o de fase acelerada, e inicio en edad infantil. En modelos *in silico*, esta alteración molecular provoca la reducción del nivel de proteína CHS1/LYST, probablemente atribuible a la severa perturbación en el potencial electrostático de la proteína. El paciente 2, era un adulto homocigoto para una nueva mutación *missense*

c.961 T> C, p.Cys258Arg y, como se ha reportado para la mayoría de mutaciones missense en el SCH, exhibió la forma "adolescente" de la enfermedad clínicamente moderada. Comparativamente, el estudio *in silico* de la mutación mostró un menor efecto sobre la estructura y potencial electrostático de la proteína CHS1/LYST.

De acuerdo a nuestros hallazgos en esta patología, se debería revisa la idea de que las mutaciones *missense* en *LYST/CHS1* causan sólo la forma clínicamente leve de la enfermedad SCH. En cada caso, el análisis, *in silico* o modelo celular, del efecto de la mutación sobre la proteína CHS1/LYST puede ser relevante para anticipar su repercusión clínica. Anticipar la gravedad de la enfermedad es muy importante para el manejo de los pacientes, e identificar precozmente a aquellos que se beneficiarían de la inscripción en un protocolo de trasplante.

3.3. Capítulo III:

3.3.1. Síndrome de Hermansky-Pudlak: Descripción General de las Características Clínicas y Moleculares. Identificación de una nueva variante HPS – 1

El síndrome de Hermansky-Pudlak (HPS) es un trastorno raro autosómico recesivo que afecta a la formación y/o trasporte de orgánulos relacionados con los lisosomas (lisosome related organelles, LRO), incluidos los gránulos δ de las plaquetas. HPS causa hipopigmentación oculocutánea, diátesis hemorrágica y colitis granulomatosa o fibrosis pulmonar. Hasta la fecha, no existe un tratamiento curativo; el tratamiento clínico depende de la gravedad de los síntomas. Un diagnóstico precoz de pacientes con HPS podría mejorar su calidad de vida y la gestión clínica de éstos. Sin embargo, la

ausencia de una prueba de la función plaquetaria específica, la amplia heterogeneidad molecular, y la falta de correlación genotipo-fenotipo dificultan el diagnóstico.

Se han identificado 9 subtipos diferentes de HPS como resultado de mutaciones en 9 genes (*HPS1* a *HPS9*) que codifican para proteínas implicadas en la formación y trasporte de LROs. La caracterización molecular de los pacientes y los conocimientos derivados de modelos animales de HPS contribuyen a ampliar el conocimiento sobre la biogénesis y función de los LROs.

En esta Tesis estudiamos y diagnosticamos molecularmente a un paciente con HPS tipo 1, que es el segundo caso de HPS caracterizado en España. Los estudios de agregación plaquetaria y secreción de serotonina, confirmaron el defecto moderado de la función de sus plaquetas, y los estudios de microscopia electrónica mostraron la ausencia de gránulos densos en este paciente con albinismo oculocutáneo tirosinasa negativo. El estudio molecular identificó una mutación sin sentido en homocigosis (c.844 G>T, p.Glu204Stop) en el exón 7 del gen *HPS1*. Esta variante molecular no se había descrito hasta ahora en la literatura.

3.3.2. Marcadores de Microsatélites como Herramienta para el Mapeo Rápido de Autocigosidad en el Síndrome de Hermansky–Pudlak. Identificación de la Segunda Mutación Causante del Fenotipo HPS7 en una Paciente Diagnosticada en Edad Avanzada

El segundo paciente con HPS caracterizado en esta Tesis ha sido una mujer inglesa de edad avanzada (77 años) procedente de una familia consanguínea. La mujer presenta diátesis hemorrágica, albinismo oculocutáneo, reducción de la agudeza visual y nistagmus a lo largo de su vida y colitis granulomatosa. Los estudios de función plaquetaria mediante agregación y lumiagregometría detectaron un defecto en la secreción de gránulos δ en la paciente. La historia clínica y los hallazgos funcionales de laboratorio eran concordantes con el diagnóstico de HPS.

