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TITLE PAGE

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

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Running head
Effects of absence of PTGS1 in platelets

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ABSTRACT (247 words/250)

We have identified a rare missense variant on chromosome 9, position 125145990 (GRCh37), in exon 8 in *PTGS1* (the gene encoding cyclo-oxygenase 1, COX-1, the target of anti-thrombotic aspirin therapy). We report that in the homozygous state within a large consanguineous family this variant is associated with a bleeding phenotype and alterations in platelet reactivity and eicosanoid production.

Western blotting and confocal imaging demonstrated that COX-1 was absent in the platelets of three family members homozygous for the *PTGS1* variant but present in their leukocytes. Platelet reactivity, as assessed by aggregometry, lumi-aggregometry and flow cytometry, was impaired in homozygous family members, as were platelet adhesion and spreading. The productions of COX-derived eicosanoids by stimulated platelets were greatly reduced but there were no changes in the levels of urinary metabolites of COX-derived eicosanoids. The proband exhibited additional defects in platelet aggregation and spreading which may explain why her bleeding phenotype was slightly more severe than those of other homozygous affected relatives.

This is the first demonstration in humans of the specific loss of platelet COX-1 activity and provides insight into its consequences for platelet function and eicosanoid metabolism. Notably despite the absence of thromboxane A_2 (TXA_2) formation by platelets, urinary TXA_2 metabolites were in the normal range indicating these cannot be assumed as markers of *in vivo* platelet function. Results from this study are important benchmarks for the effects of aspirin upon platelet COX-1, platelet function and eicosanoid production as they define selective platelet COX-1 ablation within humans.

INTRODUCTION

Platelets are central to the processes of hemostasis and thrombosis, the latter of which can lead to cardiovascular events such as myocardial infarction or stroke. At sites of vascular injury, platelets are activated upon interaction with collagen, von Willebrand factor (VWF) and fibrinogen and undergo shape change. In order to form a platelet plug, platelets first adhere, then pseudopodia extend from the surface. Following this, lamellipodia spread between these protrusions, resulting in a fully spread platelet within 30 min[1,2]. Platelets release the contents of their dense and α-granules, reinforcing activation and leading to the recruitment of further platelets to form a hemostatic plug in a positive feedback loop[3]. A second feedback loop comprises liberation of arachidonic acid (AA) from membrane phospholipids by phospholipase A_2 (PLA_2) to form thromboxane A_2 (TXA_2).

AA is the substrate for three groups of eicosanoid-producing enzymes: lipoxygenase (LOX) which leads to hydroxyeicosatraenoic acids (HETEs) and leukotrienes, cytochrome P450 (CYP450) which leads to epoxyeicosatrienoic acids (EETs) and cyclo-oxygenase (COX) which leads to prostanoids. COX exists in two isoforms, the constitutively expressed COX-1 (more precisely known as prostaglandin endoperoxide synthase 1, *PTGS1*) and the (generally) inducible COX-2 (*PTGS2*), which both convert AA into prostaglandin (PG) G_2 via an oxygenation reaction and then PGH_2 via a peroxidase reaction[4–6]. In platelets, PGH_2 is then converted by thromboxane synthase to the pro-aggregatory TXA_2[7]. TXA_2 is a key part of the positive feedback loop mentioned above. Irreversible blockade of platelet COX-1 by aspirin abolishes the production of TXA_2 by platelets, explaining its efficacy in anti-thrombotic prophylaxis[8]. Because of aspirin's short half-life within the body and its irreversible effects upon COX, a low dose (75-100 mg per day) demonstrates a more selective effect upon platelets than upon the rest of the body, where nucleated cells can regenerate COX-1 protein[9–11].

Here, we describe the first case of autosomal recessive inheritance of a rare variant in *PTGS1* which reproduces the selective anti-platelet effect of aspirin and provides insight into the normal balance of prostanoid production.

METHODS

Additional methods can be found in the Supplemental Methods.

Ethics and consent

The proband and relatives were enrolled in the National Institute for Health Research (NIHR) BioResource under the Bleeding, Platelet and Thrombotic Disease domain after providing informed written consent[12,13]. The NIHR BioResource projects were approved by Research Ethics Committees in the UK and appropriate national ethics authorities in non-UK enrolment centres. Extensive phenotyping included coding of clinical and laboratory phenotypes with Human Phenotype Ontology (HPO) terms and collection of numerical and family history data was performed as described previously[12]. Healthy volunteer studies were approved by the NHS St. Thomas' Hospital Research Ethics Committee (07/Q702/24). Healthy volunteers and family members abstained from non-steroidal anti-inflammatory drug (NSAID) use for two weeks before sample collection.

Genotyping

The proband and her parents underwent whole genome sequencing (WGS). Variants were called and annotated as described previously[13]. In all other family members variants in *PTGS1* were called by Sanger sequencing[14]. Furthermore, the variant was expressed in cells and COX-activity was measured using a Clark type oxygen electrode[15].

Sample collection

Midstream flow urine was collected and stored for subsequent eicosanomic analysis[16]. Blood was collected by venepuncture into trisodium citrate (BD Diagnostics, UK). Platelet-rich plasma (PRP) was obtained by centrifugation at 175 x g for 15 min. Platelet-poor plasma (PPP) was obtained by centrifugation of PRP at 12000 x g for 2 min. COX-1 protein presence was determined by Western blotting in platelets and confocal microscopy in

platelets and leukocytes. In addition, the number of platelet-monocyte and platelet-neutrophil aggregates were quantified to assess whether this variant modulates interactions of platelets with other blood cells using an ImageStream®[X] imaging flow cytometer (Merck Millipore, UK).

Platelet function studies

Platelet reactivity by light transmission aggregometry (LTA) and Optimul methods was completed within 2 h of blood collection[17,18]. In parallel, ATP release and P-selectin levels were determined to establish markers of platelets release and activation respectively and platelet spreading on collagen-coated surfaces were performed[19].

Data analysis

Statistical summaries are presented as mean±s.d. One-way ANOVA was performed using GraphPad Prism version 8.1.1 for Mac OS X (GraphPad Software, CA, USA) where appropriate. Statistically significant differences in means are presented as *$P<0.05$, **$P<0.01$, ***$P<0.001$ or ****$P<0.0001$. Percentiles of control values were generated, and pedigree member data was compared to this where only one value was obtained.

RESULTS

Whole genome sequencing and phenotyping of the pedigree

The proband, a female of Iranian descent, aged 37 at enrolment (Figure 1, IV-1) was referred to the haemophilia outpatient clinic because of perioperative bleeding following a sinus operation. She had a history of cystic fibrosis (CF), C6 complement deficiency resulting in chronic infections, β-thalassemia trait and normoprolactinemic galactorrhoea. She had more extensive haemoptysis than expected from her CF and also suffered from frequent nosebleeds. At presentation she had a normal platelet count of 234×10^9/L.

Upon taking the family history we appreciated that she was part of a large consanguineous family. Moreover, her mother (III-2) and maternal aunt (III-1) also had a clinical bleeding phenotype including easy bruising and menorrhagia. The two uncles (III-4 and III-5) and a cousin (IV-2) did not have any clinical bleeding (Figure 1A-B). Depending on the severity of bleeding, the proband received desmopressin, tranexamic acid and, very occasionally, platelets.

