Pathogenetic and clinical study of a patient with thrombocytopenia due to the p.E527K gain-of-function variant of *SRC*

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SUPPLEMENTAL DATA of the paper entitled "PATHOGENETIC AND CLINICAL STUDY OF A PATIENT WITH THROMBOCYTOPENIA DUE TO THE p.E527K GAIN-OF-FUNCTION VARIANT OF SRC"

FIGURE S1

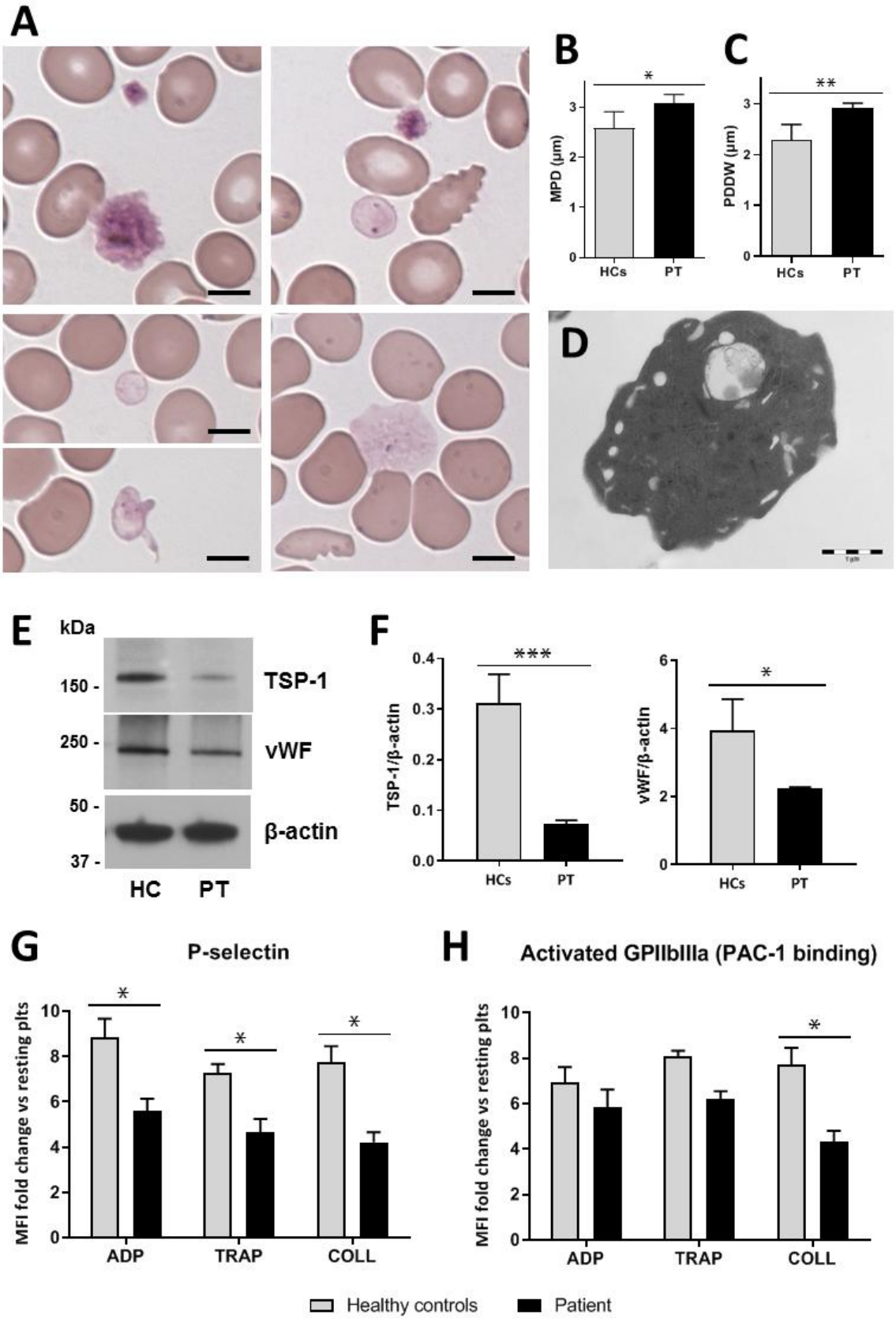
A
B
C
MPD (µm)
PDDW (µm)
HCs
PT
D
E
kDa
150 -
250 -
50 -
37 -
TSP-1
vWF
β-actin
HC
PT
F
TSP-1/β-actin
vWF/β-actin
G
P-selectin
H
Activated GPIIbIIIa (PAC-1 binding)
MFI fold change vs resting plts
ADP
TRAP
COLL
Healthy controls
Patient