El diagnóstico molecular se realizó a partir de ADN usando marcadores microsatélites (STRs) flanqueando los genes conocidos de HPS. Con esta herramienta se pretende mapear rápidamente las zonas homocigotas susceptibles de portar la mutación responsable de esta enfermedad. Sorprendentemente sólo los marcadores del gen *HPS7*se encontraron en homocigosis. Se secuenciaron los exones y regiones flanqueantes y se detectó la mutación c.177 G>A, p.Trp59Stop, en el exón 4, siendo ésta la segunda variante molecular responsable del fenotipo de HPS-7 descrita en la literatura.

3.4. Capítulo IV: Una crioaglutinina plaquetaria atípica de tipo IgM induce la agregación dependiente de GPVI en plaquetas humanas

Las crioaglutininas plaquetarias (PCAs) causan pseudotrombocitopenia, que se define como un bajo recuento de plaquetas en el hemograma, debido a la aglutinación o agrupamiento de las plaquetas *ex vivo.* Este fenómeno se da hasta en el 0.15% de los hemogramas, y su no reconocimiento puede complicar el diagnóstico clínico de estos enfermos, e incluso llevar a un manejo médico inadecuado.

En la gran mayoría de casos la pseudotrombocitopenia se debe a anticuerpos, de tipo IgM o IgG, que reconocen epitopes expuestos en proteínas superficiales de las plaquetas por la combinación del efecto quelante del EDTA, el anticoagulante usado en los tubos de hemograma, y la temperatura no fisiológica (18-22ºC) a la que están los tubos en el laboratorio. Más raramente, la pseudotrombocitopenia ocurre también a 37ºC y/o en presencia de otros anticoagulantes como citrato o heparina. En cualquier caso, hasta la fecha, los mecanismos y consecuencias de las PCAs en los individuos afectados no están bien definidos.

En esta Tesis estudiamos una paciente remitida para estudio en nuestro centro con un historial de sangrado leve y trombocitopenia crónica moderada y con una aparente alteración severa de la agregabilidad plaquetaria. En la paciente identificamos una pseudotrombocitopenia atípica no dependiente de EDTA y temperatura dependiente. Nuestro objetivo fue caracterizar su mecanismo de acción. Los estudios extensos, agregación, activación, inmunológicos y de microscopía, de plaquetas de la enferma y de plaquetas alogénicas tratadas con plasma o suero de la paciente revelaron que esta enferma ha desarrollado una inmunoglobulina atípica de tipo IgM, que se

comporta como aglutinina plaquetaria fría (temperatura dependiente).

De forma singular esta PCA induce la activación y agregación de plaquetas autólogas o alogénicas. Este efecto es temperatura dependiente y se puede bloquear con EDTA y con un anticuerpo contra la integrina $\alpha_{IIb}\beta_3$. El análisis de citometría de flujo de las plaquetas no estimuladas de la paciente reveló, además de niveles elevados de IgM, un aumento de la expresión de marcadores de activación de plaquetas (CD62 y CD63), y una expresión de GPVI baja. En el plasma de la enferma se obtuvieron niveles altos de TxA_2 y de GPVI soluble, también sugerentes de activación plaquetaria *ex vivo*. El suero inactivado de la paciente incubado con plaquetas de un donante sano, indujo la disminución del número de plaquetas, pero no ocurrió lo mismo en el caso de un paciente deficiente de $\alpha_{IIb}\beta_3$ (TG) y otro paciente con afibrinogenemia (carece de fibrinógeno). En las plaquetas alogénicas, el plasma de la paciente provocó la activación plaquetaria medida por el cambio de forma (observado por microscopía electrónica), un aumento en la expresión de CD62 y CD63, liberación-^{14}C de la serotonina, y la producción de TxA_2. La activación no fue inhibida por la aspirina, cangrelor (inhibe el receptor P2Y12 de ADP) ni por el bloqueo del receptor Fc (FcγRIIA). Por el contrario sí fue suprimida por inhibidores de Src (dasatinib) y Syk (PRT318), y también por una proteína de fusión GPVI-Fc soluble. Las plaquetas de un paciente deficiente en GPVI no se aglutinaron ni activaron tras la incubación con el plasma de la paciente.

