A novel genetic variant in *PTGS1* affects N-glycosylation of cyclooxygenase-1 causing a dominant–negative effect

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Supplemental Material

Patients, blood sampling, and DNA collection

The proband was a 13-year-old teen from Asiatic origin adopted by a Spanish family, who was enrolled in the Spanish multicenter project "Functional and Molecular Characterization of Patients with Inherited Platelet Disorders".[1,2] This project followed the Helsinki Declaration and had the approval of the Ethics Committees of the Instituto de Investigación Biomédica (IBSAL, Salamanca, Spain) and Hospital Universitario Reina Sofía (Murcia, Spain). The patient, and control subjects, provided written informed consent. Clinical data were reviewed by the investigator team and bleeding symptoms were scored using ISTH bleeding assessment tool (ISTH-BAT).[3,4]

Venous blood was 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 España, Spain). 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

The patient DNA was analysed by high-throughput sequencing (HTS)-gene panel using a MiSeq Illumina platform (Illumina, CA, USA).[2,5] 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).[6] The PTGS1 variant identified in the index case by HTS-gene panel[2] was confirmed by means of Sanger sequencing in an ABI 3130 automated sequencer. The following specific forward and reverse primers were designed using Primer3 (http://bioinfo.ut.ee/primer3/)

PTGS1 5F: cttgtcaccgttatttttgctctct

PTGS1 5R: atctgtaaagacccaaacacagaga

Genomic DNA was amplified with the Fast Start High Fidelity PCR System (Roche, Basel, Switzerland) following the manufacturer's instructions, with some variations in the annealing temperature. DNA sequences were evaluated using Chromas Lite v2.1.1 (Technelysium, South Brisbane, Australia) and DS Gene v1.5 (Accerlys, San Diego, CA) software. Data were analyzed using annotations of genome version hg19/GRCh37. COX-1 sequence alignment with 12 species was done by using Uniprot and visualized with Jalview software.[7] The new human COX-1 crystal structure (https://www.rcsb.org/structure/6Y3C) was visualized in the Chimera software (UCSF, CA, USA).

Platelet aggregation

Platelet-rich-plasma (PRP) and platelet poor plasma (PPP) from citrate whole blood were prepared by stepwise centrifugation (140 × g, 15 min; 1000 × g, 10 min, respectively). Light transmission aggregometry (LTA) in PRP (2 x 10^{11} platelets/L) was performed as described[1] 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: 1.6 mM arachidonic acid [AA; Sigma-Aldrich, UK], 10 µM ADP [Chronolog, UK], 10 µg/ml Collagen [Nycomed, Austria], 25 µM protease-activated receptor agonist peptide (PAR1p) (TRAP-6; Bachem, Austria], 10 µM U46619 (Cayman Chemical, UK) and 1.25 mg/mL Ristocetin [Helena Bioscience, UK]

Platelet flow cytometry

Platelet expression of membrane glycoproteins (GPs) GPIa (integrin α2, CD49b), GPIbα (CD42B), GPIX (CD42Aa), GPIIb (integrin subunit αIIb, CD41a), GPIIIa (β3, CD61) and GPVI was evaluated by flow cytometry in citrated whole blood diluted 1:10 in sterile phosphate-buffered saline (PBS) using specific antibodies (all from BD Biosciences, Madrid, Spain). To analyze platelet granule secretion and $\alpha_{IIb}\beta_3$ activation, diluted PRP (~20 × 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 analysed using BD Accuri™ C6 software[8].

Platelet TXB_2 synthesis

LTA assays were stopped by adding 100 μmol/L diclofenac sodium (Sigma-Aldrich, UK) and 100 U/mL heparin (Leo Laboratories, UK). Samples were centrifuged (500 x g, 2 min) and supernatants were collected and stored at -80°C until TXB_2 measurement by ELISA (Cayman Chemical, MI, USA).

Eicosanomic profile in whole blood

Citrated whole blood was incubated with phosphate buffered saline (PBS), collagen (30 μg/mL) or PAP1p-6 (25μM) (37°C, 30 min) under stirring conditions in the aggregometer. Stimulation was ended with 100 μmol/L diclofenac sodium (Sigma-Aldrich, UK) and 100 U/mL heparin (Leo Laboratories, UK). Plasma supernatants (2000 x g, 5 min) were collected and stored frozen until use for total eicosanoids measurement by gas chromatography–tandem mass spectrometry as described elsewhere.[9,10] Briefly, after extraction with ethyl acetate, samples were passed through Maestro A columns (Tecan, Switzerland) under gravity flow, columns were washed with acetonitrile, samples were dried by vacuum centrifugation at 37°C and reconstituted in 30% ethanol. Platelet eicosanoids were then separated by reverse-phase HPLC on a 1 x 150 mm, 5 μm Luna C18 (2) column (Phenomenex, CA, USA) and quantified using an MDS Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems-ThermoFisher Scientific) with negative-mode electrospray ionization and multiple reaction monitoring.