Sequencing demonstrated that the proband (IV-1), her mother (III-2) and aunt (III-1) were homozygous for a variant on chromosome 9, position 125145990 (GRCh37), altering a guanine to a cysteine in exon 8 of *PTGS1*. This variant resulted in a missense substitution of tryptophan to serine at amino acid 322 (Figure 1C). The variant had a Combined Annotation Dependent Depletion (CADD) score[20] of 31.0 and was absent from the Genome Aggregation Database (gnomAD)[21]. Using Alamut® Visual, the new variant has been shown to be highly conserved with a phyloP score of 9.88. III-3, III-4 and III-5 were heterozygous (GC) for the alternate allele while IV-2 was homozygous for the reference allele (CC), where C represents the wild-type and G represents the mutant allele. III-6 and III-7 were unavailable for genotyping. The proband had an additional biallelic mutation (chromosome 7, position

117175467) causing a splice donor variant in the cystic fibrosis transmembrane conductance regulator (CFTR) gene causing CF.

COX-1 protein in platelets and leukocytes

COX-1 protein in platelet lysates from the proband and her homozygous relatives was absent. In III-3 and III-4, expression was present but reduced and was at normal levels in III-5 and IV-2 (Figure 2A). The absence of COX-1 protein in platelets from the proband and homozygous relatives (Figures 2Ci, Figure 2Di) compared to a healthy control (Figures 2Bi) was confirmed with immunohistochemical analysis. COX-1 expression, however, was retained in leukocytes from all those tested (Figure 2Cii, Figure 2Dii). The variant did not affect COX enzyme activity as shown in kinetic analysis of isolated recombinant protein (Wild-type, K_m = 7.9±0.8 µmol/L; W322S, K_m = 14.1±1.1 µmol/L, Supplementary Figures 1A-B). The variant also had no effect on COX-activity after inhibition by aspirin (Supplementary Figure 1C). Though there was no appreciable phenotypic difference in the quality of interactions observed, there was a reduction in the number of platelet interactions with monocytes in the proband that was not found in other family members (controls, 34.8±19.2%; proband, 7.5%; homozygous relatives, 27.4±9.0%; unaffected relatives, 32.8±10.6%; Figure 3A, Figure 3C). There was no change in platelet-neutrophil interactions (Figure 3B, Figure 3D).

The role of PTGS1 recessive variant on platelet reactivity

Platelet reactivity is measured *in vitro* by aggregation and release experiments. Aggregation responses to arachidonic acid (AA; 1 mmol/L) in the proband and her homozygous relatives were reduced compared to control from 65±7% to 4±1%; responses to collagen (1 and 3 µg/mL) reduced from 64±13% to 17±10% and from 67±8% to 20±11%, respectively; and responses to adenosine diphosphate (ADP; 10 µmol/L) reduced from 62±11% to 38±13%. Interestingly, the proband also had a greatly reduced response to ristocetin (1.5 mg/mL; control, 69±10%; proband, 7%) which was normal in all family members tested. Other than a

reduced epinephrine (10 µmol/L) response, which was found in all family members, there was no difference in other LTA responses of the unaffected family members compared to control (Figure 4A). These findings were also reflected using Optimul aggregometry where AA responses were absent, and collagen and epinephrine responses were severely blunted in the proband and homozygous relatives (Supplementary Figure 2). TRAP-6 amide (25 µmol/L)-stimulated ATP release was normal in all family members. AA (1 mmol/L) and collagen (3 µg/mL)-stimulated secretion, however, was below the 20th percentile in the proband and homozygous relatives (Figure 4B). Upon activation, platelets express P-selectin and undergo shape change and spreading. U46619 (0.5 µmol/L)-induced P-selectin expression was similar in all individuals (Figure 4C).

The effect of the PTGS1 variant on platelet spreading

The number of platelets with filopodia were increased in the proband and homozygous family members (control, 9±6%; proband, 36±17%, p<0.001; homozygous relative, 36±4%, p<0.01). *PTGS1* variant was also associated with a reduction in fully spread platelets (control, 45±6%; proband, 9±9%, p<0.001; homozygous relatives, 22±10%, p<0.001). Adherent platelets and number of lamellipodia were similar across all individuals tested (Figure 5). In addition, there were fewer platelets from both the proband and the homozygous relative that adhered to the fibrinogen-coated coverslips (control, 26±4%; proband, 8±2%, p<0.001; homozygous relatives, 13±3%, p<0.01).

The role of PTGS1 variant on eicosanoid production by stimulated whole blood and basal urine metabolites

Incubation of blood from healthy volunteers with collagen or TRAP-6 amide greatly increased the levels of TXB_2 (a stable breakdown product of TXA_2), 11-dehydro-TXB2 (11-dH-TXB$_2$, a dehydrogenation product of TXB_2)[22], PGE_2, PGD_2, 15-HETE, 11-HETE and 12-HETE. In the *PTGS1*-deficient proband 12-HETE production was unaffected but there was an absence of TXB_2, PGE_2, PGD_2 and 15-HETE (Figure 6 A-B; Supplemental Table S1).

Despite the fact that platelets are able to synthesize PGD_2, PGE_2 and TXA_2 from PGH_2, urinary metabolites for these enzymes were unchanged in the proband and homozygous relatives compared to normal reference ranges. As expected, PGI_2 metabolites, generated by PGI_2 prostacyclin synthase from PGH_2 in endothelial cells only were all within the standard range (Figure 6 C-F, Supplemental Table S1). Indeed, leukocytes and endothelial cells are additional sources of PGD_2 and PGE_2 products, respectively.

DISCUSSION

We report autosomal recessive inheritance of a homozygous rare missense variant in *PTGS1* associated with an aspirin-like platelet phenotype. This phenotype provides the opportunity to definitively assess the roles of platelet COX-1 in human platelet function, including the production of eicosanoids. This cannot be assumed from exposure of platelets from other humans to aspirin *in vivo* or *in vitro* as aspirin has effects at sites other than platelet COX-1[23].

The 965G>C variant of *PTGS1* found in the family reported here is absent from gnomAD. In the identified pedigree, however, three of the 8 family members studied were homozygous due to consanguinity and three were heterozygous for the variant. Interestingly, since this is a missense variant outside both the functional sites of the COX enzyme the phenotype was unexpected. Within the homozygous carriers, despite similar reproducible platelet aggregation, we saw minor differences in bleeding phenotype, which reflects the clinical heterogeneity of presentation of some of the rare platelet disorders. This is also consistent with observations that while millions of people take aspirin daily to prevent secondary cardiovascular events, and this increases their risk of bleeding, the vast majority do not suffer from major spontaneous bleeding. Similarly, mice with a deficiency in COX-1 exhibit impaired haemostasis but only after being challenged by the tail-bleeding assay[24,25].

As above, data derived from the three pedigree members with the homozygous variant demonstrated a consistent effect upon platelet function and eicosanoid profile, irrespective of other clinical differences. Notably, the proband had an additional diagnosis of CF and administration of COX-inhibitors, which have anti-inflammatory effects, has been shown to inhibit the decline of lung function[26]. However, the proband did not show any evidence of a beneficial effect accruing from the absence of her platelet COX-1, in keeping with our

understanding of the anti-inflammatory effects of NSAIDs being mediated primarily via inhibition of COX-2[27].