Figure S1. Morphology, content in α-granules secretory proteins, and functional response of platelets of the investigated patient. (A): Peripheral blood smears, May-Grünwald-Giemsa (MGG) staining. Representative examples of platelets of the patient, which presented anisocytosis, macrocytosis with large platelets, and reduced α-granules content with about 30% of hypo- or agranular platelets. Scale bars, 5 μm. **(B)**: Mean platelet diameter (MPD) was measured on MGG-stained blood smears through software-assisted image analysis according to a previously reported method [1]. The data obtained in the patient (PT) on three different occasions were compared with those previously measured in a cohort of 55 consecutive healthy controls (HCs) [1]. Data are presented as means ± SD. **(C)**: Platelet diameter distribution width (PDDW) was calculated with the same method [1] as a measure of platelet anisocytosis. **(D)**: Transmission electron microscopy analysis of patient's platelets. Representative example of platelets presenting a markedly reduced content in α-granules. Scale bar, 1 μm. **(E-F):** Immunoblotting analysis of patient's platelet content in thrombospondin 1 (TSP-1) and von Willebrand factor (vWF). Lysates were obtained from washed resting platelets of the patient (PT) and healthy controls (HCs). β-actin was used as loading control. **(E)**: Representative image of immunoblotting experiments. **(F)**: Densitometric analysis of the bands obtained with two separate experiments (means ± SD). Patient's samples were processed in parallel with those of 4 healthy controls. TSP-1 and vWF levels were expressed as the TSP-1/β-actin and vWF/β-actin ratio, respectively. **(G-H):** Flow cytometry investigation of activation response of patient's platelets to stimulation with adenosine diphosphate (ADP), thrombin receptor activating peptide (TRAP), and collagen. Platelet surface expression of P-selectin and of the activated form of GPIIbIIIa (PAC-1 antibody binding), was measured after incubation with ADP (5 μM), TRAP (25 μM), and collagen (COLL) (4 μg/mL), or vehicle alone (HEPES buffer). Platelet activation is expressed as the ratio between mean fluoresce intensity (MFI) measured after stimulation with each agonist and MFI measured after incubation with the buffer alone (resting platelets, plts). The values obtained in the patient were compared with those of 4 healthy controls processed in parallel. Data are expressed as means ± SD. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ by two-tailed Student t test.