Immunofluorescence studies in platelets and leukocytes

The immunostaining of platelets and leukocytes was performed essentially as described before.[11] Briefly, platelet-rich-plasma was fixed with 4% paraformaldehyde in PBS at room temperature for 15 min. Then, platelets were washed with PBS-ACD (22.0 g/L citric acid, trisodium salt, dihydrate; 7.3 g/L citric acid, anhydrous; and 24.5

g/L D-(+)-Glucose), (pH 6.1,) and resuspended in 1% BSA (Sigma-Aldrich). For leukocyte analysis, the interface between PRP and red blood cells was separated, Lyse/Fix solution (BD Bioscience, Germany) was added and leukocytes were isolated by centrifugation (2000 x g, 5 min) and diluted in saline. Platelets or leukocytes were spotted onto glass coverslips at 37°C for 90 min and then blocked with the blocking buffer (0.2% Triton X-100, 2% donkey serum and 1% BSA) for 60 min. Following this, cells were incubated with anti-COX-1 (Cell Signaling Technology, UK; 4841S; 1:100), anti-α-tubulin (Sigma-Aldrich, T5168; 1:200) and anti-CD45 (Abcam, UK, ab8216; 1:200) antibodies overnight.

Cells were washed and incubated for 60 minutes with Alexa 647-conjugated secondary antibody (donkey anti-rabbit polyclonal IgG H+L; Life Technologies; A-31573; 1:500) and Alexa 488-conjugated secondary antibody (donkey anti-mouse polyclonal IgG H+L; Life Technologies; A-21202; 1:500) to detect COX-1, α-tubulin and CD45, respectively. Samples were incubated with DAPI (25 μg/mL; Life Technologies) for 10 minutes to stain leukocyte nuclei. The coverslips were then mounted onto glass slides and visualized with oil immersion objectives (CFI Plan Apochromat 40X, N.A.1.4, working distance 0.26 mm – 63x for platelets and 40x for leukocytes) on a confocal laser scanning microscope (LSM 880 with Airyscan, Zeiss, UK). Acquisition and image processing were performed using the ZEN software (Version 2.35spi, Zeiss) and ImageJ (Version 1.51a, National Institutes of Health, USA)

Cell models for mutant COX-1

To assess the effect of COX-1 genetic variants we established HEK 293T and HEK 293 cells models. These cells were selected as they hardly express COX-1.[12] pcDNA3.1+/C-(K)-DYK vectors containing wild-type or mutant (c.428A>G [p.Asn143Ser]) cDNA g of *PTGS1* were commercially available (OHu14933D, Genscript). A Ser145Ala construct was prepared by PCR-based site-directed mutagenesis on the commercial pcDNA3.1 wild-type vector, by using the Stratagene QuikChange Site-Directed Mutagenesis kit with appropriate primers as described.[13]

HEK 293T or HEK 293 cells (ATCC, LGC Standards S.L.U. Spain) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum.

HEK 293 T cells (2 x 10^5 /well) were transiently transfected with vectors expressing wild-type or mutant (p.Arg143Ser or p.Ser145Ala) COX-1 using Lipofectamine 3000

(ThermoFisher Scientific). Comparable transfection efficiencies in either condition were ensured by flow cytometry measurement of intracellular levels of recombinant COX-1 using the BD IntraSure kit (BD Biosciences) and the antibody α-DYKDDDDK*PE (BioLegend, CA, USA, Cat#637309).

HEK 293 cells (2 x 10^5 /well) were transfected with vectors expressing wild-type (Asn143) or Ser143 mutant COX-1 as above, also containing the neomicine resistance gen. Cell were grown in a geneticine (G418, ThermoFisher Sicentific) containing media for 20 days. Resistant and stable transfected cell clones were isolated and grown separately. Finally, cells expressing either the wild-type or the Ser143 mutant COX-1 were transiently transfected with different amounts of vector (0.25-3 μg) expressing the alternative COX-1 protein.

In either case, at 48h post-trasnfection, cells were washed with PBS and splitted for the stimulation experiment and for preparation of protein lysates. In stimulation experiments, cells (25 x10^4 for each transfection condition) were incubated with PBS or 500 μM AA for 30min at 37 °C, centrifuged (5 min, 1000 x g) and the supernatants were recovered and stored frozen at -80°C until TXA_2 determination by ELISA (Cayman Chemical).