While COX-1 protein was expressed at normal levels in platelets from the family member with the CC genotype, it was absent in those with the GG genotype. Conversely, COX-1 was still expressed in the leukocytes irrespective of genotype. This may indicate that the *PTGS1* variant is not expressed by megakaryocytes or that the variant affects the stability of the protein; i.e. that the COX-1 protein degrades more rapidly and then cannot be replenished within platelets because they lack transcriptional machinery, akin to what is observed in erythrocytes in G6PD deficiency[28]. Inheritance of one copy of the mutant allele resulted in variable but never absent platelet COX-1 protein levels which were sufficient to sustain function. When the variant was expressed and characterized, the recombinant protein was found to have normal enzyme activity which is consistent with the findings that in homozygous family members urinary COX-1 metabolites where within the normal range; i.e. implying that despite the *PTGS1* variant, COX-1 activity in tissues other than the platelet was preserved. Due to constraints in sample availability, we were unable to investigate COX-1 protein levels in other nucleated cell types in the homozygous family members.

Platelet reactivity in the homozygous family members was consistent with that seen in previous studies in the presence of aspirin *in vitro* and *in vivo*[18,29,30]. In particular, aggregation responses to collagen, epinephrine and ADP using both light transmission and Optimul aggregometry were reduced and responses to AA were absent but were normal to U46619 (TXA_2 analogue). Homozygous knock-out mice for *PTGS1* show similar impairment in platelet aggregation[31,32]. ATP release from dense granules induced by collagen was impaired in platelets from homozygous family members which is similarly concordant with an aspirin-like defect[29].

Platelet spreading in all homozygous family members was impaired. Specifically, the number of actin-rich filopodia was increased, though the number of platelets which reached the point of being fully spread was lower. Indeed, the number of platelets which adhered to the fibrinogen-coated surface was significantly reduced, indicating a dysfunction in the process leading to formation of a stable platelet plug which could increase the risk of bleeding. This evidence suggests that either this variant or an unknown defect carried by these family members is associated with a dysfunction in the signalling mechanisms required for sufficient spreading. Whilst we did not directly compare platelet spreading from the homozygous family with that of low-dose aspirin-treated healthy subjects, other groups have found that aspirin does not have a significant effect on spreading[33].

The proband had a more severe bleeding phenotype than the other family members homozygous for the *PTGS1* variant which might be attributable to an additional diagnosis of cystic fibrosis and antibiotic use[34] but is more likely due to an additional dysfunctional pathway[35,36]. Indeed, ristocetin-induced platelet aggregation was impaired even though von Willebrand factor antigen (VWF:Ag) and function (VWF:RCo) levels were in the normal range (83.2 IU/dL and 71.7 IU/dL respectively). No coagulation defect was identified which could contribute to bleeding: prothrombin time (PT, 9.6 seconds) and activated partial thromboplastin time (APTT, 28 seconds) were in the normal range and the proband's factor VIII level was 0.98 IU/mL, above the minimum required for normal haemostasis. Furthermore, no variants were found in *GP1b* or *P-selectin* in the proband. Interestingly, the proband and homozygous relatives had significant changes in platelet spreading on collagen where 70% fewer platelets adhered than samples taken from controls, a response which is dependent upon platelet integrin $\alpha_{IIb}\beta_3$ (which also binds VWF and fibrinogen). Also, of the platelets that did adhere, fewer reached the stage of being fully spread.

As expected, COX-1 deficient platelets in whole blood failed to produce any COX-derived prostanoids, namely PGE_2, PGD_2, 11-HETE, 15-HETE and the stable metabolite of TXA_2

(TXB$_2$) following exposure to platelet agonists[32,37–39]. Notably, the individuals supplying these platelets had thromboxane metabolite (TX-M) levels within the normal range indicating that urinary TX-M is not a valid or reliable measure of platelet function; contrary to its frequent use for this purpose. This finding supports our recent report that in humans basal TX-M is not derived from platelets but from other sources such as the kidneys[16], and provides further rebuttal to challenges of this interpretation[40]. As urinary TX-M levels are reduced in humans consuming low dose aspirin[8], the findings also demonstrate that low dose aspirin is not specific for platelets and inhibits COX at other sites. Previous studies have measured the urinary eicosanoid profile in CF patients and reported higher levels of TX-M than in healthy comparators. This is in agreement with our findings in the proband who had higher levels than other homozygous family members. This implies that the elevated production of TXA$_2$ in CF leading to increased TX-M cannot be explained by increased platelet activation[41]. Indeed, COX-2 inhibitors reduced urinary TX-M levels in CF patients consistent with a source other than platelet COX-1[42].

Previous cases have been reported variants in *PTGS1* which have been associated with autosomal dominant inheritance of enhanced bleeding, some impairment of platelet aggregation and changes in protein levels. There have been no reports of absence of COX-1 protein and/or ablation of associated eicosanoid production as reported here[39,43,52,53,44–51]. Nance *et al.* identified a pedigree with a non-synonymous variant in the signal peptide of *PTGS1* (rs3842787; c.50C>T, p.Pro17Leu) that segregated with an aspirin like platelet function defect. The proband also carried a variant in the *F8* causing haemophilia A (rs28935203; c.5096A>T; p.Y1699F). The affected family members with both variants had more severe bleeding than expected from mild haemophilia A alone. In this study, extensive platelet function testing was performed demonstrating impaired platelet aggregation induced by AA, epinephrine and low dose ADP and reduced platelet TXB$_2$ release[50]. Two compound heterozygous cases have been reported. The first in a patient with post-procedural bleeds and an aspirin-like defect who carried two high frequency variants (R8L and P17L) which

had previously been reported not to have an effect on function[31,52]. Analysis of the second case identified a rare variant (c.337C>T, p.Arg113Cys; gnomAD frequency 6.134×10^{-5}) in compound heterozygosity with a common variant (c.1003G>A, p.Val481Ile; gnomAD frequency 0.007) which was classified as probably pathogenic and accompanied reduced plasma TXB_2 levels [51]. Finally, Bastida *et al.* reported two cases with variants in *PTGS1* (c.35_40delTCCTGC, p.Leu13_Leu14del and c.428A>G, p.Asn143Ser) by sequencing 82 patients with an inherited platelet disorder on their high-throughput sequencing platform to investigate the unknown molecular pathology. They did not, however, perform in-depth platelet phenotyping[53]. Consequently, none of these previous reports describe complete loss of platelet PTGS1 function.

In conclusion, we describe the first case of a well characterized family with autosomal recessive inheritance producing an aspirin-like platelet function defect due to a rare variant in *PTGS1*. This case models the specific loss of platelet COX-1 activity and provides a benchmark of COX-1's role in platelet function and eicosanoid metabolism.