FIGURE S2

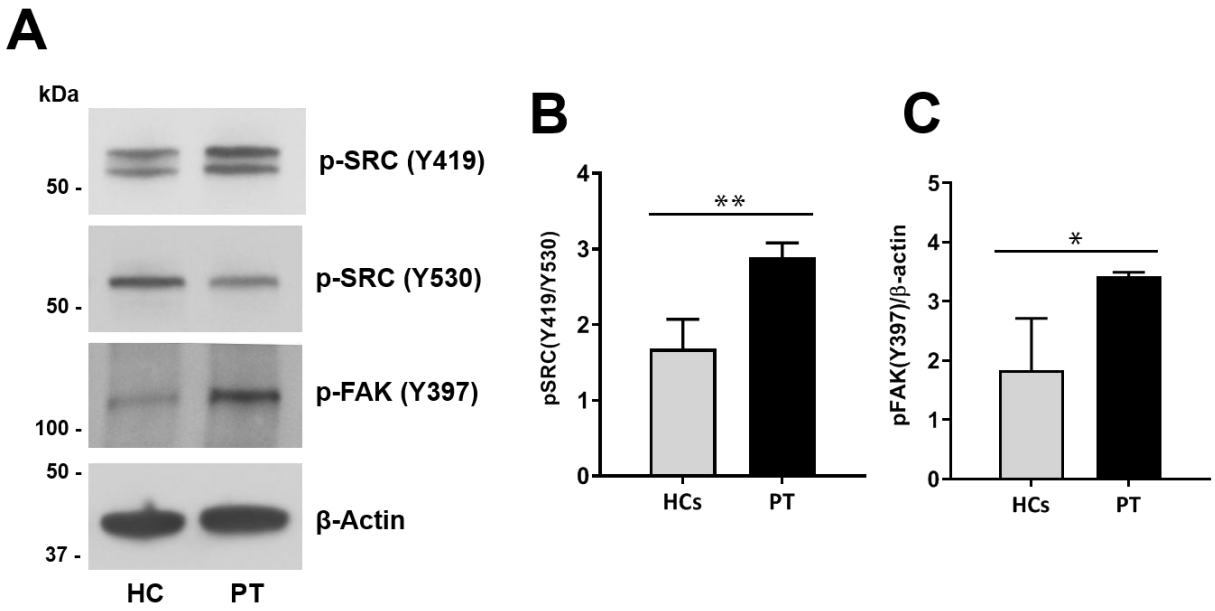

Figure S2. Activation status of SRC and FAK in platelets of the investigated patient. Lysates were obtained from washed resting platelets of the patient (PT) and healthy controls (HCs). Immunoblotting was performed with an antibody recognizing SRC phosphorylated at tyrosine 419 (SRC activation tyrosine, Y419) and an antibody recognizing SRC phosphorylated at tyrosine 530 (SRC inhibitory tyrosine, Y530). Membranes were also incubated with an antibody against the activated form of the tyrosine kinase FAK (phosphorylated at tyrosine 397, Y397). β-actin was used as loading control. **(A)**: Representative images of the immunoblotting experiments. **(B)**: Densitometric analysis of SRC phosphorylation. SRC activation status is expressed as the SRC phospho-Y419/phospho-Y530 ratio normalized to β-actin. **(C)**: Densitometric analysis of FAK activation expressed as the FAK phospho-Y397/ β-actin ratio. Data are expressed as means ± SD. ** $p<0.01$, and * $p<0.05$ by two-tailed Student t test.