Western blot analysis

Standard western blotting procedures were used.[8] Briefly, washed platelets (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, containing protease inhibitors (cOmplete™ Protease Inhibitor Cocktail, Sigma-Aldrich, UK) were lysed with Triton X-100 (0.5% v/v). Proteins were separated by 8% SDS–PAGE and transferred to polyvinylidene fluoride membranes. Blots, were stepwise incubated with primary antibodies against COX-1 and β-actin (Cell Signaling Technology, cat# 4841S and (Sigma-Aldrich, cat# A3854 respectively) followed by secondary horseradish peroxidase–conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich, cat# 12-348).

Proteins were detected by chemiluminescence (ECL prime; GEHealthcare).For N-glycosylation analysis, lysates from cell transfections were denatured for 5 min at 100°C in 150 mM sodium phosphate buffer, pH 7.4, supplemented with 2% SDS and 1M β-mercaptoetanol.[14] Samples were chilled on ice and digested at 37°C for 15h with

0.6 U N-glycosidase F (PNGase-F, Roche Diagnostics GmbH, Germany) in the presence of 15% Triton X-100. Finally, cell proteins lysates, with or without N-glycosidase F treatment, were separated on 8% SDS-PAGE gels and immunoblotting was carried out as above.

Statistical analysis

All functional assays were performed in parallel with samples from the patient and from at least one healthy control. Unless specifically stated, statistical comparisons could not be performed because of the limited sample number.

