Identification of a homozygous recessive variant in *PTGS1* resulting in a congenital aspirin-like defect in platelet function

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SUPPLEMENTAL MATERIAL

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Statement of equal authors' contribution
*MVC, MAH and SS contributed equally to this work as first authors.
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Effects of absence of PTGS1 in platelets

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DETAILED METHODS

Platelet aggregometry

Final aggregation was measured in platelet-rich plasma (PRP) by light transmission aggregometry using a PAP-8E turbidometric aggregometer (Bio/Data). Responses to arachidonic acid (AA; 1 mmol/L, Sigma, UK), adenosine diphosphate (ADP; 10 µmol/L, Chronolog, UK), collagen (0.3–3 µg/mL, Nycomed, Austria), epinephrine (10 µmol/L, Labmedics, UK), Ristocetin (1.5 mg/mL, Helena Bioscience, UK), thromboxane (TX) A_2 mimetic U46619 (3 µmol/L, Cayman Chemical) or TRAP-6 amide (25 µmol/L, Bachem, Austria) were measured.

Optimul aggregometry was performed as published previously. Briefly, PRP or PPP were placed in the appropriate wells of a half-area 96-well plate containing AA (0.03–1 mmol/L), ADP (0.005–40 µmol/L), collagen (0.01–40 µg/mL), epinephrine (0.0004–10 µmol/L), ristocetin (0.14–4 mg/mL), U46619 (0.005– 40 µmol/L), TRAP-6 amide (SFLLRN, 0.03–40 µmol/L) or vehicle. Plates were mixed (1200 rpm, 37°C; BioShake IQ, Q Instruments, Germany) for 5 min, and absorbance at 595 nm was measured using a standard absorbance microplate reader (Sunrise, Tecan, Switzerland). Platelet aggregation was calculated as percentage change in absorbance.

ATP release

Chrono-Lume reagent (0.2 µmol/L luciferin/luciferase, Chronolog) was added to PRP and ADP + ATP secretion was assessed by measuring luminescence in an optical lumi-aggregometer (560 CA, Chronolog, USA) after stimulation by AA (1 mmol/L), ADP (10 µmol/L), collagen (3 µg/mL) or TRAP-6 amide (25 µmol/L). Maximum ATP release was calculated using a 2 µmol/L ATP standard and data were analysed using Chart v8.1.12 software (ADInstruments, UK).

P-selectin expression

Whole blood was added to wells of a modified Optimul plate with ADP (40 µmol/L), U46619 (0.5 µmol/L), or a combination of both. After mixing (1200 rpm, 37°C, 5 min), the aggregation was halted by the addition of anti-coagulant citrate dextrose solution (ACD; 5 mmol/L glucose, 6.8 mmol/L trisodium citrate, 3.8 mmol/L citric acid)[1]. Diluted blood was incubated in the dark with APC-conjugated CD61 and PE-conjugated CD62P antibodies (4°C, 30 min). Samples then fixed in 1% formalin and flow cytometric analysis (FACSCalibur, BD Biosciences, UK) was conducted to determine mean fluorescence intensity (MFI) values for 10,000 CD61-positive events.

ImageStream®^{X} analysis

Whole blood was fixed and erythrocytes removed using Lyse/Fix (BD Biosciences). Cell interactions were assessed using an ImageStream®^{X} Mark II imaging flow cytometer (Merck Millipore, UK) in whole blood stained with anti-CD61-FITC (platelets), anti-CD45-PerCP-Cy5.5 (leukocytes), anti-CD14-APC (monocytes) and anti-CD66b-Pacific Blue (neutrophils). Samples were fixed and diluted in a formalin/phosphate-buffered saline solution containing dextrose and bovine serum albumin (BSA). The number of platelet-monocyte and platelet-neutrophil aggregates were quantified using IDEAS® software (Merck Millipore, UK).