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FIGURE LEGENDS

Figure 1. (A) Pedigree of the affected family, in which black, white and grey symbols indicate presence of the bleeding phenotype, absence of the bleeding phenotype and unknown bleeding phenotype, respectively. The genotype, where known, is shown under each symbol, where G is the mutant allele and C is the reference allele. Double lines indicate consanguinity and strike-through lines are used to indicate deceased individuals. (B) Human Phenotype Ontology (HPO) annotation of the three affected family members. (C) A ribbon diagram of the crystal structure of aspirin-acetylated COX-1 showing the location of the variant for the proband which results in a missense substitution of tryptophan to serine at amino acid 322.

Figure 2. COX-1 protein in control, proband and relatives. (A) Western blots and quantification of COX-1 and GAPDH expression in platelet lysates, isolated from the controls, the proband (IV-1), homozygous (III-1 and III-2) and unaffected (III-3, III-4, III-5 and IV-2) family members. Representative immunohistochemical analysis of COX-1 expression in (B) control, (C) proband and (D) homozygous relative (i) platelets and (ii) leukocytes. Washed platelets were identified by tubulin (green) staining and COX-1 (magenta) was present in control but not in the proband or affected relative. In washed leukocytes, nuclear staining was confirmed by DAPI (blue), LAMP-3 (green) and COX-1 (magenta) was expressed in all samples.

Figure 3. Analysis of unstimulated whole blood acquired using an ImageStream[X] Mark II incorporating a x60 objective lens. Scale bars represent 7 μm and identified (A) platelet-monocyte and (B) platelet-neutrophil aggregates. Percentage of (C) platelet-monocyte (CD14+) and (D) platelet-neutrophil (CD66b+) aggregates are quantified. Platelets identified by anti-CD61, leukocytes by anti-CD45, monocytes by anti-CD14 and neutrophils by anti-CD66b.

Figure 4. Effect of *PTGS1* variant on platelet aggregation, secretion, and adhesion responses. (A) Aggregation responses to arachidonic acid (AA; 1 mmol/L), adenosine diphosphate (ADP; 10 µmol/L), collagen (0.1-3 µg/mL), epinephrine (10 µmol/L), ristocetin (1.5 mg/mL), U46619 (3 µmol/L) and TRAP-6 amide (25 µmol/L) and (B) ATP secretion to AA (1 mmol/L), ADP (10 µmol/L), collagen (3 µg/mL) and TRAP-6 amide (25 µmol/L). n = 20 (healthy controls; range with median); n = 1 (proband); n = 2 (homozygous relatives); n = 4 (unaffected relatives). (C) P-selectin expression as measured by flow cytometry in whole blood stimulated by ADP (40 µmol/L), U46619 (0.5 µmol/L) or ADP plus U46619.

Figure 5. Platelet spreading on fibrinogen-coated surfaces in (A) a control, (B) the proband and (C) a homozygous relative with (D) quantification of adhered platelet, filopodia, lamellipodia, fully spread platelet frequency and total platelets per field of view.

Figure 6. Contribution of *PTGS1* to eicosanoid synthesis in whole blood and urine. Platelet-derived eicosanoid levels in whole blood from healthy volunteers or from the proband stimulated with collagen (30 mg/mL) (A) or TRAP-6 amide (30 µmol/L) (B). Levels are expressed as increase over levels in vehicle-treated blood. Urinary C) PGD_2 and (D) PGE_2 (C) PGI_2 and (D) TXA_2 metabolite levels in proband, homozygous and unaffected relatives. n = 4 (healthy volunteers); n = 1 (proband), n = 2 (homozygous relatives), n = 3 (unaffected relatives). Normal control ranges are indicated.