FIGURE S3

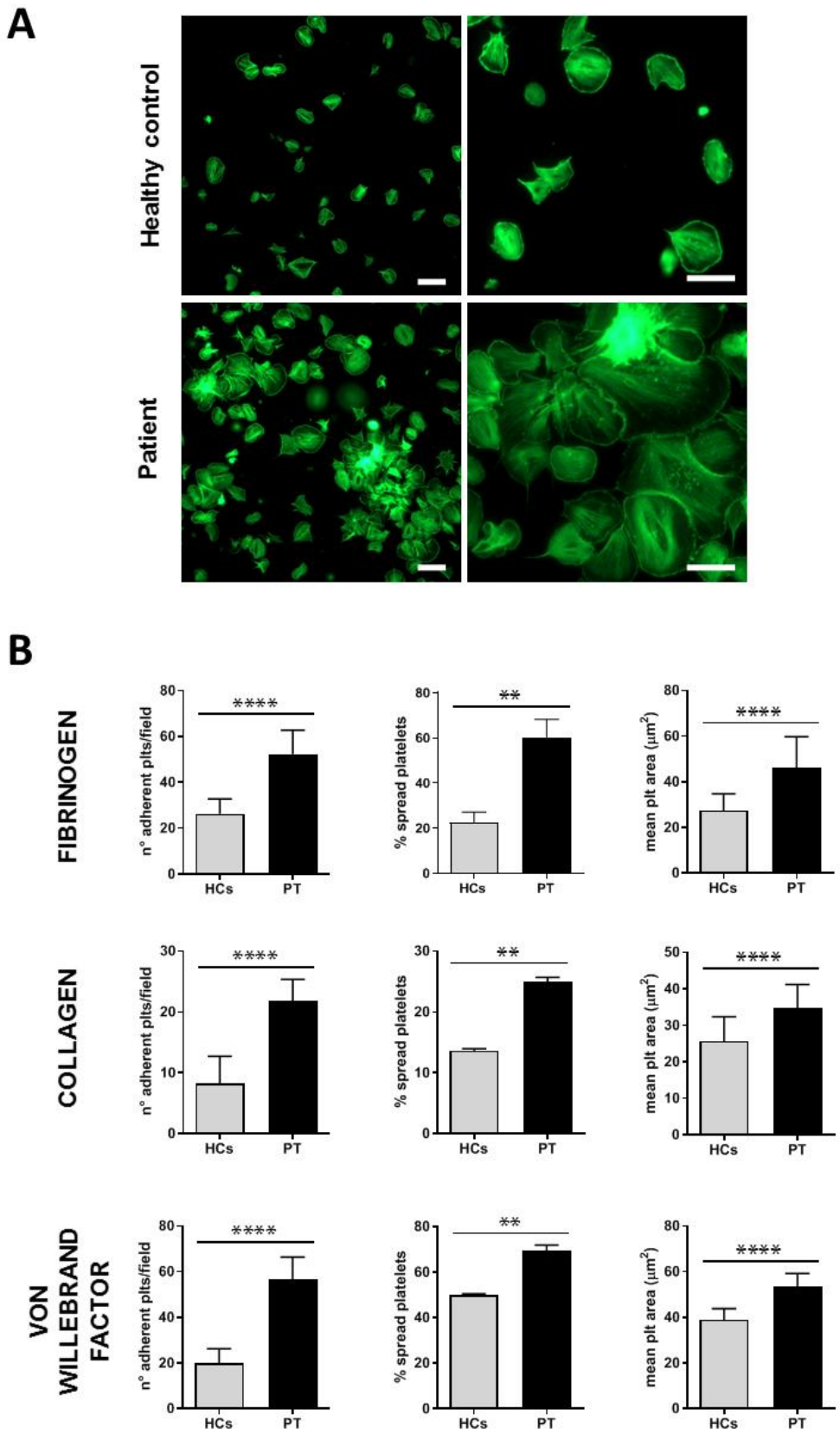
A
Healthy control
Patient
B
FIBRINOGEN
COLLAGEN
VON WILLEBRAND FACTOR
n° adherent plts/field
% spread platelets
mean plt area (µm²)
HCs
PT
****
**

Figure S3. Adhesion and spreading of platelets of the investigated patient on extracellular matrix proteins. Aliquots of 1 x10^8 washed platelets were incubated for 45 minutes at 37°C on glass coverslips previously coated with fibrinogen, type I collagen, or von Willebrand factor. Specimens were then fixed and stained for polymerized actin (phalloidin, green). Adhesion and spreading were investigated by fluorescence microscopy and software-assisted image analysis. Samples of the patient (PT) were processed in parallel with those of 4 healthy controls (HCs). **(A)**: Representative example of platelet adhesion and spreading on fibrinogen. Scale bars, 10 µm. **(B):** Platelet adhesion on each extracellular matrix protein was quantified as the number of adherent platelets (plts) per microscopic field. Platelet spreading was measured as the percentage of spread platelets with respect to the number of total adherent platelets, and as the average value of the area covered by each platelet (plt) (µm^2). The data reported with the histograms are expressed as means ± SD. **** $p < 0.0001$, and ** $p < 0.01$, by two-tailed Student t test.