En conjunto, estos datos proporcionan la primera evidencia de una PCA de tipo IgM que provoca verdadera activación y agregación plaquetaria, y la diana de esta aglutinina es la GPVI, uno de los receptores plaquetarios de colágeno. La persistencia de esta actividad PCA y una cifra baja de plaquetas ($\approx$100 x10^9 plaquetas /L) en la enferma durante un período de seguimiento de 5 años, sugieren un papel patológico de esta PCA

en el desarrollo de la trombocitopenia crónica de la paciente y su clínica de sangrado leve.

4. Conclusiones

10. Gracias a esta tesis hemos impulsado un proyecto de colaboración para la promoción y el diagnóstico funcional y molecular de los pacientes españoles y portugueses con sospecha clínica de trastornos plaquetarios congénitos. Este proyecto ha permitido el diagnóstico preciso de un elevado número de pacientes, que se beneficiará de un tratamiento médico más específico.
11. Esta tesis comprende el estudio de 70 pacientes con sospecha clínica de un trastorno plaquetario congénito, y representa la cohorte más grande de este tipo de pacientes investigado en la Península Ibérica.
12. Nuestro estudio demuestra que mientras que una estrategia para el envío de muestras de sangre a una instalación distante puede no ser ideal y supone limitaciones técnicas, es válido para la identificación funcional de los pacientes con fenotipos graves, tales como aquellos con TG y SBS, ya que se pueden observar grandes diferencias en las respuestas funcionales de las plaquetas a agonistas fuertes entre los pacientes con trastornos severos y controles paralelos. En el caso de los defectos plaquetarios leves, tales como defectos en la secreción de las plaquetas y la transducción de señales, esta estrategia puede seguir siendo válida, siempre que las condiciones de toma de muestras de sangre y de envío sean las adecuadas.
13. Entre las muestras de los pacientes que habían sido remitidos a nuestro laboratorio, la sospecha clínica de un trastorno hereditario en particular fue confirmado por estudios de plaquetas especializadas en el 67% de los casos, mientras que en el 33% restante no

se ratificó la sospecha clínica. Por lo tanto, nuestro estudio demuestra que el diagnóstico correcto de los trastornos hereditarios de las plaquetas puede no ser tan sencillo, incluso en trastornos severos como TG y SBS, y apoya el beneficio de los laboratorios especializados de plaquetas para la confirmación diagnóstica en estos pacientes. El diagnóstico erróneo de los trastornos hereditarios de las plaquetas no fue sin consecuencias, ya que un número relativamente alto de pacientes fueron sometidos a esplenectomía indebida.

14. Un alto porcentaje de pacientes (40%) alcanzaron un diagnóstico a nivel molecular, el 28,8% con TG, y el 17,7% con SBS. La caracterización molecular de doce pacientes con TG reveló dieciséis mutaciones a lo largo de los dos genes que codifican para el receptor $\alpha_{IIb}\beta_3$, la mitad de ellos no habían sido descritos previamente. En el caso de BSS, se identificaron seis mutaciones en ocho individuos, y tres de estas mutaciones fueron descritas aquí por primera vez. Estos datos amplían la variabilidad molecular de estos trastornos.
15. Se identificaron dos nuevas variantes de SCH en este estudio. Describimos por primera vez un caso de la forma severa de la enfermedad debido a una mutación *missense* en homocigosis, con características que son, probablemente, una consecuencia directa de la elevada inestabilidad de la proteína mutante. Esto indica que en esta enfermedad, los intentos de relacionar el genotipo y fenotipo clínico requieren un conocimiento molecular del efecto real de la mutación.
16. Este estudio describe el segundo caso de la caracterización molecular de un paciente HPS en España, mediante la adopción de un enfoque clásico para obtener un diagnóstico molecular de *HPS1*. También se identificó una mutación nueva en el gen

HPS7 en otro paciente HPS, para ello se empleó el mapeo de marcadores microsatélites con el fin de detectar rápidamente el gen candidato para la secuenciación por Sanger.