Figure 1

A
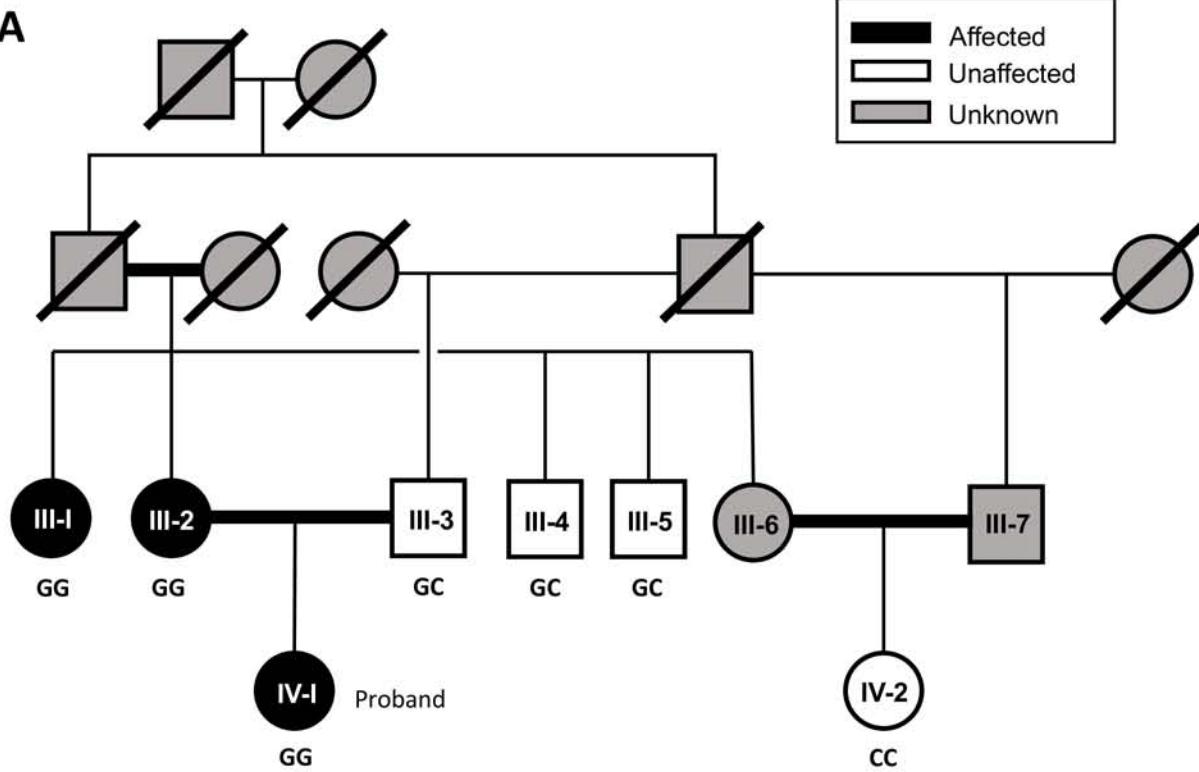
B

C

Figure 2

A

COX-1 GAPDH

COX-1 GAPDH

COX-1:GAPDH ratio (arbitary units)
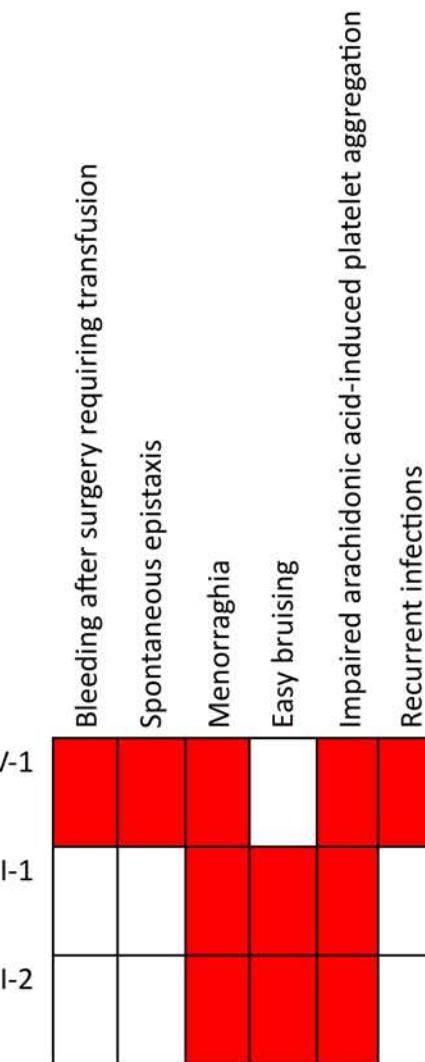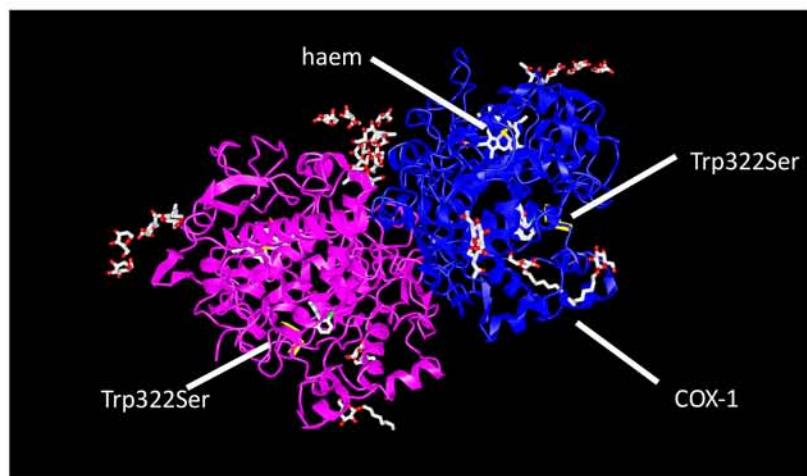
- ● Controls
- ● Proband
- ● Homozygous relatives
- ● Unaffected relatives
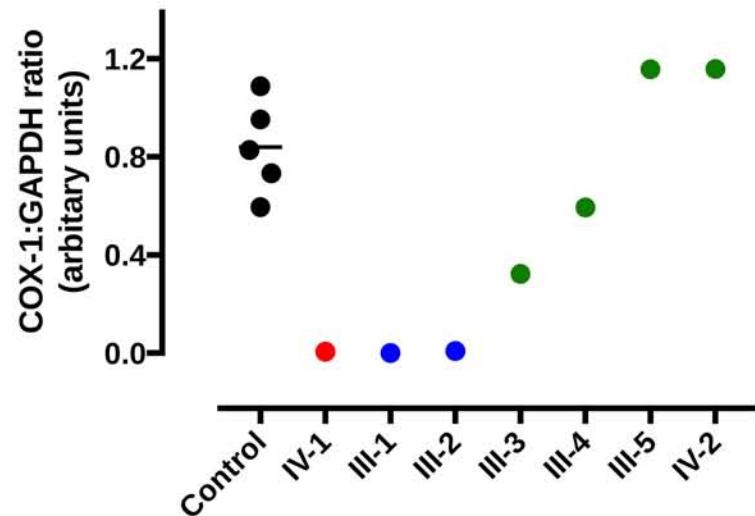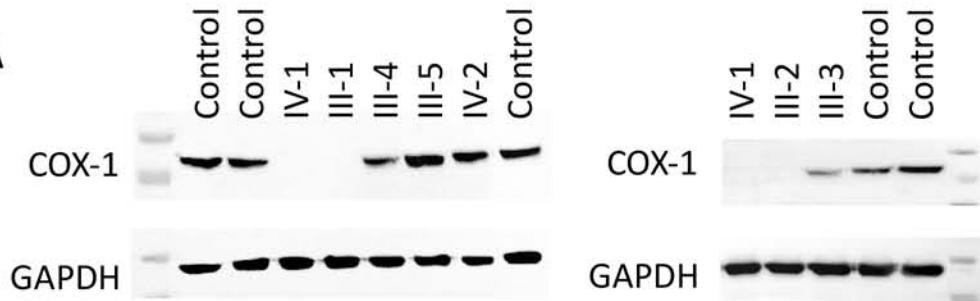
Platelets **Leukocytes**

Unrelated Control
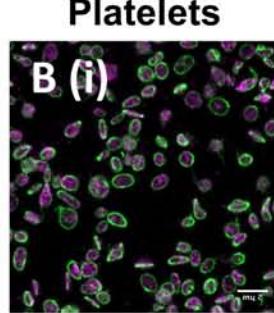
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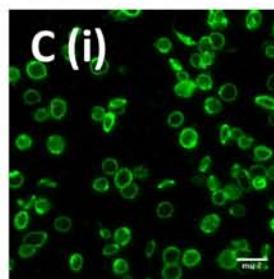
Homozygous relative
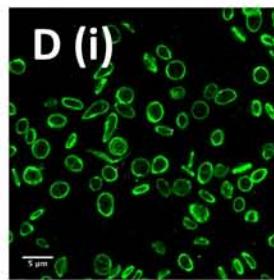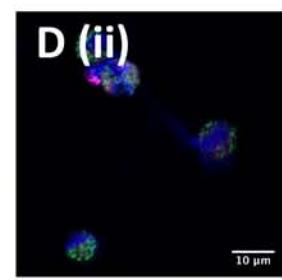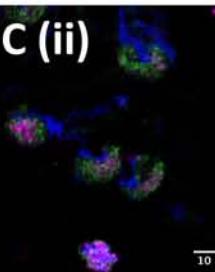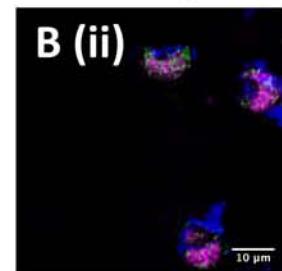
COX-1 Tubulin   COX-1 DAPI LAMP-3