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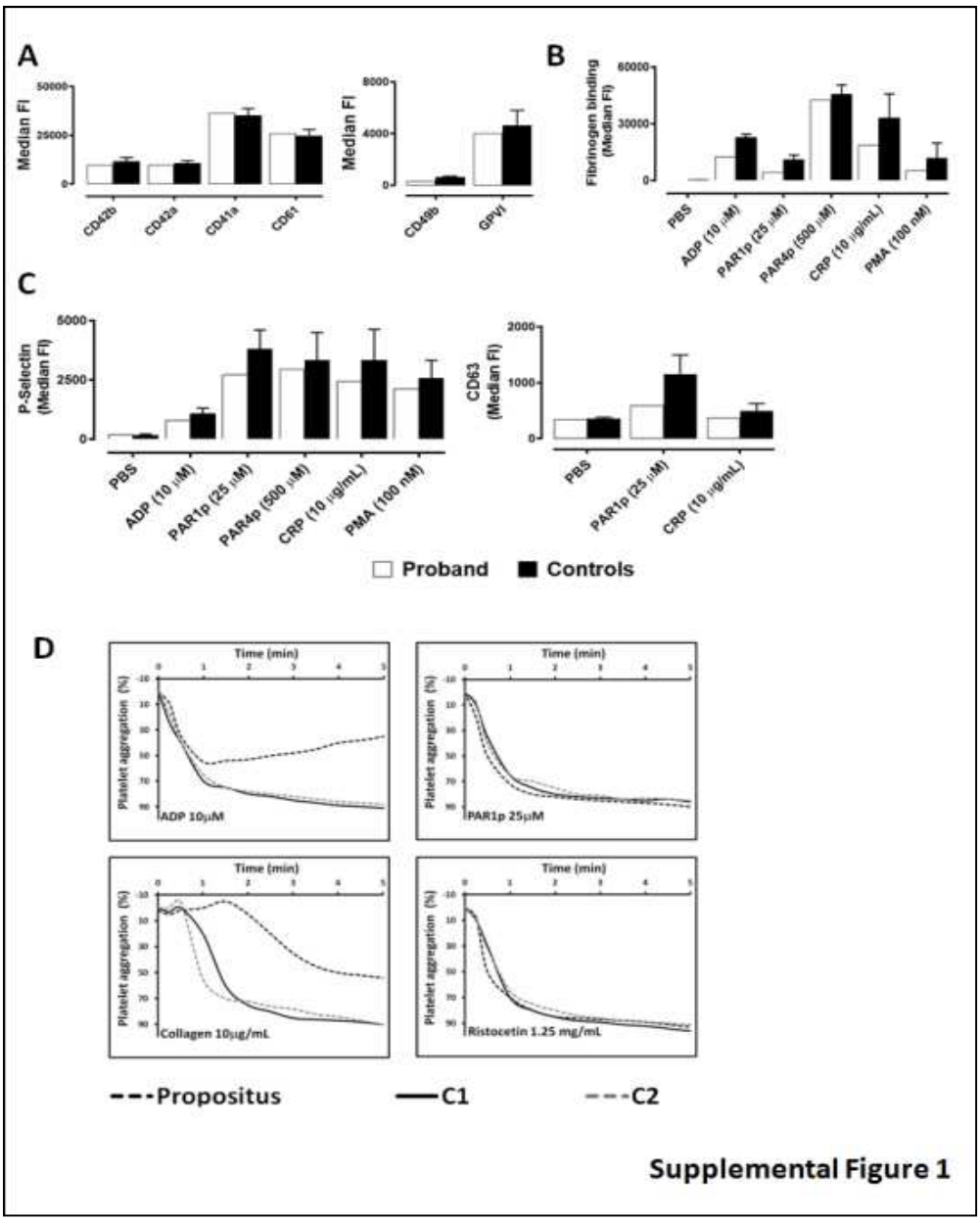
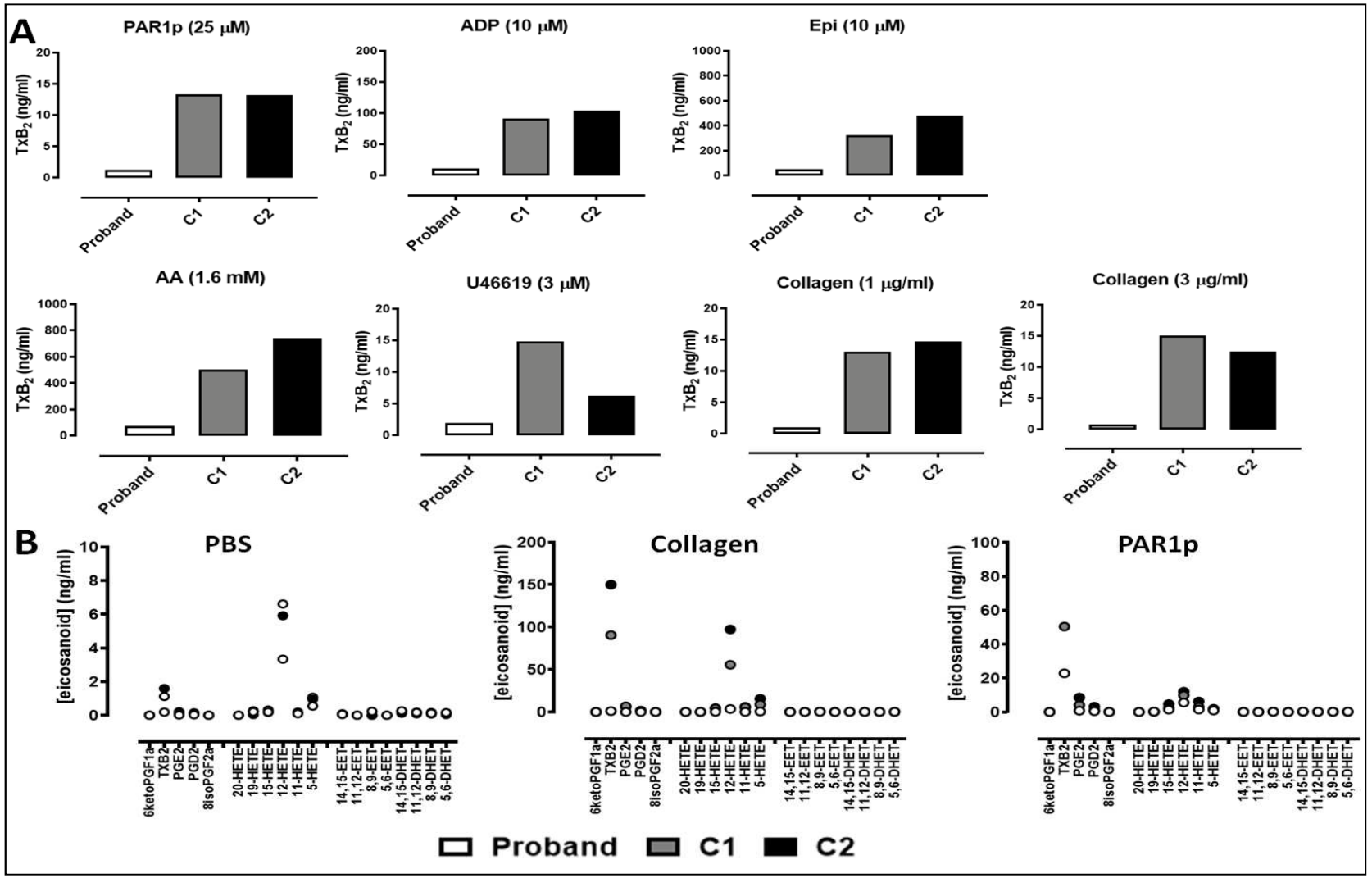
Supplemental Figure 1. The proband's platelet function phenotype