17. El análisis de otros casos individuales con sospecha clínica de trastorno hereditario de plaquetas, nos ha llevado a alcanzar un diagnóstico molecular de CAMT en un paciente, THC2 en otro caso, y tres MYH9-RD.
18. En el estudio del paciente con trombocitopenia crónica y tendencia hemorrágica leve, sin signos de cualquier otro trastorno, ponemos de manifiesto la primera evidencia de un autoanticuerpo IgM que causa la activación y agregación de plaquetas autólogas y alogénicas dependiente de la temperatura, a través de la interacción con receptor plaquetario de colágeno GPVI. Estas características persisten en la paciente durante un período de seguimiento de 5 años, apoya un papel causal en la trombocitopenia crónica y el sangrado.

En resumen, esta tesis destaca la necesidad de mejorar el diagnóstico de los trastornos plaquetarios congénitos a nivel funcional y molecular. Se debe considerar el establecimiento de criterios estandarizados para el diagnóstico de los trastornos plaquetarios congénitos con el fin de evitar la actual heterogeneidad entre las distintas instalaciones, y la creación de centros de referencia para favorecer el diagnóstico correcto de estas enfermedades.

Resumen en Inglés

1. Introduction

1.1. Platelet and Hemostasis

Platelets play an essential role in the maintenance of the vascular integrity and in the prevention of bleeding. Under normal physiological conditions, platelets, due to their small size, circulate in close contact to the endothelium of the blood vessel wall. After injury, in the initiation phase, platelets adhere to exposed subedothelium, through the interaction of the GPIb/IX/V complex to von Willebrand Factor (vWF) linked to vascular collagen, direct binding to collagen by the collagen receptors GPVI and $\alpha_2\beta_1$ integrin, interaction of integrin $\alpha_{IIb}\beta_3$ to soluble vWF. All these interactions allow the formation of a platelet monolayer at site of vascular injury. Moreover, binding of platelet receptors to their corresponding ligands induce intraplatelet signaling from inside to outside platelet (inside-out) and from outside to inside (outside-in). This signaling promotes platelet activation events such as shape change, intracellular calcium mobilization, thromboxane (TxA2) generation and release of granules content.

In the extension phase, soluble agonists such as ADP, TxA2, epinephrine and thrombin, released and/or synthesized by adherent platelets at site of vascular damage, acting in an autocrine and paracrine manner, join their specific membrane receptors triggering new waves of intraplatelet signaling that potentiate platelet activation and incorporation of new platelet to the growing thrombus in the process known as platelet aggregation that is essentially mediated by the integrin $\alpha_{IIb}\beta_3$. Finally the stabilization of the thrombus over the damaged area is accomplished through close contact between platelets within the thrombus, favoring direct or indirect links between molecules of

adjacent platelet and increased concentration of platelet agonists and bioactive compounds inside the thrombus.

1.2. Inherited Platelet Disorders

This hemostatic platelet function may be compromised by acquired or congenital defects that affect either the production/destruction of platelets, or the expression or the function of any of the elements involved in platelet physiology, causing bleeding diathesis in patients with these alterations.

Congenital platelet disorders are grouped in inherited thrombocytopenias, in which genetic defects cause an abnormal decrease in the number of platelets in the blood; and thrombopathies or inherited disorders of platelet function. Some inherited thrombocytopenias are associated with hereditary thrombopathies or vice versa.

In the first group, we include syndromic inherited thrombocytopenias in which platelet affectation associates to a range of other alterations. The most prominent are the Wiskott-Aldrich syndrome, thrombocytopenia with absent radius, MYH9-associated disease, DiGeorge Syndrome and Paris-Trousseau syndrome or Jacobsen. The non-syndromic thrombocytopenias include X-linked thrombocytopenia, thrombocytopenia type 2, congenital amegakaryocytic thrombocytopenia, familial platelet disorder with associated myeloid malignancy and thrombocytopenia associated with mutations in *GATA-1*.