Western blotting

Platelet protein lysates were prepared as described[2]. Protein concentration was quantified with a Bradford analysis and 25 µg of platelet lysates was loaded on a 10% Bis-Tris gel (Bio-Rad, CA, USA). Protein fractions were resolved by SDS–polyacrylamide gel electrophoresis, and blots were incubated with anti-COX-1 antibody (Cell Signaling Technology, The Netherlands) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (clone 4G5, Fitzgerald Industries International). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (Dako, Denmark) and chemiluminescent blots

were imaged with the ChemiDoc MP imager and the ImageLab software version 4.1 (Bio-Rad) was used for image acquisition.

Production of variant W322S and wild type human COX-1 and COX and peroxidase activity assays

Oligos used for site-directed mutagenesis were purchased from Integrated DNA Technologies (Coralville, IA, USA). A pFastBac-1 vector containing the gene for human (hu) COX-1 was used to insert six histidine residues at rTEV cleavage site between Ala-24 and Gly-25, which is directly after the signal sequence. Subsequently, this construct was used to engineer the W322S mutant construct using the QuikChange Mutagenesis kit II (Agilent Technologies). Both constructs were sequence verified. Baculovirus generation and expression was carried out in sf21 insect cells as previously described[3]. For purification, the cell pellet from a 2L culture of insect cells was resuspended in buffer A (40 mmol/L HEPES, pH 7.4, 250 mmol/L sucrose, and 1 mmol/L dithiothreitol), lysed using a Microfluidizer, and clarified by centrifugation at 10000 *x g* for 15 min. The supernatant was layered over buffer B (40 mmol/L HEPES, pH 7.4, 1.3 mol/L sucrose, and 1 mmol/L dithiothreitol) at a ratio of 3:1 (v/v) supernatant to buffer B and subsequently centrifuged at 140000 *x g* for 60 min to isolate microsomal membranes. Microsomal membrane was resuspended in 50 mmol/L Tris, pH 8.0, 300 mmol/L NaCl, 10 mmol/L imidazole, 5 mmol/L 2-mercaptoethanol, 15% (v/v) glycerol and solubilized by adding dodecyl maltoside ($C_{10}M$; Affymetrix) to a final concentration of 0.87% (w/v). The solubilization mixture was stirred overnight at 4°C, followed by centrifugation at 140000 *x g* for 75 min. Ni-NTA affinity chromatography using a HiTrap™ HP Chelating column (GE Healthcare) was then utilized to produce purified wild type and W322S huCOX-1 in 25 mmol/L Tris, pH 8.0, 150 mmol/L NaCl and 0.15% (w/v) $C_{10}M$ for kinetic characterization.

COX activity was measured using a Clark type oxygen electrode as described[3]. The assays were performed at 37°C utilizing cuvettes containing 100 mmol/L Tris, pH 8.0, 1 mmol/L phenol, 5 µmol/L Fe^{3+}-protoporphyrin IX, AA (Cayman Chemical) as the substrate. Reactions

were initiated via the addition of 20 µg of wild type or W322S huCOX-1 and activity was recorded as the maximal rate of oxygen consumption. For inhibition studies, 5 µmol/L wild type or W322S was incubated with aspirin (500 µmol/L) before measurement of COX activity over 30 min. Peroxidase activity was measured by monitoring the oxidation of guaiacol. K_m and V_{max} values were determined by measuring oxygen uptake using AA (1-100 µmol/L) and fitting the data to the Michaelis-Menton equation using GraphPad Prism. All measurements were carried out in duplicate±s.d.