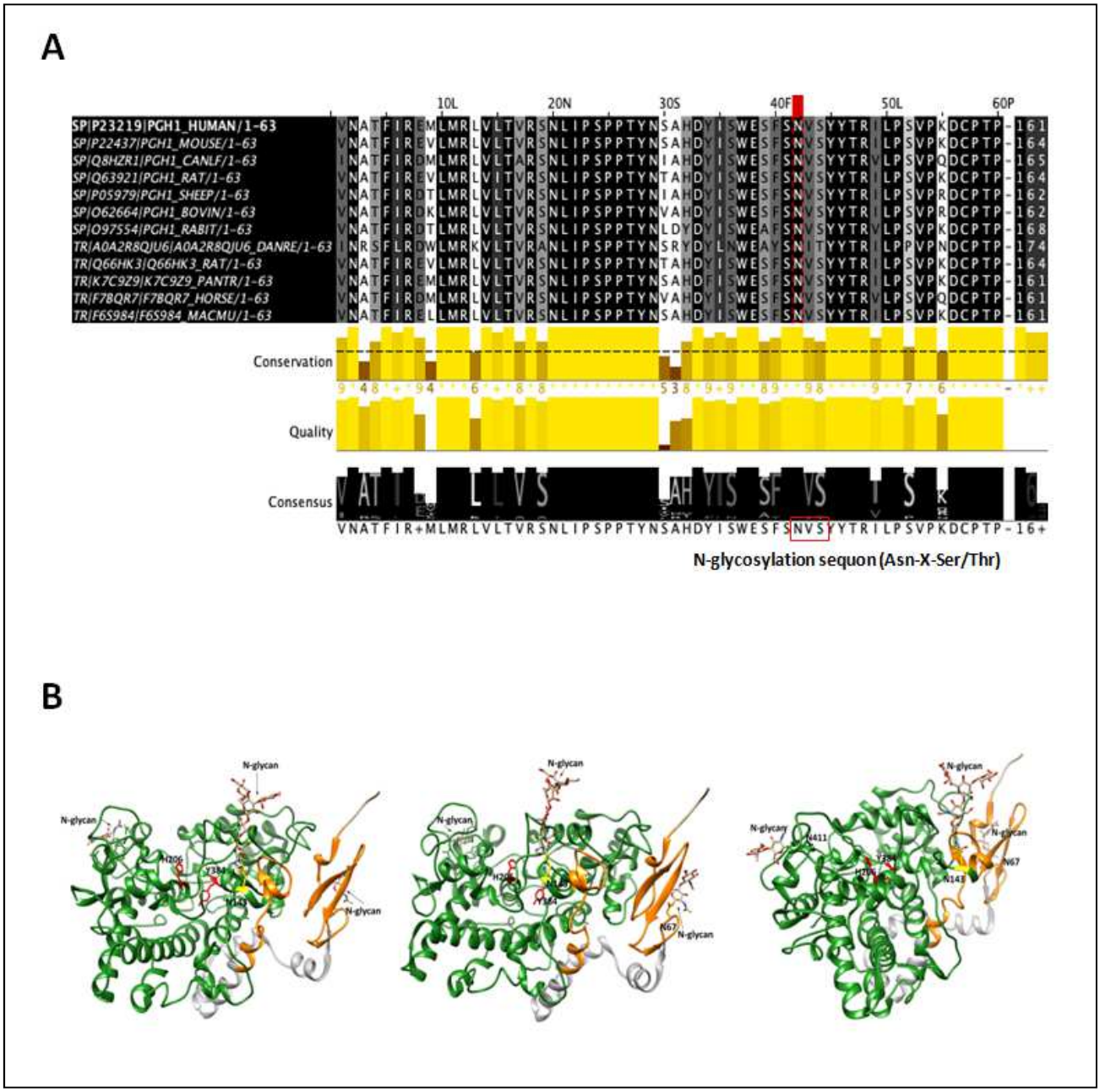
A) GPs expression profiles were assessed by flow cytometry in diluted whole blood from the proband and healthy controls (n=2) assayed in paralel. The fluorescently labelled antibodies used are reported in Supplemental Material. Platelets were incubated under static conditions in the presence of PBS or platelet agonists and of fibrinogen-Alexa 488 **(B)**, anti-CD62P or CD63 monoclonal antibodies **(C),** to evaluate fibrinogen binding and the release of α–and δ–granules, respectively, by flow cytometry. In A-C graphs the median fluorescence intensity is shown; mean ± SD of values in the two controls. **D)** Platelet aggregation in response to the indicated agonists was evaluated in PRP from the index case and two healthy controls (C1 and C2)

Supplemental Figure 2: Thromboxane A_2 release in platelet-rich-plasma and eicosanoid production in whole blood from the proband and time-matched controls.



