Src-related thrombocytopenia - a fine line between a megakaryocyte dysfunction and an immune-mediated disease

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Supplemental Material

Patients, blood sampling, and DNA collection

Venous blood samples were drawn into either 7.5% K3 EDTA tubes (for complete blood counts [CBC] and DNA purification) or buffered 0.105 M sodium citrate (for functional studies). CBC were performed using a Sysmex® XS1000i hematological counter (Sysmex, Sant Just Desvern, Spain), and the IPF was measured using Sysmex XE-2100. DNA was isolated using a DNeasy blood and tissue kit, (Qiagen, Germany) and quantified using a Qubit 2.0 fluorometer (ThermoFisher Scientific, CA, USA).

Molecular analysis by HTS gene panel and Sanger sequencing, and *BTK* nanopore sequencing

DNAs from two siblings (Cases #3 and #6) were analysed by high throughput sequencing (HTS) using an Ion Torrent PGM platform (Thermo Fisher Scientific, Waltham, MA. USA).[1] Sequences were annotated as reported[1] using annotations of genome version hg19/GRCh37. The identified genetic variants were assessed according to the standards of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG).[2] The *SRC* c.1579G>A variant (p.E527K) identified by HTS-gene panel was confirmed and segregated in other family member by means of Sanger sequencing in an ABI 3130 automated sequencer. The specific forward and reverse primers were designed using Primer3 (http://bioinfo.ut.ee/primer3).

- SRC-14F CACCCCACTTTCCTCACC
- SRC-14R CGGGAGCTGGACAGAGTC

In selected family members (cases 3,6,7,9,10,11,12 and 13), the *BTK* gene was analyzed by long-range polymerase chain reaction (LR-PCR) and nanopore sequencing in an Oxford Nanopore flow cell (Oxford Nanopore FLO-MIN106D R9 version) in the MinION device and MinKnow software (Oxford Nanopore), essentially as described.[3] Appropriate sequence could be obtained in 15 out of 22 kb of the gene.

Platelet aggregation

Platelet-rich-plasma (PRP) and platelet poor plasma (PPP) from citrate whole blood were prepared by centrifugation (140 × g, 15 min; 1000 × g, 10 min, respectively). Light transmission aggregometry

(LTA) in PRP (150-350 x 10^{11} platelets/L) was performed as described[4] by using an Aggrecorder II aggregometer (Menarini Diagnostics, Florence, Italy). Time course changes in the maximal percentage of light transmission of PRP over baseline PPP were recorded for 300 seconds upon stimulation with the following platelet agonists: 1.5 mM arachidonic acid (AA; Diagnostica Stago, Barcelona, Spain), 5 µM ADP (Merck Life Science S.L.U.[Sigma-Aldrich], Madrid, Spain), 25 µM protease-activated receptor agonist peptide (TRAP or PAR1) (Merck Life Science S.L.U), 2 µM collagen-related peptide (CRP) (CambCol Laboratories, Cambridge, UK) and 1.25 mg/mL ristocetin (Merck Life Science S.L.U).

Platelet flow cytometry

Platelet expression of membrane glycoproteins (GP) GPIa (integrin α2, CD49b), GPIbα (CD42b), GPIX (CD42a), GPIIb (integrin subunit αIIb, CD41a), GPIIIa (β3, CD61) and GPVI was evaluated by flow cytometry in diluted (1:10 in Tyrode buffer) citrated whole blood, using specific antibodies (all from BD Biosciences, Madrid, Spain). To analyze platelet granule secretion and $\alpha_{IIb}\beta_3$ activation, diluted PRP ($\sim 20 \times 10^9$/L platelets) was incubated under static conditions (30 minutes at room temperature [RT]) with Tyrode's buffer, as control for non-stimulated platelets, or with agonists in the presence of anti-CD41*APC (as a platelet marker), fibrinogen-Alexa488 (Thermo Fisher, Madrid, Spain) and anti-CD62*PE (α-granule secretion) or anti-CD63*PE (dense granule secretion) (BD Biosciences). Reactions were stopped with 4% paraformaldehyde (PFA) (v/v) (15 min, RT), samples were diluted with PBS and then run in a BD Accuri™ C6 device (BD Biosciences, Ann Arbor, MI, USA). The median fluorescence intensity (MFI) of CD41a positively stained cells (platelets) was analyzed using BD Accuri™ C6 software.[5]