FIGURE S4

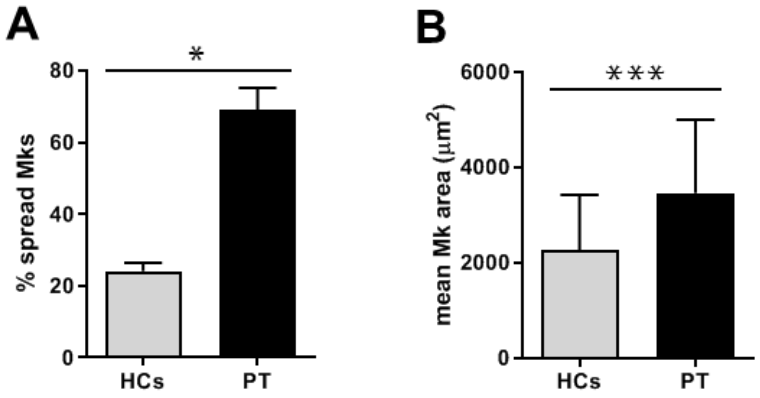

Figure S4. Spreading of megakaryocytes of the investigated patient on type I collagen. Megakaryocytes (Mks) of the patient (PT) and 3 healthy controls (HCs) were incubated on type I collagen-coated coverslips for 16 hours at 37°C and 5% CO_2, fixed and stained with phalloidin. **(A-B):** Spreading was measured through image analysis as the percentage of spread Mks with respect to the total number of Mks (A), and the mean area covered by each Mk (μm^2) (B). The data reported with the histograms are expressed represent as means ± SD. *** p <0.001, and * p<0.05 by two-tailed Student t test.

SUPPLEMENTAL TABLES

Table S1. Main parameters of the blood cell count of the investigated patient carrying the p.E527K mutation of SRC.

	Age 2 yrs (at diagnosis)	**Age 3.5 yrs**	**Age 4.5 yrs**
Hgb (gr/dL)	12.4	9.6	7.3
MCV (fL)	77.6	78.3	79.1
WBC ($x10^9$/L)	7.3	5.7	5.8
Neu ($x10^9$/L)	2.8	2.9	2.7
Platelets ($x10^9$/L)	71	50	19
MPV (fL)[1]	10.9	9.1	nr

Note: [1] mean platelet volume evaluated using an automated blood cell counter, normal range 6.4 - 8.0 fL.
Abbreviations: yrs, years; Hgb, hemoglobin; Neu, neutrophils; nr, not reported by the automated counter.

Table S2. Results of the study of *in vitro* platelet aggregation in the investigated patient, maximal extent (percentage).

ADP, 5 µM	**TRAP, 25 µM**	**Collagen, 4 mg/mL**	**Collagen, 20 mg/mL**	**Ristocetin, 1.5 mg/mL**
81	79	10	60	100

Note: Normal ranges: ADP, 43-86%; TRAP, 70-100%; collagen, 66-90% ; ristocetin, 67-90%.

Table S3. Results of flow cytometry investigation of surface expression of the major platelet glycoproteins (GP) in the investigated patient.

GPIbα (clone SZ2)	GPIbα (clone MB45)	GPIbIX (clone SZ1)	GPIIbIIIa (clone P2)	GPIIIa (clone VIPL2)
181	174	186	105	111

Note: data are expressed as the percentage of mean fluorescence intensity compared to two healthy controls processed in parallel.

Table S4. Summary of the clinical features of the individuals carrying the p.E527K germline variant of SRC described so far.

Report [n. of families / n. of described patients]	**Turro et al.**[2] [1/4]				**De Kock et al.**[3] [1/1]	**Present report** [1/1]
Patient ID [1]	13	19	31	35	P	Present patient
Age at diagnosis (yrs)	55	40	35	26	5	2
Thrombocytopenia [platelet count, $x10^9$/L]	yes [33]	yes [124-188]	yes [51-81]	yes [55-88]	yes [41-50]	yes [19-100]
Bleeding symptoms	yes	nr	yes	yes	yes	yes
Platelet morphology	Anisocytosis, macrocytosis, α-granule deficiency	Anisocytosis, macrocytosis, α-granule deficiency	Anisocytosis, macrocytosis, α-granule deficiency	Anisocytosis, macrocytosis, α-granule deficiency	Anisocytosis, macrocytosis, α-granule deficiency	Anisocytosis, macrocytosis, α-granule deficiency
Platelet function	nd	Delayed platelet aggregation after stimulation with collagen	nd	Delayed platelet aggregation after stimulation with collagen	Defective platelet aggregation after stimulation with collagen	Defective platelet aggregation and GPIIbIIIa activation after stimulation with collagen
Myelofibrosis	yes, diagnosed at age 22 yrs	no	yes, diagnosed at age 35 yrs	yes, diagnosed at age 17 yrs	no	yes, no signs at diagnosis, but developed at age 3.5 yrs
Osteoporosis	yes	nr	yes	no	no	no
Facial dysmorphism	no	nr	yes	yes	yes	no
Premature edentulism	no	nr	yes	yes	no	no
Other phenotypes	none[2]	none[2]	none[2]	none[2]	Autism, intellectual disability, joint hyperlaxity, esotropia	none