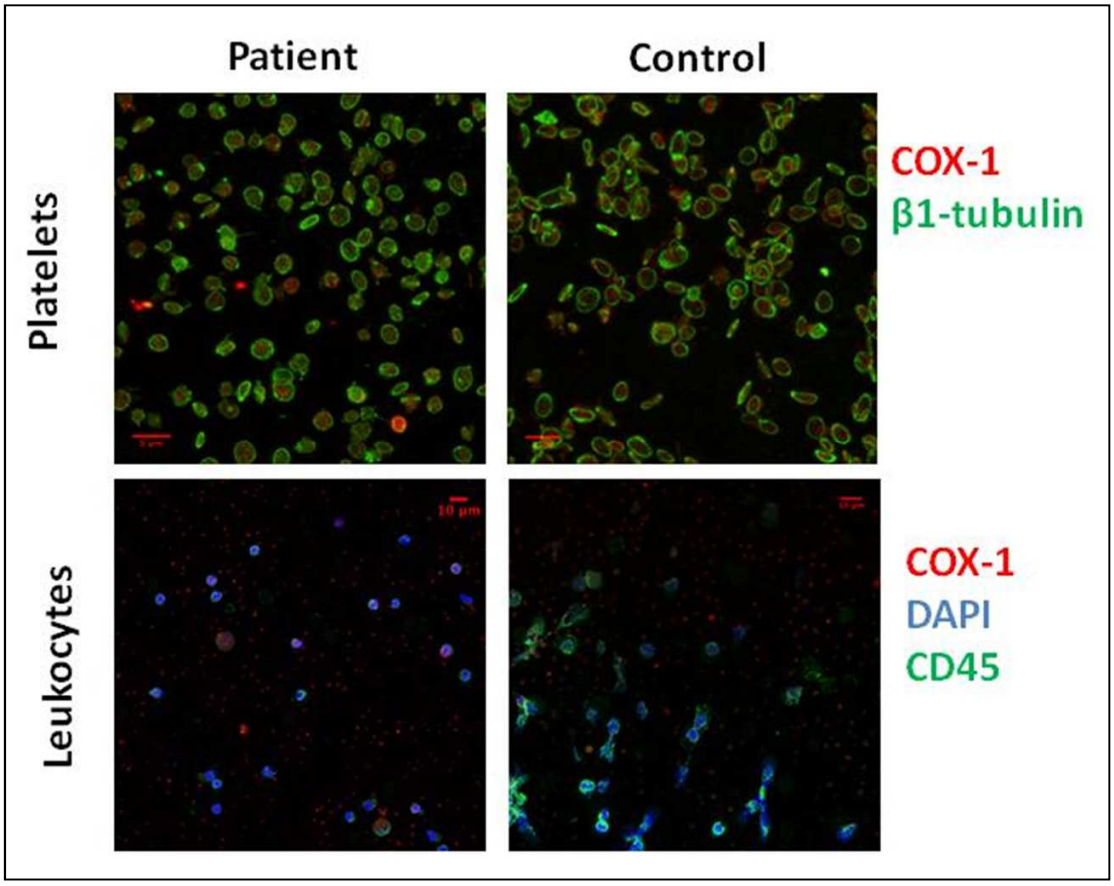
A) TXB_2 levels (ng/mL) in supernatants of platelet aggregation reactions from the proband and time-matched controls (C1 and C2) measured by ELISA. **B)** Eicosanoid levels determined by LC-MS in the proband and controls (C1 and C2) whole blood, at basal level (PBS) and following incubation with collagen (30 mg/mL) or TRAP-6 amide (30 µmol/L).

Supplemental Figure 3. Schematic representation for the conservation of the Asn143 N-glycosylation residue in the Cox-1 sequence and its location in the COX-1 tridimensional structure.