Immunofluorescence assays in blood smears

Immunofluorescence staining of α-granule proteins (thrombospondin, vWF, and P-selectin), δ-granule markers (LAMP-1, LAM-2 and CD63), β1-tubulin, Src and phosphorylated-Src were performed in blood smears as recently reported.[6,7] In brief, standard blood smear were prepared by smearing 3–4 µL blood drop in superfrost 76 x26 mm microscope slides (Menzel-Gläser, Linea LAB, Badalona, Spain). These blood films are stable for several days and were shipped by regular mail even to Prof A Greinacher´ lab for centralized staining. Slides were fixed and permeabilized with ice-cold acetone (-20ºC; 2–5 min), and stained with primary antibodies. The following antibodies were used: anti-thrombospondin antibody (ab85762, Abcam, Cambridge, UK); anti-P-Selectin (555522, BD Biosciences, San Jose, CA, USA); anti-vWF(A0082, Dako, Waldbronn, Germany); anti-LAMP1(sc18821, Santa Cruz Biotechnology, Heidelberg, Germany); anti-LAMP2 (sc18822, Santa

Cruz); anti-CD63 (558019, BD Biosciences); anti-β1-tubulin (T4026; Merck Life Science, Darmstadt, Germany). After washing the blood smears were incubated with the appropriate secondary antibody: ALEXAFluor 568 (goat anti-rabbit) (A11011, Invitrogen, Thermo Fisher Scientific) or ALEXAFluor 488 (goat anti-mouse) (A11001, Invitrogen,Thermo Fisher Scientific, Dreieich, Germany).

Western blot analysis

Washed platelets (0.5-1 × 10^9 platelets/mL) were resuspended in modified Tyrode's HEPES buffer (134 mmol/L NaCl, 2.9 mmol/L KCl, 0.34 mmol/L Na_2HPO_4, 12 mmol/L $NaHCO_3$ and 1 mmol/L $MgCl_2$ and 20mmol/L HEPES, pH 7.4; all Sigma, UK), pH 7.4, and lysed by addition of sodium dodecyl sulfate (SDS) reducing sample buffer. Proteins in whole cell lysates were separated by 8% SDS–polyacrylamide gel electrophoresis for 60 minutes and transferred to polyvinylidene fluoride membranes (Millipore, Merck KGaA, Germany). Blots were incubated with the primary antibodies followed by secondary horseradish peroxidase–conjugated IgG antibody (Merck Life Science). Enhanced chemi-luminescence reaction (BioRad GE Healthcare, UK) was used to detect proteins using clear-blue X-ray film.

Transmission Electron Microscopy Assays

Electron microscopy was used to examine platelet morphology and ultrastructure as described.[8] Briefly, PRP was fixed in 1.25% glutaraldehyde (Electron Microscopy Sciences, Hatfield, Pennsylvania) for 45 minutes at room temperature. After fixation, samples were washed, post-fixed in 1% osmium tetroxide (Sigma-Aldrich) for 2 hours at 4°C and then treated with 2% uranyl acetate veronal for 2 hours at 4°C. Then, samples were dehydrated with graded ethanol and propylene solutions and embedded in Epon (Taab Laboratories, Reading, UK). Embedded samples were sectioned with an Ultracut E ultramicrotome (Reichert, Vienna, Austria) and stained with uranyl acetate and lead citrate (Merck, Darmstadt, Germany). Platelet sections were observed with a Philips/FEITecnai12 transmission electron microscope (FEI; Hillsboro, Oregon, United States) at 80 kV.

Protein phosphorylation in lymphocytes by flow cytometry

Human peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples by Ficoll-Paque (GE Healthcare Life Sciences) density centrifugation method (400 x g, 30 minutes). PBMCs (50µL) were stained with anti 6µL of anti-CD19 (BD) at room temperature for 20 minutes, and stimulated with 20 µg/mL soluble anti-IgM antibody (Jackson Immunoresearch Labs, West

Grove, USA) at 37 °C for 5 minutes. Cells were fixed and permeabilized using the Intrasure kit (BD) (for fixing: 40µL of reagent A; for permeabilizing 20µL of reagent B), and incubated with P-Syk, P-BTK and P-ERK (Cell Signaling Technology) antibodies (15µL of dilution 1/20). Following a washing step, a goat anti-rabbit IgG (H+L) secondary antibody*alexa fluor 633 (Invitrogen) was added (50µL from dilution 1/50), and the analysis was performed using a BD ACCURI cytometer.