Notes: [1] Patient identification in the original report. [2] Intellectual disability and behavior abnormalities were noted in some patients of this family, but these neurological manifestations were not investigated in details.[3] **Abbreviations:** n. = number. nd = not determined. nr = not reported. yrs = years

SUPPLEMENTARY METHODS

Analysis of platelet size on peripheral blood smears

Platelet diameters were measured by software-assisted image analysis of May-Grünwald-Giemsa-stained peripheral blood smears, according to a previously published protocol [1]. Mean platelet diameter and platelet diameter distribution width (the 97.5th to 2.5th percentiles difference of platelet diameters measured in each sample) were calculated as reported [1]. Image analysis was carried out through the Axiovision 4.6 software (Carl Zeiss, Oberkochen, Germany). Analysis of patient's samples was performed on the slides prepared on three different occasions. Data are expressed as means ± SD.

Platelet aggregation

Platelet aggregation was studied according to the densitometric method of Born as previously reported. [4] Collagen was purchased from Mascia Brunelli (Milan, Italy). Adenosine diphosphate (ADP) and ristocetin were from Sigma-Aldrich (St Louis, MO). Thrombin receptor activating peptide (TRAP) was purchased from Tocris (Bristol, UK).

Flow cytometry of platelet glycoproteins expression

Aliquots of whole blood were incubated for 30 minutes in the dark at room temperature with the following FITC-conjugated monoclonal antibodies (moAbs) from Immunotech (Marseille, France): P2 against GPIIb in the intact complex with GPIIIa (CD41); SZ2 against GPIbα (CD42b); MB45 against GPIbα (CD42b); SZ1 recognizing GPIX (CD42a) when correctly complexed with GPIbα; mouse IgG1 isotype control. FITC-conjugated VIPL2 against glycoprotein GPIIIa (CD61) was from Immunostep (Salamanca, Spain). PE-conjugated P2 against CD41 (Immunotech) was used to gate platelets. Platelets were analyzed using a FC-500 flow cytometer (Beckman Coulter, Brea, CA, USA). Data obtained in the patient were expressed as the percentage of mean fluorescence intensity compared to two healthy controls processed in parallel.

Flow cytometry investigation of platelet activation

Platelet activation in response to different agonists was investigated by flow cytometry as reported. [5] Briefly, aliquots of whole blood were incubated with moAbs and ADP 5 µM (Sigma-Aldrich), TRAP 25 µM (Tocris), collagen 4 µg/mL (Mascia Brunelli), or vehicle HEPES buffer alone, for 10 minutes at 37°C, and fixed with paraformaldehyde. The following moAbs were used: FITC-conjugated PAC-1, which specifically binds

to the activated conformation of GPIIbIIIa (Becton Dickinson, San Josè, CA, USA); FITC-conjugated CLB-Thromb/6 against P-selectin (Immunotech); PE-conjugated P2 against CD41 (Immunotech). Platelets were gated by CD41 expression. Platelet activation was expressed as the ratio between mean fluorescence intensity (MFI) measured after stimulation with each agonist and MFI measured after incubation with the buffer alone. Data obtained in patient's samples with two separate set of experiments were compared with those of 4 healthy controls processed in parallel.

Immunoblotting assays

General procedures of immunoblotting analysis of lysates of resting platelets have been previously described in detail.[6] Briefly, whole platelet lysates were prepared and dissociated under reducing conditions, loaded on the gradient AnyKd gels, and transferred to nitrocellulose (Biorad, Hercules, CA, USA).[6] Membranes were probed with the following antibodies: mouse A6.1 against Thrombospondin-1 (Abcam, Cambridge, UK); rabbit polyclonal against von Willebrand Factor (Dako, Santa clara, CA, USA); rabbit D49G4 against Phospho-SRC Tyr419 (Cell Signaling Technology, Massachusetts, USA); rabbit polyclonal against Phospho-SRC Tyr530 (Cell Signaling); rabbit D20B1 against Phospho-FAK Tyr397 (Cell Signaling); mouse AC-15 against β-actin (Sigma-Aldrich). The appropriate HRP-conjugated secondary antibodies (Dako) were used for detection. Protein bands were visualized by an enhanced chemiluminescence method (ECL, GE Healthcare, Waukesha, WI, USA). Patient's samples were collected on two different occasions and processed in parallel with those of 5 healthy controls. Densitometric analysis was performed by Image J software (https://imagej.nih.gov/ij/).