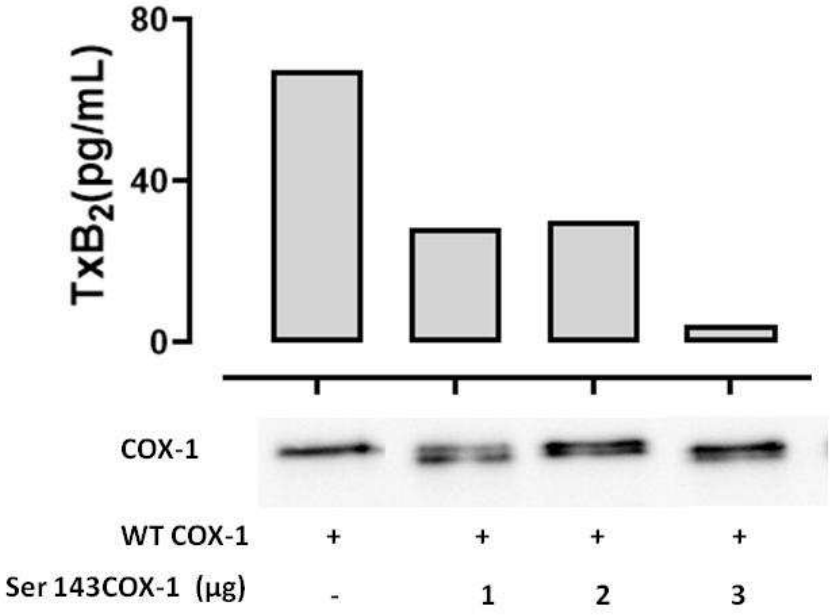
A) Amino acid sequence alignment of COX-1 from different species is represented and the mutated residue (in a red square) is conserved among species. The affected sequence is also highlighted. **B)** The tridimensional structure of human wild-type COX-1 protein was visualized by using the crystal by *Scilimati A et al* (PDB:6y3c) (results to be published) in the Quimera software. N-glycosylation residue 143 is shown in yellow; catalytic resides Y384, H205 are shown in red; glycans are marked with arrows; different domains of COX-1 are coloured with the same pattern used in panel A.

Supplemental Figure 4: Immunofluorescence analysis of COX-1 in platelets and leukocytes from the patient with the novel *PTGS1* variant p.Asn143S and time-matched control.



In platelets tubulin is represented in green and COX-1 in red. Leukocytes were identified using CD45 (green), as surface marker, and DAPI (blue) as a nuclear marker, while COX-1 is represented in red. Images were acquired with Airyscan laser scanning confocal microscope. Bars represent 5 µm for platelets and 10 µm for leukocytes.

Supplemental Figure 5. The Ser143 COX-1 mutant has a dominant-negative effect on the enzymatic activity of native COX-1.



HEK 273 cells were transfected with vector expressing wild-type Asn143 COX-1 and the neomicine resistance gen and grown in the presence of geneticine. Resistant and stable transfected cell clones were isolated and then transfected with different amounts of Ser143 mutant COX-1. Cells were washed and splitted for preparation of protein lysates and for AA (500uM) stimulation and TXA_2 production measurement by ELISA. A double COX-1 protein is seen in co-transfected cells.

Supplemental Table 1. Blood parameters and clinical bleeding symptoms in the heterozygous carrier of the novel variant p.Asn143Ser in COX-1

Age	**13 yr**
Blood Parameters	
WBC, $\times 10^9$/L	5.73
RBC, $\times 10^{12}$/L	3.89
Hb, g/dL	12.2
Hct (%)	33.3
Platelets, $\times 10^9$/L	206
MPV, fL	11.4
PFA-100 CT (s)	
Collagen/epinephrin	>300
Collagen/ADP	120s
ISTH-BAT score	6
Bleeding symptoms	-Epistaxis -Easy bruising and petechiae -Menorrhagia -Bleeding after surgery

WBC=white blood cells; RBC=red blood cells; Hb=hemoglobin; Hct=hematocrit; MPV=mean platelet volume; PFA-100= platelet function analyzer; CT=closure times. ISTH-BAT: International Society on Thrombosis and Haemostasis –Bleeding Assessment tool.

Supplemental Table 2. Agonist-induced eicosanoid production in whole blood determined by LCMS. Total eicosanoid levels in whole blood from healthy controls (C1 and C2) or from the proband following incubation with vehicle (PBS), collagen (30 µg/mL) or PAR1p (TRAP-6 amide) (25 µM).