Supplemental Table S1. Analysis of platelets from SRC-RT patients by transmission electron microscopy

	Control	Carriers
Platelet area (μm^2)	2.70 ± 1.39	6.63 ± 4.85[a]
Maximum diameter (µm)	2.75 ± 1	3.71 ± 1.29[b]
Minimum diameter (μm^2)	1.43 ± 0.54	2.44 ± 1.04[a]
Number of granules per (μm^2)	3.57 ± 0.3	1.86 ± 0.61
Total area OCS per platelet area (μm^2)	1.22 ± 0.48	2.30 ± 1.33

Electron microscopy images were analyzed with Leica QWin Pro V3 software in samples from 1 control and 2 patients (cases #3, and 7). Between 2-3 sections (10-20 platelets) were assessed with the macro in ImageJ. Mann– Whitney test was used in all experiments. Results are presented as mean ± SD; a $p \leq 0.001$ compared to control; b $p \leq 0.01$ compared to control. Abbreviation: OCS, open canalicular system

Supplemental legends to figures

Figure S1: Platelet count level over time of one p. E527K Src variant carrier (case 3) and correlation with corticosteroid treatment and splenectomy. The course of platelet counts for a follow-up period of 25 years show that platelets were ≥50x10^9/l in 3/8 (27.3%) determinations before steroids, in 11/17 determinations (64.7%) during the exposure period of 16 months to corticosteroids, and in 16/16 determinations (100% of cases) after splenectomy was performed. Blue bars indicate the dose (mg/day) of methylprednisolone (left axis); the continuous line reflect platelet counts (right axis).

Figure S2: Platelet morphology analysis in six family member carriers of the p. E527K Src variant. The upper two panel show images of May-Grünwald-Giemsa stained blood smears of a healthy volunteer and of the seven family members that carry the gain-of-function p. E527K Src variant. The lower panels show the platelet size distribution plots provided by hematological counter (Sysmex® XS1000i) in EDTA blood samples from these individuals.

Figure S3. Immunofluorescence staining on blood smears. Panels show markers of platelet α-granule markers (thrombospondin, P-selectin and vWF), in 6 members of the pedigree (cases #3, #6, #7, #9, #11, #12) carrying the Src p.E527K variant. Scale bars correspond to 2 μm.

Figure S4: Immunofluorescence staining of the platelet dense granule markers, and of the cytoskeletal protein β1-tubulin. Conventional blood smears from a healthy volunteer and carriers of the Src p.E527K (cases #3, #6, #7, #9, #11, #12) stained with specific antibodies against the δ-granule markers LAMP-1, LAM-2 and CD63, and the cytoskeletal protein β1-tubulin