Figure 3
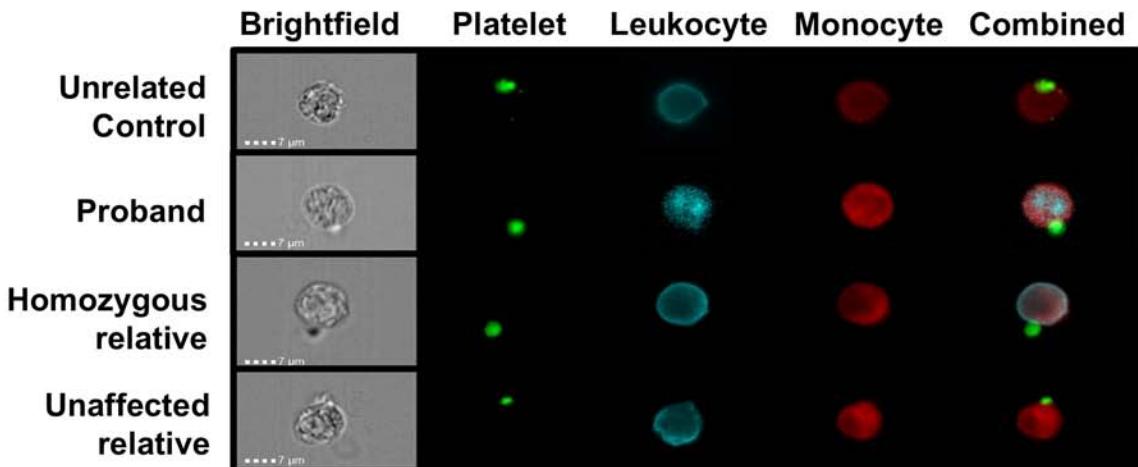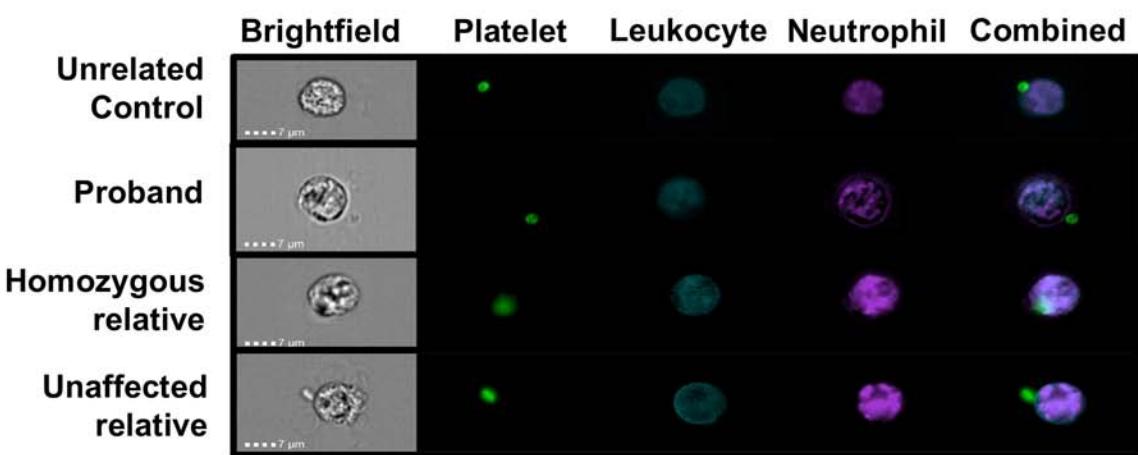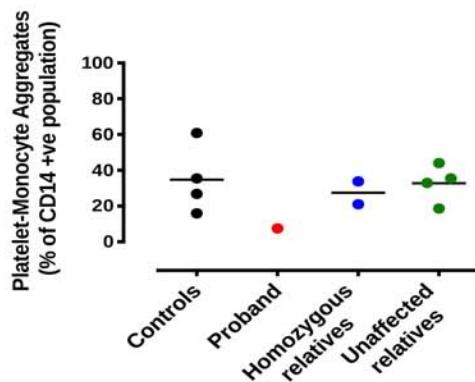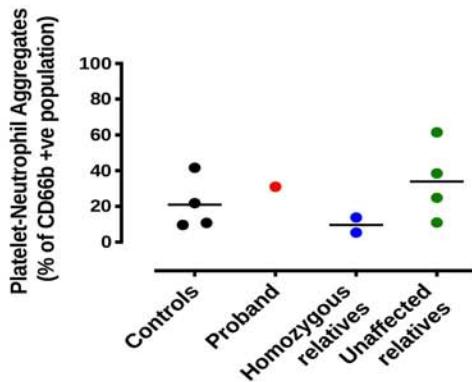
Figure 4