On the other hand, thrombopathies can be classified according to the element of platelet physiology that is affected. Thus, a first group of thrombopathies includes those defects in different platelet membrane receptors, which mainly will affect the initial phase of platelet adhesion and thrombus formation at the site of vascular injury. The

most severe and best known inherited platelet receptor defects are the Bernard-Soulier Syndrome (BSS) and the Glanzmann's thrombasthenia (GT). The BSS is due to pathological molecular changes in *GP1BA*, *GP1BB* and *GP9* genes encoding the proteins of the GPIb/IX/V complex, the primary platelet receptor for vWF. Very rare mutations in *GP1BA* also result in a supranormal affinity of the receptor GPIb/IX/V to vWF and cause the platelet-type von Willebrand disease (PT-vWD). Alterations along the *ITGB3* and *ITGA2B* genes cause failure of expression and/or functionality of the main platelet fibrinogen receptor, the integrin $\alpha_{IIb}\beta_3$, leading to GT. Currently, a relatively large number of patients with BSS and GT (almost a thousand) have been described. In contrast, genetic alterations in other platelet receptors are much less frequent (less than 25), and include few patients with defects in P2Y12 receptor, one of the ADP receptors, TP, the TxA2 receptor, $\alpha_2\beta_1$ integrin, and GPVI, collagen receptors, or the epinephrine receptor ADRA2A. So far, no patients have been identified bearing mutations in the thrombin receptors (PAR-1 and PAR-4).

A second group of thrombopathies comprises those inherited defects in the formation or the content of the platelet granules, most often affecting the extension and stabilization phases of thrombus formation. Among the alterations affecting α granules are the Grey Platelet Syndrome and the Quebec Syndrome, and among defects of formation and/or trafficking of δ granules are the Hermansky-Pudlak Syndrome (HPS), the Syndrome Chediak-Higashi (CHS) and the Griscelli syndrome.

Other congenital defects that can impair platelet function are those affecting proteins with major roles in intraplatelet signaling leading to the activation of platelets. This group includes congenital deficiencies of several enzymes (nucleotide cyclases, phospholipases, cyclooxygenases, kinases), G proteins (Gαq, Gαi, Gαs), some

transcription factors and proteins of platelet cytoskeleton such as talin and Kindlin-3.

Finally, there is a miscellany of other inherited thrombopathies including the Scott Syndrome, caused by mutations in genes encoding key proteins for the flip-flop of anionic phospholipids in the membrane during platelet activation. This exposure of negatively charged phospholipids at the platelet surface is critical for the formation of the tenase complex on platelets, and prothrombin and their procoagulant activity.

1.3. Epidemiology, Clinical Manifestations and Diagnosis of Congenital Platelet Disorders

Congenital platelet disorders are a rare and heterogeneous group of diseases. Despite being rare, their true prevalence is unknown and recent studies suggest that, overall, platelet abnormalities may be as frequent as Von Willebrand disease, which is the most prevalent bleeding diathesis (0.1-1% of the population). Clinical symptoms related to bleeding are highly variable; because most patients have a mild to moderate bleeding tendency, many of them are not diagnosed during childhood. However, after trauma or surgery extensive and unexpected bleeding can take place. Prompt diagnosis is desirable to prevent bleeding associated with high bleeding risk situations, such as dental procedures, surgery or childbirth, and to avoid inappropriate medical and pharmacological treatments. In addition to bleeding, different types of platelet disorders also display other relevant clinical manifestations affecting different organs and tissues (hearing impairment, renal, cardiac, cognitive, musculoskeletal fibrosis, immunodeficiency, etc.).

The diagnosis of congenital platelet disorders requires a thorough clinical investigation, a laboratory study with general biochemistry and coagulation tests, blood count and smear, with special attention to the platelet count and morphology. The analysis

of the functional status of platelets is important and is performed with various tests such as the classical light transmission platelet aggregation, lumiaggregometry, flow cytometry to measure surface glycoproteins and platelet activation, immunoassays and other biochemical analysis of released substances or proteins by electrophoretic methods. For the study of patients with abnormalities of platelet granules, electron microscopy is a very useful tool.

In addition to platelet function tests, which are the most used in clinical laboratories, a complete and unequivocal diagnosis of platelet disorders is achieved with the identification of the genetic defect in each patient. Detection of the molecular alteration is important for a better medical management of affected patients, to avoid inappropriate treatment, to provide genetic counseling, and to fulfill the growing information demand of patients about their illnesses.

When the functional study identifies a specific congenital platelet disorder, such as BSS or GT, molecular analysis is easier and consists in PCR amplification of the candidate gene(s) ,its sequencing by the Sanger's method, and comparison of the sequence obtained with that of reference reported in public and reliable databases (http://www.ensembl.org/index.html).