References

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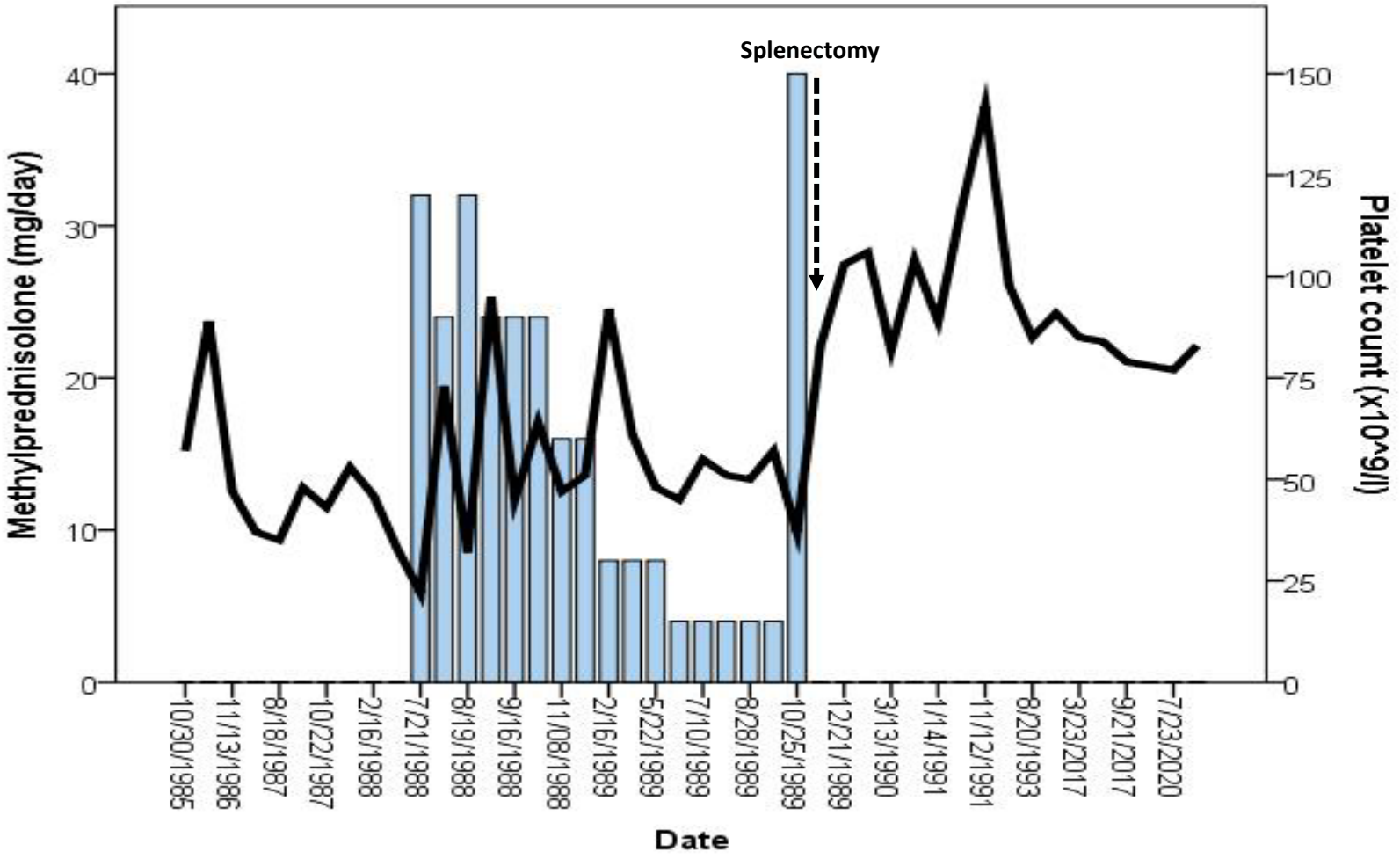
Splenectomy
Methylprednisolone (mg/day)
Platelet count (x10^9/l)
Date
0
10
20
30
40
0
25
50
75
100
125
150
10/30/1985
11/13/1986
8/18/1987
10/22/1987
2/16/1988
7/21/1988
8/19/1988
9/16/1988
11/08/1988
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10/25/1989
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3/23/2017
9/21/2017
7/23/2020