Figure 5

Figure 6

A. Whole blood, Collagen

B. Whole blood, TRAP-6 amide

C. Urinary [PGD-M] (ng/mg creatinine)

D. Urinary [PGE-M] (ng/mg creatinine)

E. Urinary [PGI-M] (ng/mg creatinine)

F. Urinary [TX-M] (ng/mg creatinine)
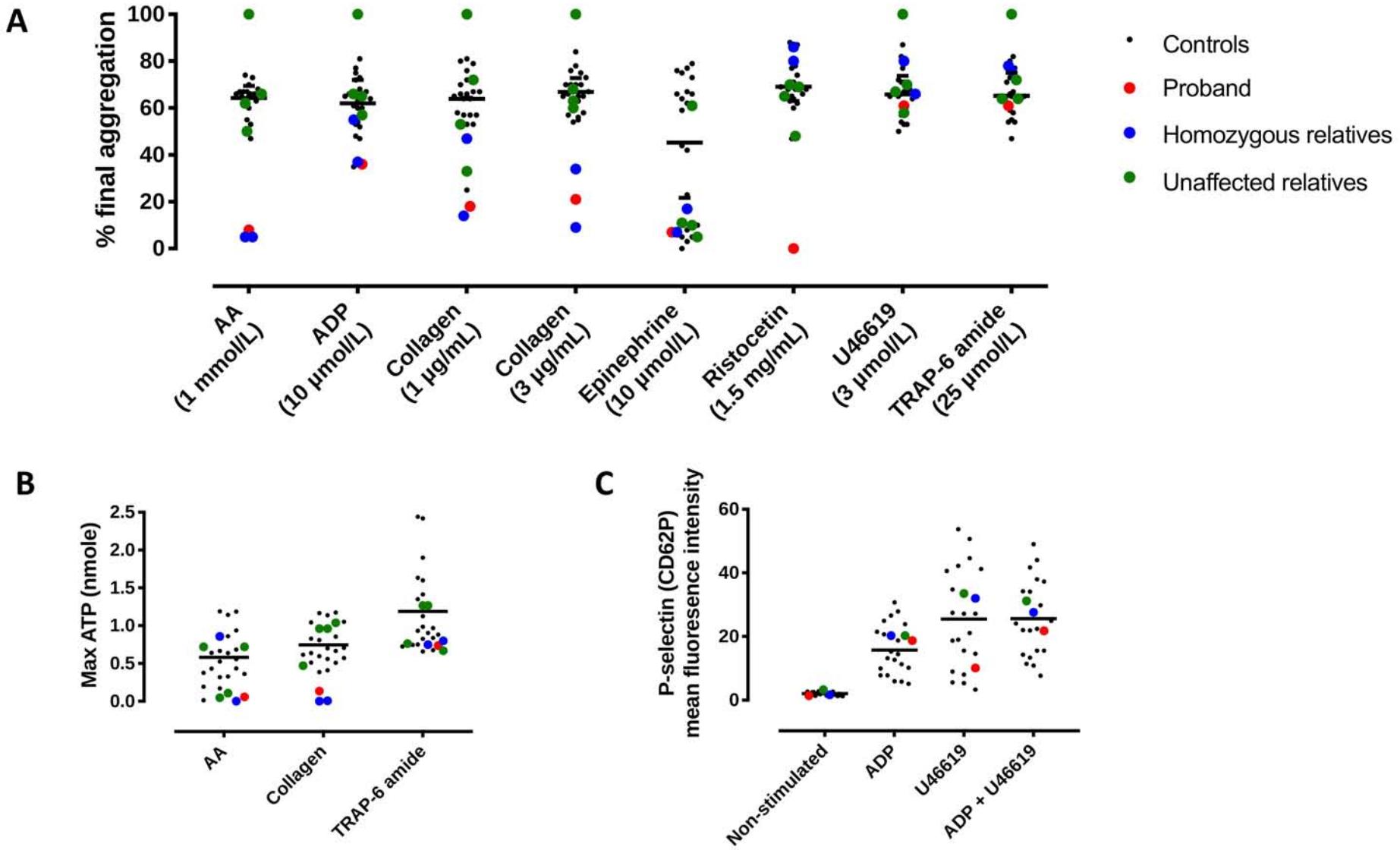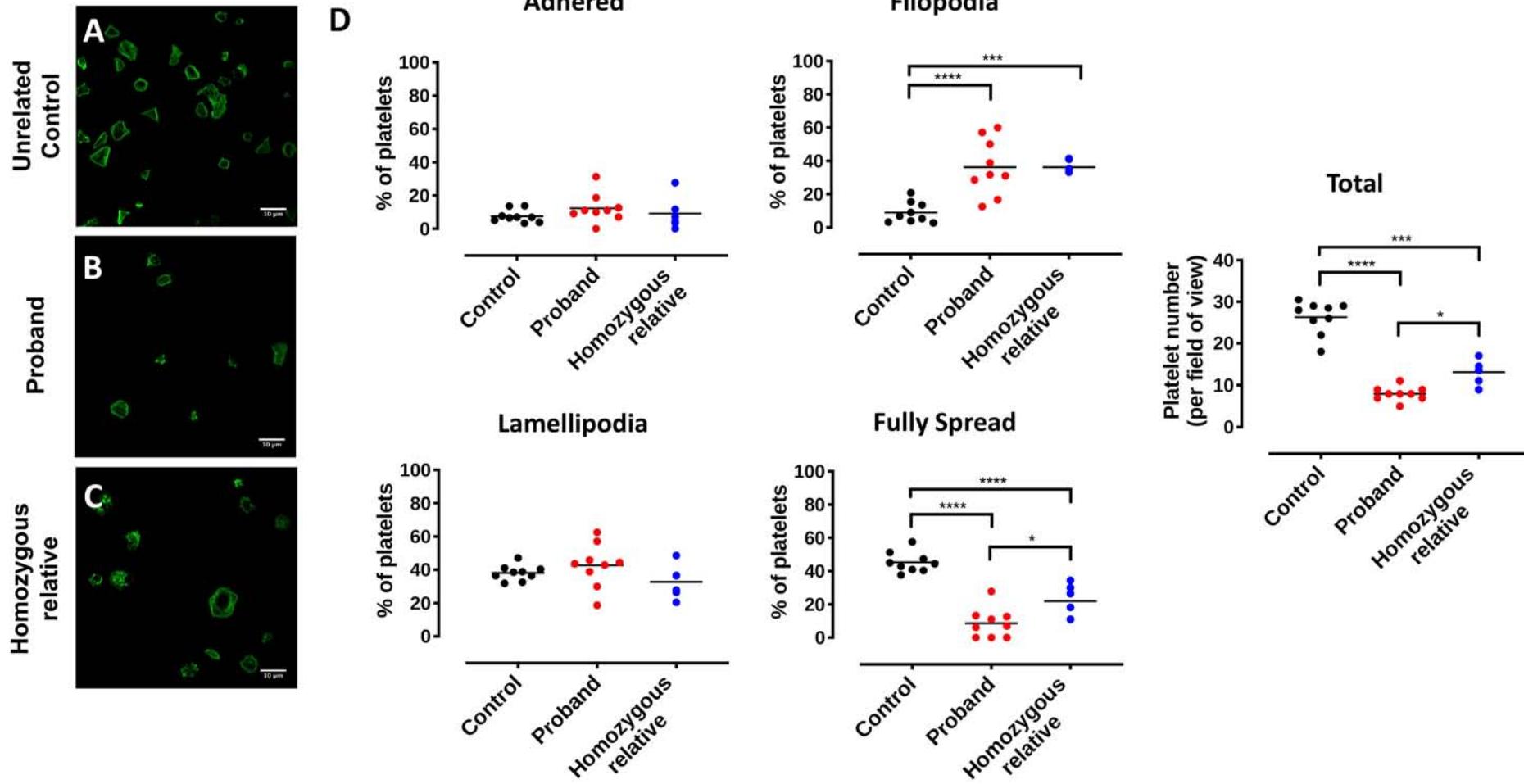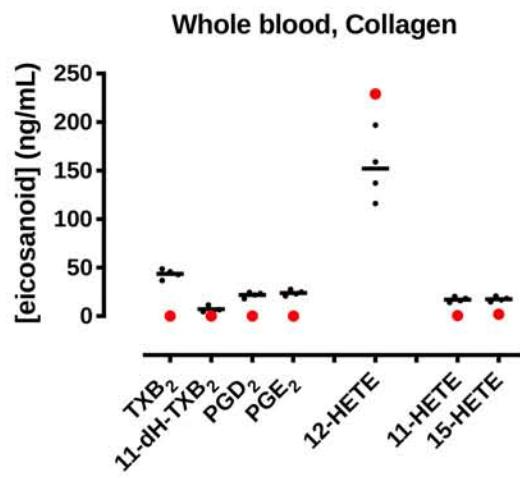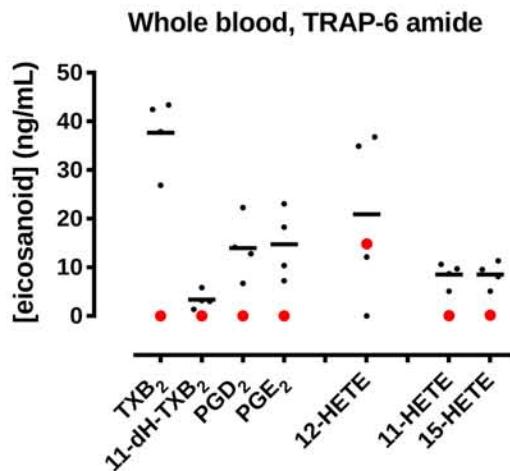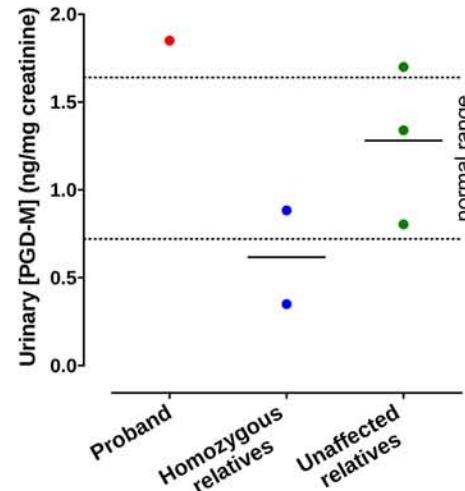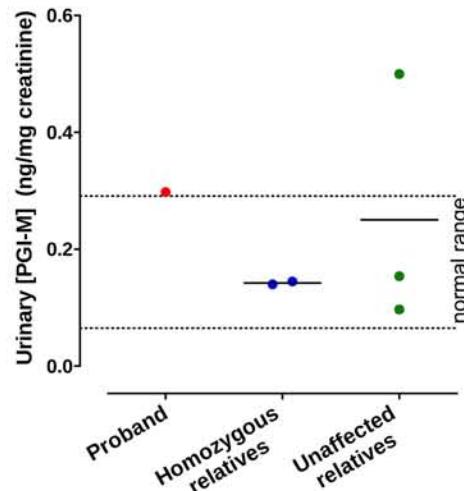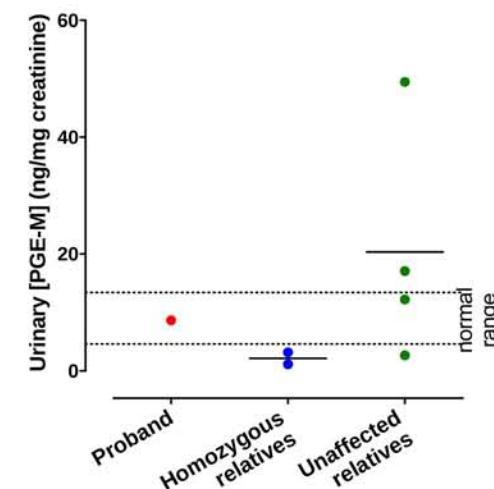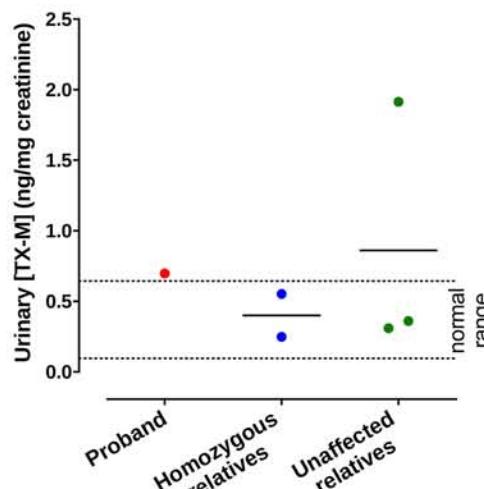
• Controls   • Proband