Detecting COX-1 protein expression using confocal imaging

PRP was fixed with paraformaldehyde (4%) in PBS at room temperature for 15 min. Platelets were then pelleted, washed with ACD (pH 6.1) and resuspended in BSA (1%). In other experiments, the interface between PRP and red blood cells was taken, Lyse/Fix was added and leukocytes were isolated by centrifugation (2000 *x g*, 5 min) before being resuspended in saline. Platelets or leukocytes were spotted onto glass coverslips and incubated at 37°C for 90 min. Coverslips were then rinsed with PBS and blocking buffer (0.2% Triton-X100, 2% donkey serum and 1% BSA) was added for 60 min. Following this, platelets were incubated with anti-COX-1 (Cell Signaling Technology) and anti-tubulin (Sigma-Aldrich, UK) overnight. Leukocytes were incubated with anti-COX-1, anti-DAPI for DNA (ThermoFisher Scientific, UK) and anti-LAMP-3 for neutrophils (Hybridoma Bank, USA). After wash with PBS, slides were incubated Alexa Fluor® secondary antibodies (ThermoFisher Scientific).

The coverslips were mounted onto glass slides and visualised 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) equipped with 5 lasers: diode 405-30 (405 nm), Argon (458/488/514 nm), DPSS 561-10 (561 nm), HeNe633 (633 nm), Chameleon (680-1080 nm). Acquisition and image processing were performed using the ZEN software (Version 2.35spi, Zeiss) and ImageJ (Version 1.51a, National Institutes of Health, USA).

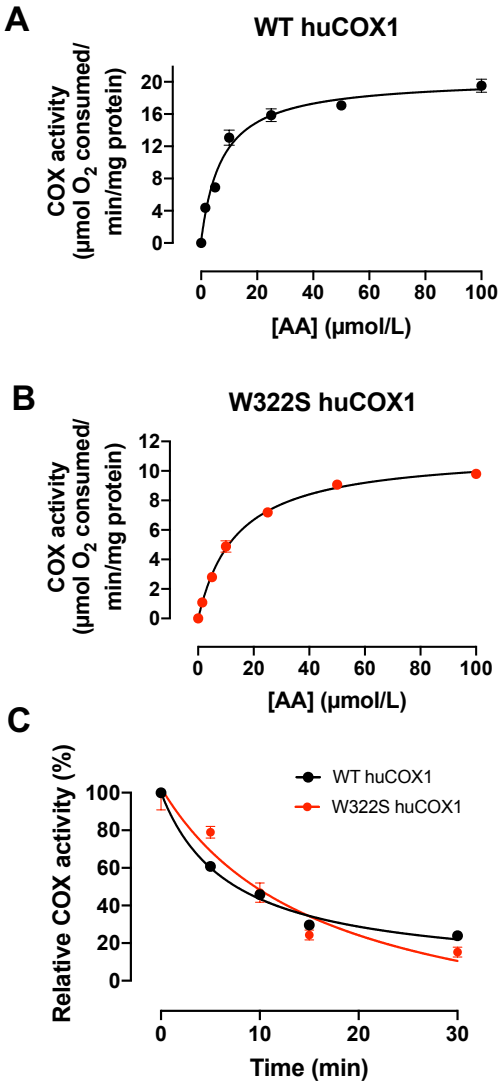
Spreading

Washed platelets were resuspended at $2x10^8$/ml and placed onto coverslips coated with collagen (100 µg/mL). Adherent platelets were fixed with paraformaldehyde (0.2%) and permeabilised with Triton-X100 (0.2%). After washing with PBS, platelet F-actin was stained with Alexa Fluor® 488 phalloidin (ThermoFisher Scientific) and mounted onto slides. Slides were visualised as above. Platelets were scored by two independent markers as adhered, exhibiting filopodia or lamellipodia or as fully spread and expressed as percentage of all platelets.