However, in many cases platelet function studies provide nonspecific patterns compatible with alterations in a large number of structural proteins, receptors and other proteins involved in platelet signaling pathways. The molecular diagnosis of these congenital disorders is a major challenge and new molecular approaches, increasingly accessible, are needed. These new approaches include linkage analysis, to identify the chromosomal region in which the gene of interest is located, and next generation sequencing (NGS), which enables massive parallel sequencing of DNA fragments from a single sample. Using the

latter technique we can sequence the exome or the whole genome. With these molecular approaches, in recent years it has been possible to identify the genes associated with diseases such as Gray Platelet Syndrome and thrombocytopenia with absent or radio.

2. *Objectives*

Considering that full characterization of inherited platelet disorders is hampered by the variability and low specificity of platelet function tests, the complexity and yet limited accessibility of molecular diagnosis, and, in Spain, by the lack of highly specialized reference centers for the study of these diseases, the aims of this Thesis have been:

6. To facilitate access to the specialized functional and molecular diagnosis of inherited platelet disorders, for the medical community of the Iberian Peninsula, by promoting and initiating a collaborative project for the recruitment and study of patients with a previous diagnosis or clinical suspicion of either inherited thrombocytopenias or congenital disorders of platelet function.
7. To perform a quantitative re-evaluation of bleeding diathesis in the recruited patients by means of a common bleeding scale.
8. To complete a detailed and standardized analysis of platelet function in the samples from recruited patients, aiming to confirm or discard a functional diagnosis.
9. To achieve a molecular diagnosis in the individuals with a confirmed laboratory diagnosis.
10. To establish potential relationships between clinical and/or laboratory phenotype and genotype, in different types of inherited platelet disorders.

3. Results

3.1. Chapter I: Functional and Molecular Characterization of Inherited Platelet Disorders in the Iberian Peninsula: Results from a Collaborative Study

The diagnostic evaluation of inherited platelet disorders (IPDs) is complicated and time-consuming, resulting in a relevant number of undiagnosed and incorrectly classified patients. In order to evaluate the spectrum of IPDs in individuals with clinical suspicion of these disorders, and to provide a diagnostic tool to centers not having access to specific platelets studies, we established the project "Functional and Molecular Characterization of Patients with Inherited Platelet Disorders" under the scientific sponsorship of the Spanish Society of Thrombosis and Hemostasis.

Subjects were patients from a prospective cohort of individuals referred for clinical suspicion of IPDs as well as healthy controls. Functional studies included light transmission aggregation, flow cytometry, and when indicated, Western-blot analysis of platelet glycoproteins, and clot retraction analysis. Genetic analysis was mainly performed by sequencing of coding regions and proximal regulatory regions of the genes of interest.

Of the 70 cases referred for study, we functionally and molecularly characterized 12 patients with Glanzmann Thrombasthenia, 8 patients with Bernard Soulier syndrome, and 8 with other forms of IPDs. Twelve novel mutations were identified among these patients. The systematic study of patients revealed that almost one-third of patients had been previously misdiagnosed.

Our study provides a global picture of the current limitations and access to the

diagnosis of IPDs, identifies and confirms new genetic variants that cause these disorders, and emphasizes the need of creating reference centers that can help health care providers in the recognition of these defects.

3.2. Chapter II: Chediak-Higashi syndrome: description of two novel homozygous missense mutations causing divergent clinical phenotype and review of the literature

Chediak-Higashi syndrome (CHS) is a rare autosomal recessive disease resulting from mutations in the LYST/CHS1 gene, which encodes for a 429 kDa protein, CHS1/LYST, that regulates vesicle trafficking and determines the size of lysosomes and other organelles. To date 59 different mutations have been characterized, and a reasonably straightforward phenotype-genotype correlation has been suggested. We describe two patients on opposite ends of the CHS clinical spectrum with novel missense mutations. We characterized these patients in terms of their mutations, protein localization and expression, mRNA stability, and electrostatic potential. Patient 1 is the first report of a severe early-onset CHS with a homozygous missense mutation (c.11362 G>A, p.G3725R) in the LYST/CHS1 gene. This molecular change results in a reduction at the CHS1 protein level, not due to an mRNA effect, but probably attributable to the remarkable serious perturbation in the electrostatic potential. Patient 2, who exhibited the adolescence form of the disease, was found to be homozygous for a novel missense mutation c.961 T>C, p.C258R, with minor effect on the structure of the CHS1/LYST protein. Re-examining accepted premises of missense mutant alleles being reported among patients with clinically mild forms of the disorder should be done, and attempts to link genotype and clinical phenotype require identifying the actual molecular effect of the mutation.