SUPPLEMENTAL MATERIAL

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

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Statement of equal authors' contribution
*MVC, MAH and SS contributed equally to this work as first authors.
**MAL and TDW contributed equally to this work as shared last authors
[#]Collaborative group

Running head
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 2×10^8/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].
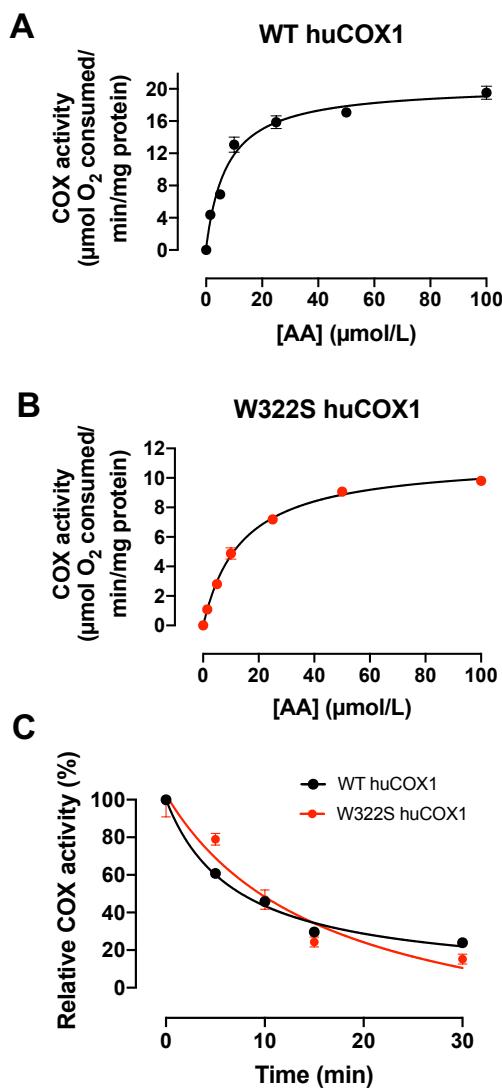
Supplementary Figure 1. Kinetic analysis of (A) wild type and (B) variant W322S COX-1 and (C) the relative activity in the presence of aspirin (500 µmol/L). COX activity was measured using a Clark type oxygen electrode as described in methods. K_m and V_{max} were determined by fitting the data to the Michaelis-Menton equation. All measurements were carried out in duplicate and shown as mean±s.d.
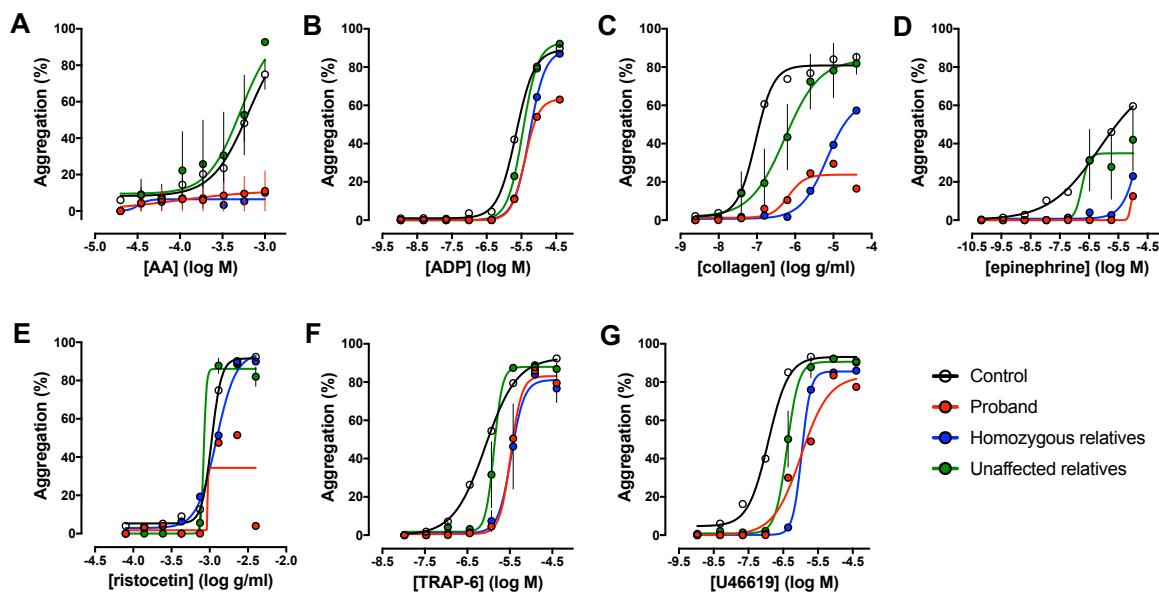
Supplementary Figure 2. Platelet aggregation measured by the Optimul method in response to (A) arachidonic acid (AA; 0.03–1 mmol/L), (B) ADP (0.005–40 µmol/L), (C) collagen (0.01–40 µg/mL), (D) epinephrine (0.0004–10 µmol/L), (E) ristocetin (0.14–4 mg/mL), (F) U46619 (0.005–40 µmol/L) and (G) TRAP-6 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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