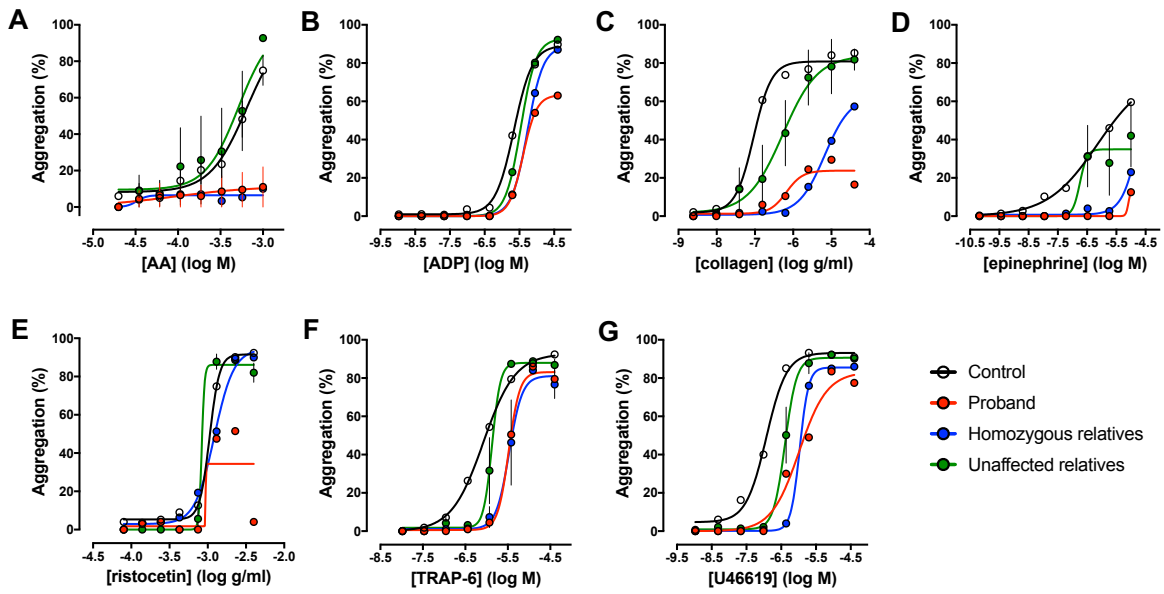
Eicosanomic analysis

Citrated whole blood was incubated with PBS, collagen (30 µg/mL) or TRAP-6 amide (30 µmol/L) at 37°C for 30 min and plasma was isolated by centrifugation at 2000 *x g* (5 min). Total eicosanoids in the conditioned plasma and urine were measured by gas chromatography–tandem mass spectrometry as previously described[4–8].

SUPPLEMENTAL FIGURES AND FIGURE LEGENDS



Supplementary Figure 1. Kinetic analysis of (A) wild type and (B) variant W322S COX-1 and (C) the relative activity in the presence of aspirin (500 µmol/L). COX activity was measured using a Clark type oxygen electrode as described in methods. K_m and V_{max} were determined by fitting the data to the Michaelis-Menton equation. All measurements were carried out in duplicate and shown as mean±s.d.



Supplementary Figure 2. Platelet aggregation measured by the Optimul method in response to (A) arachidonic acid (AA; 0.03–1 mmol/L), (B) ADP (0.005–40 µmol/L), (C) collagen (0.01–40 µg/mL), (D) epinephrine (0.0004–10 µmol/L), (E) ristocetin (0.14–4 mg/mL), (F) U46619 (0.005–40 µmol/L) and (G) TRAP-6 amide 0.03–40 µmol/L). n = 20 (healthy controls); n = 1 (proband); n = 2 (homozygous relatives); n = 4 (unaffected relatives).

SUPPLEMENTAL TABLES AND SUPPORTING INFORMATION

Table S1. Contribution of *PTGS1* to eicosanoid synthesis in whole blood stimulated with platelet activators. Total eicosanoid levels in whole blood from healthy controls or from the proband lacking *PTGS1* stimulated with vehicle (PBS), collagen (30 µg/mL) or TRAP-6 amide (30 µmol/L). n=4 (healthy controls), n=1 (proband).