Figure S1

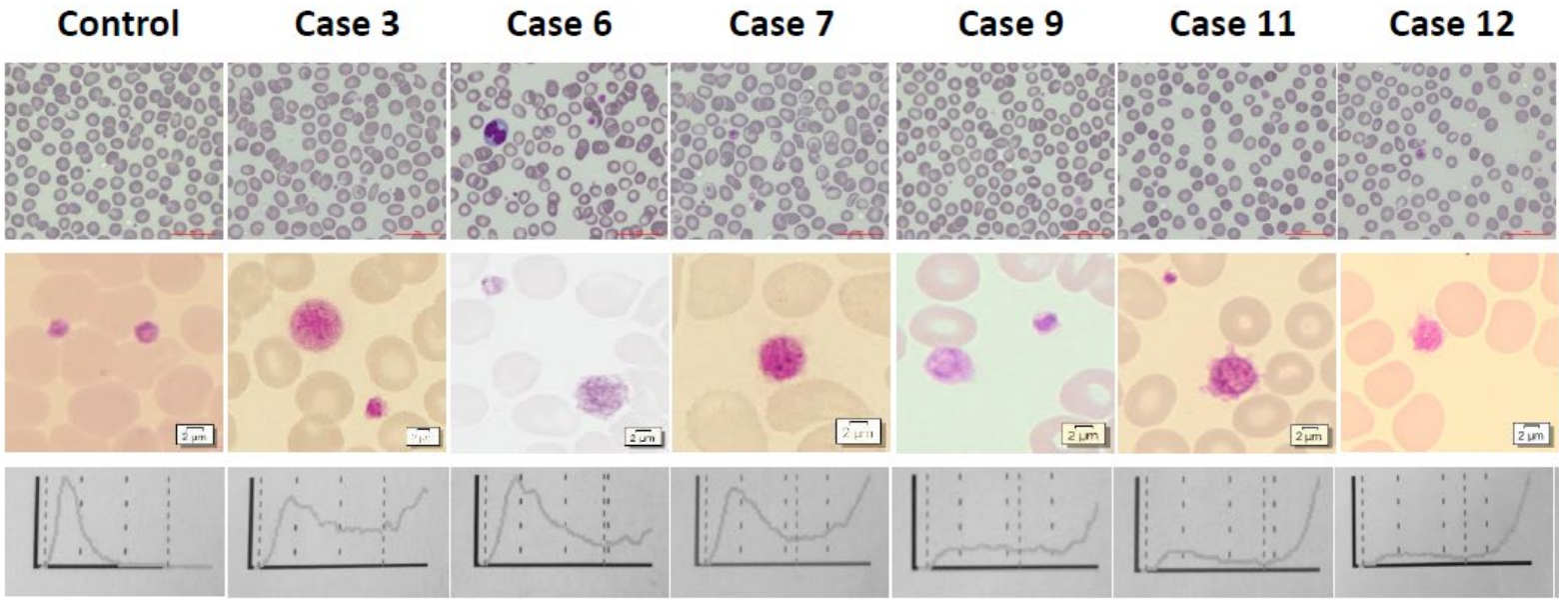

Figure S2

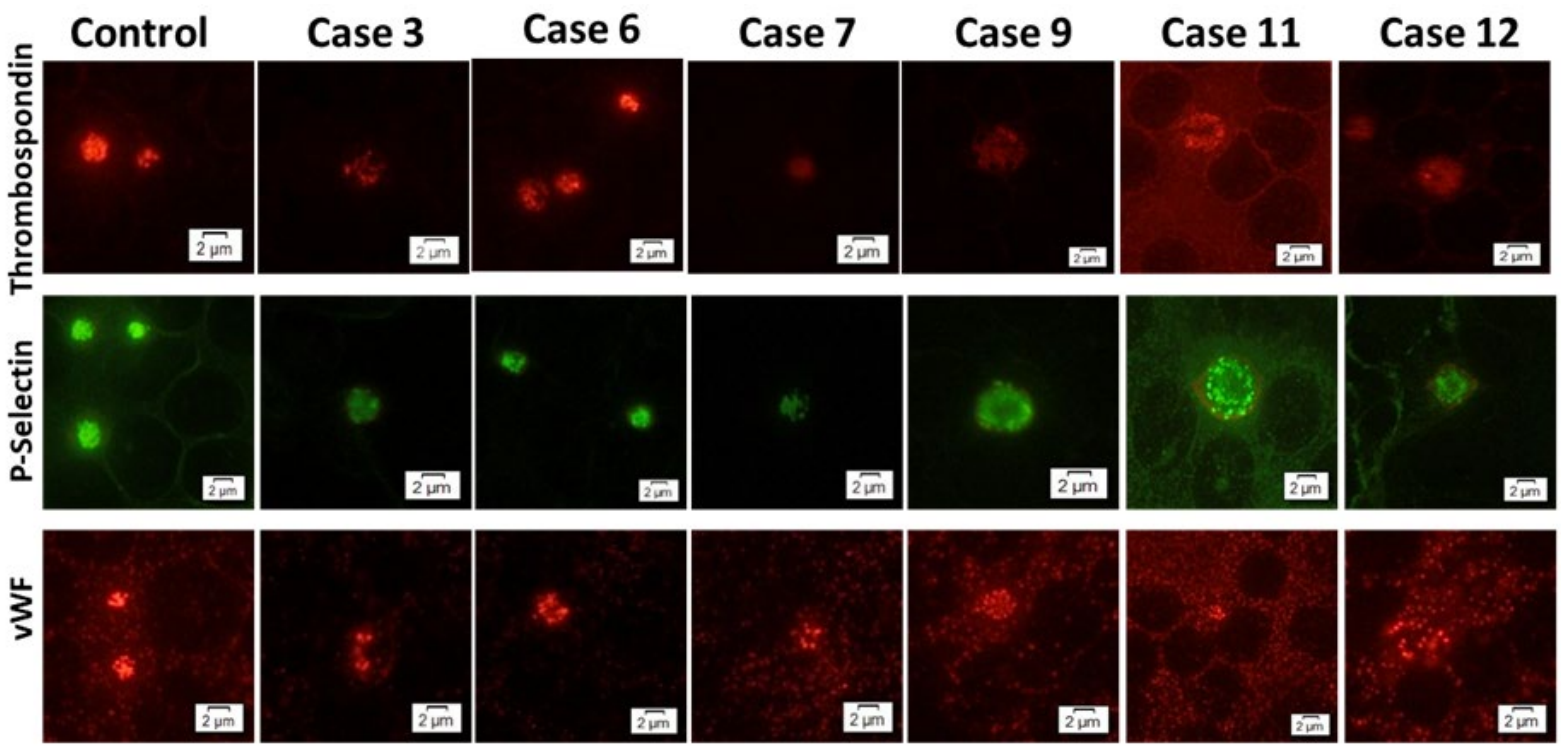
Control
Case 3
Case 6
Case 7
Case 9
Case 11
Case 12
Thrombospondin
P-Selectin
vWF
2 µm