Metabolite (ng/mL)	Patient			C1			C2		
	PBS	Collagen (30 µg/mL)	PAR1p (25µM)	PBS	Collagen (30 µg/mL)	PAR1p (25µM)	PBS	Collagen (30 µg/mL)	PAR1p (25µM)
6ketoPGF1a	0.00100	0.00367	0.00267	0.00500	0.01533	0.00200	0.00100	0.00867	0.02033
TXB2	0.19000	0.98600	22.79667	1.11633	90.45033	50.41900	1.58400	149.89300	113.25867
PGE2	0.01433	0.03200	0.76033	0.07967	6.74867	4.09833	0.20433	5.82600	8.49033
PGD2	0.02833	0.04367	0.43700	0.09233	1.48867	0.94333	0.14533	1.38967	3.12333
8isoPGF2a	0	0	0	0	0	0	0	0	0
20-HETE	0	0	0	0	0	0	0	0	0
19-HETE	0.20600	0.23233	0.19600	0.26467	0.12200	0.16300	0.04067	0.10967	0.10767
15-HETE	0.19500	0.16033	1.40067	0.30800	3.98867	2.41967	0.17133	4.51300	4.63833
12-HETE	3.33033	3.48100	5.57833	6.61000	55.63067	9.77867	5.91767	97.24267	11.97133
11-HETE	0.09600	0.11467	1.45267	0.18567	5.41400	2.99200	0.10700	6.09967	6.19967
5-HETE	0.54433	0.57367	0.88733	1.07233	8.84433	1.53967	0.95200	15.41467	1.88433
14,15-EET	0.05000	0.06333	0.06267	0.04800	0.03600	0.04667	0.09200	0.05167	0.00000
11,12-EET	0	0	0	0	0	0	0	0	0
8,9-EET	0.22833	0.28867	0.13033	0.00000	0.41633	0.22267	0.00000	0.34500	0.08933
5,6-EET	0	0	0	0	0	0	0	0	0
14,15-DHET	0.26433	0.24933	0.25900	0.24867	0.20900	0.20033	0.11800	0.12733	0.13867
11,12-DHET	0.17167	0.13200	0.15500	0.18000	0.14700	0.15667	0.08467	0.09167	0.10200
8,9-DHET	0.12233	0.14967	0.15133	0.16567	0.12000	0.10433	0.07133	0.04400	0.06967
5,6-DHET	0.15633	0.15467	0.19933	0.13933	0.11067	0.12000	0.03800	0.04167	0.06200

Supplemental Table 3. ACMG classification (A) and pathogenicity scores (B) of the p.Asn143Ser variant through different predictors.

A

Verdict: ***"Uncertain Significance"***

X PVS1	X PS1	X PS2	X PS3	☐ PS4	X PM1	√ PM2 Moderate	☐ PM3
X PM4	X PM5	X PM6	X PP1	X PP2	√ PP3 Supporting	☐ PP4	X PP5
X BA1	X BS1	X BS2	X BS3	X BS4			
√ BP1 Supporting	☐ BP2	X BP3	X BP4	☐ BP5	X BP6	X BP7	

B

Computational predictor	Classification	Score
MutationTaster	Disease causing	1
Mutation assessor	Medium	3.46
FATHMM	Tolerated	2.31
FATHMM-MKL	Damaging	0.9959
FATHMM-XF	Damaging	0.9037
LRT	Deleterious	0
DEOGEN2	Tolerated	0.4877
EIGEN	Pathogenic	0.9345
EIGEN PC	Pathogenic	0.8692
SIFT	Damaging	0.002
SIFT4G	Damaging	0.004
PROVEAN	Damaging	-4.22
MVP	Benign	0.8289
REVEL	Benign	0.546
PrimateAI	Damaging	0.4489
MetaSVM	Tolerated	-0.6806
MetaLR	Tolerated	0.1654
Polyphen	Probably damaging	0.997
Allele frequency	Not reported previously	

ACMG=American College of Medical Genetics; Allele frequency in general population gnomAD browser database (https://gnomad.broadinstitute.org/); All the information is available on the web tool (https://varsome.com/)

Supplemental Table 4. Flow cytometry determination of COX-1 expression in HEK 293 T cells transfected with wild-type COX-1 [Asn143], mutant Ser143 or Ala145 COX-1 vectors or co-transfected with wild-type and mutant Ser143 COX-1 vectors [Asn143+Ser143].

	% +α-DYKDDDDK cells	
	MEAN	SD
Asn143	48.04	13.06
Ser143	50.89	8.18
Asn143+Ser143	45.76	14.07
Ala145	49.5	5.93

HEK 293 T cells (2 x 10^5 /well) were transiently transfected with vectors expressing wild-type or mutant COX-1 using Lipofectamine 3000. Comparable transfection efficiencies in either condition were ensured by flow cytometry measurement of intracellular levels of recombinant COX-1 using the BD IntraSure kit (BD Biosciences) and the antibody α-DYKDDDDK*PE. Data show mean % of cell expressing the enzyme.