Transmission electron microscopy analysis of platelets

Transmission electron microscopy analysis of platelet ultrastructure was performed according to previously reported standard methods.[6]

Whole exome and Sanger sequencing

Whole exome sequencing on gDNA samples of the proband and raw data analysis were performed as previously described in details, using the hg19 assembly as reference. [7,8] Candidate variants where further selected among those having frequency less than 1:1000 in the gnomAD database. Candidate variants confirmation and segregation analysis were carried out by Sanger sequencing with BigDye Terminator v1.1 Cycle Sequencing Kit (ThermoFisher Scientific, Waltham, MA, USA) following manufacturer's instructions. Primer sequences are available upon request.

Platelet adhesion and spreading

Platelet adhesion and spreading were investigated according to a previously reported protocol.[5] Glass coverslips were coated with fibrinogen (Sigma-Aldrich), type I collagen purified from bovine tendon, as reported, [9] or von Willebrand Factor (Merck KGaA, Darmstadt, Germany), and blocked with bovine serum albumin (BSA), as reported.[10] Aliquots of 1 x10^8 washed platelets suspended in 2.5 mL HEPES with 1 mg/mL BSA, 5.5 mM glucose and 2 mM $MgCl_2$ were incubated on coated coverslips for 45 minutes at 37°C. After washing with buffer, specimens were fixed with paraformaldehyde 4% and stained with Alexa Fluor 488-conjugated phalloidin (Life Technologies, Carlsbad, CA).[10] At least 10 random microscopic fields at 63x magnification were acquired per each specimen to the purpose of image analysis, which was carried out through the Axiovision 4.6 software (Carl Zeiss). Platelet adhesion on each extracellular matrix protein was quantified as the number of adherent platelets per field. Platelet spreading was measured as the percentage of spread platelets with respect to the number of total adherent platelets, and as the average value of the area covered by each platelet. Patient's samples were collected and processed on two different occasions and compared with those obtained in 4 healthy controls processed in parallel.

Analysis of megakaryocyte maturation

$CD45^+$ cells from peripheral blood were separated by immunomagnetic bead selection (Miltenyi Biotech, Bologna, Italy) and cultured for 14 days according to a previously reported protocol. [11,12] Aliquots of 50 x10^3 cells were collected at the end of the culture, washed in PBS, and double-stained with the FITC-conjugated moAb HIP8 against CD41 (eBioscience, ThermoFisher Scientific) and the PE-conjugated moAb HIP1 against CD42b (Abcam). Cells were analyzed using a Navios flow cytometer (Beckman Coulter). A minimum of 10,000 events was acquired. Off-line data analysis was performed using the Beckman Coulter Navios software package. The proportion of mature megakaryocytes was measured as the percentage of CD41-positive cells co-expressing the CD42b antigen, as reported. [7,8,12] Cells were also cytospun onto glass slides, fixed with paraformaldehyde and stained with a rabbit anti-β1-tubulin antibody (kindly provided by Prof. J. Italiano Jr.). An Alexa Fluor 594-conjugated goat anti-rabbit antibody (Life Technologies) was used as secondary antibody. Hoechst (Life Technologies) was added for counterstaining nuclei. Megakaryocytes were then classified into maturation stages I to IV by morphological analysis according to standard criteria.[13,14] Patient's samples were collected and processed on two different occasions and compared with those obtained in three healthy controls processed in parallel.