Mediators (ng/mL)	Vehicle (PBS)		Collagen (30 µg/mL)		TRAP-6 amide (30 µmol/L)	
	Control	Proband	Control	Proband	Control	Proband
6ketoPGF$_{1\alpha}$	0.0 ± 0.1	0.6	0.4 ± 0.0	0.9	0.2 ± 0.0	3.4
TXB$_2$	3.7 ± 3.7	0.0	47.3 ± 1.6	0.1	41.4 ± 7.1	0.0
PGE$_2$	0.5 ± 0.4	0.0	24.6 ± 2.7	0.0	15.2 ± 7.3	0.0
PGD$_2$	1.0 ± 0.6	0.8	22.9 ± 2.3	0.7	14.9 ± 6.4	0.7
8isoPGF$_{2\alpha}$	0.0 ± 0.0	0.1	0.5 ± 0.1	0.1	0.3 ± 0.1	0.1
PGF$_{2\alpha}$	2.8 ± 0.6	1.2	6.3 ± 0.8	1.3	5.0 ± 1.2	1.2
12,13-DHOME	2.6 ± 1.7	2.3	2.5 ± 1.6	2.4	3.1 ± 2.2	1.8
9,10-DHOME	3.7 ± 3.4	7.1	3.3 ± 2.7	7.4	4.3 ± 4.1	7.4
19,20-DiHDPA	0.5 ± 0.1	1.2	0.5 ± 0.1	1.3	0.6 ± 0.2	1.3
17,18-DHET	1.5 ± 0.6	3.9	1.3 ± 0.4	4.3	1.6 ± 0.7	4.7
14,15-DHET	0.2 ± 0.0	0.3	0.2 ± 0.0	0.4	0.3 ± 0.1	0.4
11,12-DHET	0.2 ± 0.0	0.3	0.2 ± 0.0	0.3	0.2 ± 0.1	0.3
8,9-DHET	0.1 ± 0.0	0.1	0.1 ± 0.0	0.1	0.1 ± 0.1	0.1
5,6-DHET	0.1 ± 0.0	0.1	0.1 ± 0.0	0.1	0.2 ± 0.1	0.1
13-HODE	3.7 ± 3.3	3.3	5.2 ± 2.9	4.5	4.8 ± 3.5	3.7
9-HODE	4.7 ± 3.7	4.4	13.5 ± 3.8	4.5	9.0 ± 4.1	4.7
20-HETE	0.2 ± 0.0	0.3	0.3 ± 0.1	0.5	0.2 ± 0.0	0.3
19-HETE	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0
15-HETE	0.7 ± 0.5	0.3	18.3 ± 2.1	2.4	9.2 ± 3.0	0.5
12-HETE	12.8 ± 7.5	3.6	165.1 ± 28.5	232.8	32.2 ± 18.1	18.5
11-HETE	0.6 ± 0.5	0.1	17.7 ± 2.3	0.8	9.1 ± 2.7	0.2
5-HETE	0.3 ± 0.1	0.3	0.4 ± 0.1	0.4	0.4 ± 0.1	0.4
12,13-EpOME	2.8 ± 4.2	1.9	2.4 ± 3.3	1.9	3.6 ± 5.2	1.9
9,10-EpOME	1.6 ± 1.5	2.1	1.3 ± 1.1	2.1	2.0 ± 1.6	2.1
19,20-EpDPE	0.2 ± 0.0	0.3	0.2 ± 0.1	0.4	0.2 ± 0.1	0.4
17,18-EpETE	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0
14,15-EET	0.1 ± 0.0	0.1	0.2 ± 0.0	0.3	0.2 ± 0.0	0.2
11,12-EET	0.1 ± 0.0	0.1	0.1 ± 0.0	0.2	0.1 ± 0.0	0.2
8,9-EET	0.1 ± 0.0	0.1	0.1 ± 0.0	0.2	0.1 ± 0.0	0.2
5,6-EET	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0

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