Early and accurate diagnosis of the severity of the disease is extremely important for the appropriate treatment, and to early differentiate patients that would benefit from premature enrollment into a transplantation protocol.

3.3. Chapter III:

3.3.1. Hermansky-Pudlak Syndrome: Overview of clinical and molecular features and case report of a new HPS-1 variant

Hermansky-Pudlak syndrome (HPS) is a rare, autosomal recessive disorder affecting lysosome-related organelles (LRO), including dense platelet granules. HPS causes oculocutaneous hypopigmentation, bleeding diathesis and granulomatous colitis or pulmonary fibrosis. To date, there is no curative treatment and the clinical management depends on the severity of the symptoms. A prompt diagnosis of HPS patients could improve their quality of life and clinical management. However, the absence of a specific platelet function test, the wide molecular heterogeneity, and the lack of phenotype-genotype correlations hamper the rapid diagnosis. Nine different subtypes of HPS have been identified as a result of mutations in 9 genes that codify for proteins involved in formation and shuttle of the LRO. The molecular characterization of patients and knowledge derived from animal models of HPS contribute to the understanding of the biogenesis and function of the LRO.

This paper describes a patient with a novel homozygous nonsense mutation causing HPS and provides a review of the literature focusing on recent advances in the molecular characterization and physiopathology of the disease.

3.3.2. Microsatellite markers as a rapid approach for autozygosity mapping in Hermansky-Pudlak syndrome: Identification of the second HPS7 mutation in a patient presenting late in life

The second HPS patient characterized in this thesis has been an English elderly woman (77 years) from a consanguineous family. The woman presents bleeding diathesis, oculocutaneous albinism, granulomatous colitis, reduced visual acuity and nystagmus throughout her life. Platelet function studies performed on this patient (light transmission aggregometry and lumiaggregometry) detected a defect in the secretion patient's δ granules. The clinical history and functional laboratory findings were consistent with the diagnosis of HPS. Strikingly the only HPS locus that displayed homozygosis for both flanking markers and over an extended region of genetic distance was the HPS7 locus. Exons and flanking regions were sequenced and the mutation c.177 G> A, p.Trp59Stop was detected in exon 4, which is the second molecular variant phenotype responsible for HPS-7 described in the literature so far.

3.4. Chapter IV: An atypical IgM class platelet cold agglutinin induces GPVI-dependent aggregation of human platelets.

Platelet cold agglutinins (PCA) cause pseudothrombocytopenia, which is low platelet count due to ex vivo platelet clumping, complicating clinical diagnosis. Mechanisms and consequences of PCA in individuals are not well defined.

We characterized an atypical immunoglobulin (Ig)M PCA inducing activation and aggregation of autologous or allogeneic platelets via interaction with platelet glycoprotein (GP)VI.

In this thesis we investigated the effect of a plasmatic factor from a 37 year old woman with lifelong bleeding and chronic moderate thrombocytopenia using platelet aggregometry, flow cytometry, and electron microscopy.

Patient temperature-dependent pseudothrombocytopenia was EDTA-independent, but was prevented by integrin $\alpha_{IIb}\beta_3$ blockade. Flow cytometric analysis of unstimulated patient platelets revealed elevated levels of bound IgM, increased expression of platelet activation markers (CD62 and CD63), low GPVI levels and abnormally high thromboxane (TXA_2) production. Patient serum induced temperature- and $\alpha_{IIb}\beta_3$-dependent decrease of platelet count in allogeneic donor citrated platelet-rich plasma (PRP), but not in PRP from Glanzmann’s thrombasthenia (lacking $\alpha_{IIb}\beta_3$) or afibrinogenemia (lacking fibrinogen) patients. In allogeneic platelets, patient plasma induced shape change, CD62 and CD63 expression, ^{14}C-serotonin release, and TXA_2 production. Activation was not inhibited by aspirin, cangrelor (inhibits the $P2Y_{12}$ ADP receptor) nor blocking anti-Fc receptor (FcγRIIA) antibody, but was abrogated by inhibitors of Src and Syk, and by a soluble GPVI-Fc fusion protein. GPVI-deficient platelets were not activated by patient plasma. These data provide the first evidence for an IgM PCA causing platelet activation/aggregation via GPVI. The PCA activity persisted over a 5-year follow-up period, supporting a causative role in patient chronic thrombocytopenia and bleeding.