Proplatelet formation assay

Proplatelet formation was investigated at the end of the megakaryocyte culture both in suspension liquid cultures and in adhesion to fibrinogen according to a previously described protocol.[15,16] Proplatelet formation in suspension was quantified by phase-contrast microscopy at day 14 as the percentage of cells presenting at least one proplatelet with respect to the total number of cells. For the analysis in adhesion to fibrinogen, glass coverslips were coated with 100 μg/ml fibrinogen (Merck-Millipore) and aliquots of 1 x10^5 megakaryocytes were allowed to adhere at 37°C and 5% CO_2 for 16 hours. Samples were then fixed with paraformaldehyde and stained with the anti-β1-tubulin antibody as reported above. Hoechst was used for counterstaining nuclei. Proplatelet forming megakaryocytes were identified by fluorescence microscopy as the β1-tubulin+ cells displaying at least one proplatelet with respect to the total number of β1-tubulin+ cells. At least 50 fields per sample were analyzed using an Olympus IX53 microscope (Olympus Deutschland GmbH, Hamburg, Germany). Samples of the patient were collected on two different occasions and processed in parallel with those of three healthy controls.

Analysis of megakaryocyte spreading

Megakaryocyte spreading was assessed on fibrinogen and type I collagen as previously reported [13]. Briefly, megakaryocytes were allowed to adhere for 16 hours on fibrinogen-coated coverslips, as reported above, or on coverslips previously coated with 25 μg/ml type I collagen.[9] Cells were then fixed and stained with the anti-β1-tubulin antibody (see above) or with Alexa Fluor 488-conjugated phalloidin (Life Technologies) (see below). Spreading was quantified through image analysis as the percentage of spread megakaryocytes with respect to the total number of megakaryocytes, and as the average area covered by each megakaryocyte. At least 20 random microscopic fields at 20x magnification were acquired per each specimen to the purpose of image analysis, and at least 500 Mks per each sample were analyzed. Image analysis was performed through the Axiovision 4.6 software (Carl Zeiss). Data obtained in the patient's samples with two separate set of experiments were compared with those of three healthy controls processed in parallel.

Analysis of megakaryocyte podosomes

Aliquots of 1 x10^5 megakaryocytes were allowed to adhere on fibrinogen or type I collagen as reported above, fixed, and double-stained with Alexa Fluor 488-conjugated phalloidin (Life Technologies) and the rabbit D49G4 against Phospho-SRC Tyr419 (Cell Signaling Technology), which specifically recognizes the active form of SRC phosphorylated at the activation Tyrosine 419. An Alexa Fluor 594-conjugated goat anti-rabbit antibody (Life Technologies) was used as secondary antibody. Hoechst was added for counterstaining nuclei. Podosome were recognized in the green channel as polymerized actin-based focal structures.[17]

Podosome number and density were quantified by image analysis as the number of podosomes for each megakaryocyte and the number of podosomes per unit of megakaryocyte area. The Axiovision 4.6 software (Carl Zeiss) was used for image analysis. At least 20 megakaryocytes for each specimen were analyzed. Samples of the patient were processed in parallel with those of three healthy controls.

Analysis of megakaryocyte SDF1-driven migration

SDF1-driven migration of megakaryocytes on fibrinogen and type I collagen was investigated using the Transwell migration chamber system (Merck-Millipore, Milan, Italy).[15,18] Briefly, 96-well plates with polycarbonate inserts having 0.3 cm2/well membrane area with 8 µm pore size were coated with 100 µg/ml fibrinogen (Merck-Millipore) or 25 µg/ml type I collagen.[9] Aliquots of 4 x10^3 megakaryocytes suspended in 100 µL of Stem Span medium (Stem Cell Technologies) were seeded in the upper chambers, while the lower chambers were filled with 150 µL of medium with 100 ng/ml SDF1 (PeproTech, London, UK). After incubation for 16 hours at 37°C and 5% CO_2, cells remaining on the upper face of the filters were removed by a cotton wool swab. Inserts were then washed with buffer and cells on the underside of the membrane were fixed and stained for β1-tubulin as reported above. Membranes were cut out with a scalpel, mounted onto glass slides, and the number of migrated cells was counted by analyzing the entire membrane area using a Olympus BX51 microscope. The assays were performed in triplicate wells for each condition. Data obtained in the patient's samples with two separate set of experiments were compared with those of three healthy controls processed in parallel.

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