Figure S3

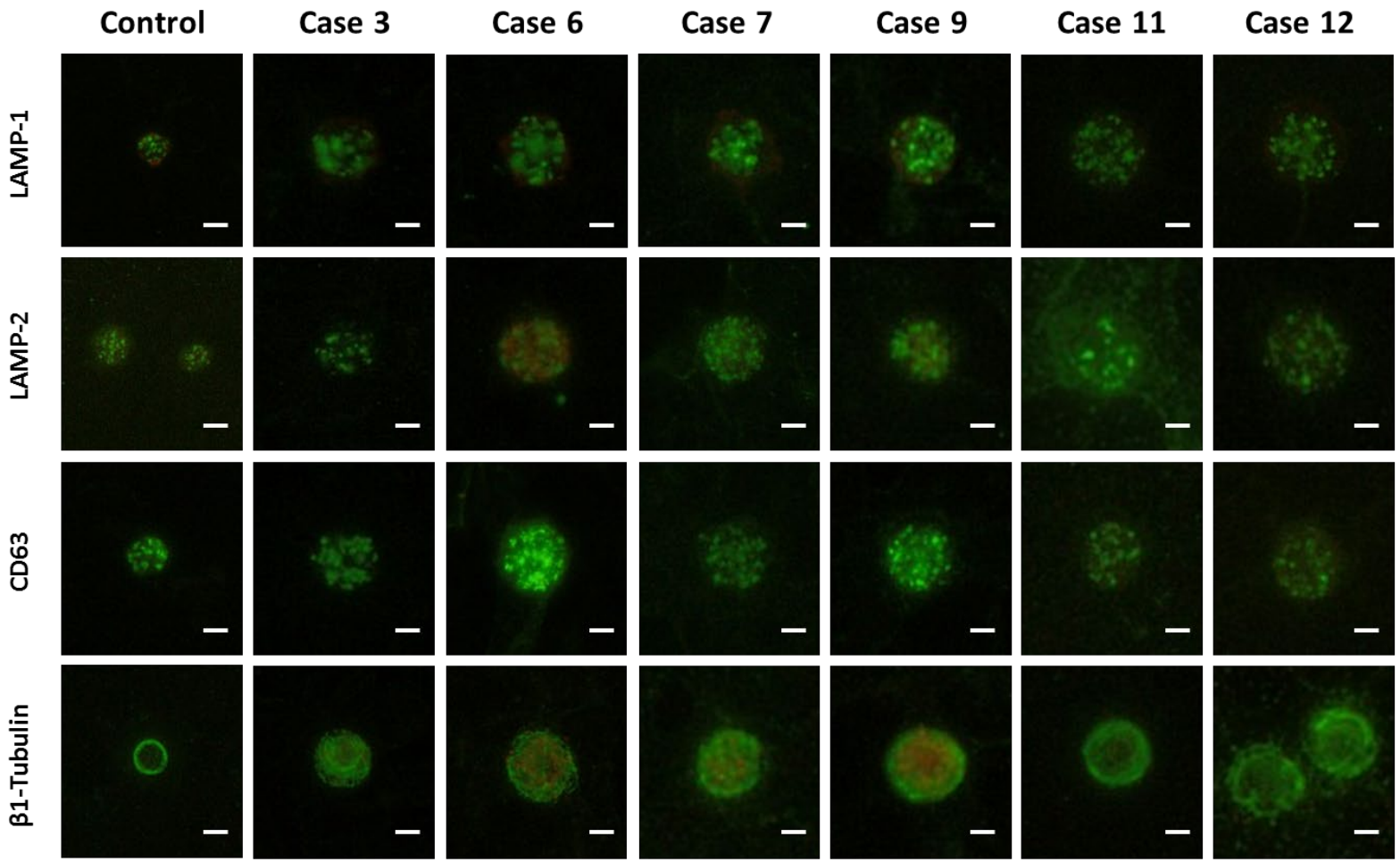
Control
Case 3
Case 6
Case 7
Case 9
Case 11
Case 12
LAMP-1
LAMP-2
CD63
β1-Tubulin

Figure S4