4. Conclusions

1. Under the scope of this Thesis we have promoted a collaborative project for the recruitment and functional and molecular diagnosis of Spanish and Portuguese patients with clinical suspicion of inherited platelet disorders. This project has permitted the accurate diagnosis of a high number of patients, which will benefit from more specific medical management.
2. This Thesis comprises the study of 70 patients with clinical suspicion of an inherited platelet disorder, and represents the largest cohort of these type of patients ever investigated in the Iberian Peninsula.
3. Our study demonstrates that while a strategy for sending blood samples to a distant facility may not be ideal and not free of technical limitations, is valid for functional identification of patients with severe phenotypes, such as those with GT and BSS, as there are big differences in platelet functional responses to strong agonists between patients with severe diseases and paired controls. In the case of mild platelet defects, such as defects in platelet secretion and signal transduction, this strategy may still be valid, provided that the conditions of blood sampling and mailing are carefully controlled.
4. Amongst samples from the patients that had been referred to our laboratory, the clinical suspicion of a particular inherited disorder was confirmed by specialized platelet studies in 67% of the cases, while in the remaining 33% the clinical suspicion was not ratified. Thus, our study demonstrates that correct diagnosis of inherited platelet disorders may not be straightforward, even in severe disorders such as GT and BSS, and supports the benefit of specialized platelet laboratories for the

diagnostic confirmation in these patients. Misdiagnosis of inherited platelet disorders was not without consequences, since a relatively high number of patients underwent undue splenectomy.

5. A high percentage of patients (40%) achieved a diagnosis at a molecular level, 28.8% with GT, and 17.7% with BSS. The molecular characterization of twelve patients with GT revealed sixteen mutations along both genes encoding for the $\alpha_{IIb}\beta_3$ receptor, half of them not reported previously. In the case of BSS, six different mutations were identified in eight individuals, and three of these mutations were firstly described in this study. These data further extent the wide molecular variability of these disorders.
6. We identified two new variants of CHS in this study. Noteworthy, we describe for the first time a case of a severe infant-onset CHS due to a homozygous missense mutation, with features that most probably are a direct consequence of the high instability of the mutant protein. This indicates that in this disease, attempts to link genotype and clinical phenotype require knowledge of the actual molecular effect of the mutation.
7. This study describes the second case of molecular characterization of a HPS patient in Spain, by means of a classical approach to reach a molecular diagnosis of *HPS1*. A novel mutation in the *HPS7* gene was also identified in an additional HPS patient following autozygosity mapping using microsatellite markers, emphasizing the potentiality of this high speed technique to identify candidate genes for Sanger sequencing in the study of disease causing mutations.

8. The analysis of other individual cases with clinical suspicion of inherited platelet disorder, led us to attain a molecular diagnosis of CAMT in one patient, THC2 in an additional patient, and MYH9-RD in three additional individuals.
9. In the study of patient with lifelong chronic thrombocytopenia and mild bleeding tendency without signs of any other disorder, we have provided the first evidence of an IgM autoantibody causing temperature-dependent activation and aggregation of autologous and allogeneic platelets, through interaction with the platelet collagen receptor GPVI. The platelet cold activity in this patient persisted over a 5-year follow-up period, supporting a causative role in her chronic thrombocytopenia and bleeding.

In summary, this Thesis highlights the need to improve the diagnosis of inherited platelet disorders at the level of the functional and by molecular analysis. Standardized criteria for diagnosis of inherited platelet disorders would overcome the present heterogeneity between facilities, and the creation of reference centers should be considered to help healthcare providers in the diagnosis of these disorders.