haematologica
Journal of the Ferrata Storti Foundation

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Received: October 27, 2025.
Accepted: February 17, 2026.

Citation: Amna Ahmed, Samantha J. Montague, Hrushikesh Vyas, Jayna Mistry, Natasha J. Pavey, Sophie R.M. Smith, Liam Griffith, Isobel Clothier, Roseanne Hudson, Emma Faulkner, Richard Buka, Patricia Bignell, Carl Fratter, Kathryn Marshall, Ben Bailiff, Natalie Poulter, Bas De Laat, Dana Huskens, Gillian C. Lowe, Steven G. Thomas and Neil V. Morgan. Scott syndrome with novel compound heterozygous pathogenic variants in ANO6 and reduced thrombin generation.
Haematologica. 2026 Feb 26. doi: 10.3324/haematol.2025.300111 [Epub ahead of print]

Scott syndrome with novel compound heterozygous pathogenic variants in *ANO6* and reduced thrombin generation

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Authorship contribution NVM designed the study; HV, JM, BB and GCL recruited the patients; AA, SJM, HV, JM, NP, SRMS, LG, IC, RH, EF, RB, PB, CF, KM, BB, NSP, BdL, DH, GCL, SGT, and NVM extracted or generated clinical or experimental data and interpreted the results; GL and NVM undertook governance of the study; AA, SJM, SGT & NVM wrote the manuscript; all authors read and approved the final version of the manuscript.

Original data are available on request from the corresponding author, Neil V. Morgan (N.V.Morgan@bham.ac.uk)

Disclosure of Conflicts of Interest: D.H., and B.d.L. are employees of Synapse Research Institute, part of Diagnostica Stago S.A.S. All remaining authors declare no conflicts of interest.

Acknowledgments The authors would like to thank the families and patients for taking part in the study. We also thank Geraldine Green and Laura Otto (University Hospitals Coventry and Warwickshire) for their help with patient information and recruitment. The work in the author's laboratories is supported by the British Heart Foundation (PG/16/103/32650, FS/18/11/33443, and PG/24/11972 to NVM, FS/PhD/22/29245 to SGT, PG/23/11230 to SJM, Birmingham Health Partners Springboard Fellowship and CRUK clinical training fellowship to HV and Zacros Project grant (to NVM). The National Institute of Health and Care Research (NIHR) Birmingham Biomedical Research Centre (NIHR203326) has supported the.

Department of Cardiovascular Sciences in the University of Birmingham where this research is based. The opinions expressed in this paper are those of the authors and do not represent any of the listed organizations.

Keywords: Scott syndrome, ANO6, bleeding, thrombin generation, annexin V

Running head

Scott syndrome with novel *ANO6* variants

Scott syndrome is a recessively inherited, mild to moderate bleeding disorder attributed to variants in *ANO6*. To date very few cases have been reported worldwide, although we believe that it is under-reported due to a lack of platelet procoagulant activity screening in standard clinical platelet function assays. Here we present a patient with bleeding diathesis with two novel heterozygous variants in *ANO6,* little to no thrombin generation and reduced phosphatidylserine (PS) exposure which is indicative of Scott syndrome. This study highlights the importance of genetic screening as a first line of diagnosis for Scott syndrome followed by confirmatory thrombin generation studies. This study also provides sufficient support for the case of *ANO6* as a definitive clinically relevant gene.

Inherited platelet disorders comprise an extremely heterogeneous group which can be associated with reduced platelet number and/or dysfunction, making genetic diagnosis challenging. Some patients experience frequent and debilitating bleeding episodes throughout their lifetime; therefore, a precise genetic diagnosis provides significant clinical benefit for the patients and offers the possibility of accurate counselling, clinical management and personalised treatment (1).

In Scott syndrome, defective phospholipid scrambling leads to reduced formation of a procoagulant surface on activated platelets (2,3). Genetic studies have implicated variants in *ANO6* (encoding anoctamin-6), a calcium-activated chloride channel responsible for phospholipid scrambling and apoptotic membrane dynamics (4). Thrombin generation is a central event in haemostasis which is dependent on both coagulation factors and the provision of the PS-dependent procoagulant surface by activated platelets. PS exposure on platelet membranes allows the assembly of tenase and prothrombinase complexes, greatly accelerating thrombin formation (5). Because of the reduced PS exposure, patients with Scott syndrome have a reduced capacity for thrombin generation and the subsequent formation of a stable fibrin clot.

Clinically, affected individuals most often present with mild to moderate bleeding symptoms, including mucocutaneous bleeding, heavy menstrual bleeding, epistaxis, and prolonged bleeding after surgery or trauma. Standard haematological investigations in these patients are usually normal, with platelet count, morphology, and functional responses such as aggregation, within reference ranges. Therefore, diagnosis requires more specialised assays that demonstrate impaired procoagulant ability.

These findings suggest an autosomal recessive mode of inheritance and provide insight into the molecular mechanisms underlying defective platelet procoagulant activity. However, the number of pathogenic variants described to date has been limited, leaving uncertainty as to whether *ANO6* can be definitively regarded as a clinically relevant pathogenic gene in the diagnostic setting (6).

We present an index case (II:2) of a 60-year-old male presenting with a clinically significant bleeding tendency (ISTH-BAT score of 8 – **Supplementary Table 1)** which included excessive bleeding after surgery. Interestingly, neither parent of II:2 had any known history of excessive bleeding (**Figure 1A)**. II:2 was recruited to the UK Genotyping and Phenotyping of Platelets (GAPP) study (approved by the UK National

Research Ethics Service by the Research Ethics Committee of West Midlands (06/MRE07/36)) for genetic studies and platelet function testing (7) following informed consent. Initial investigations including platelet count, coagulation screen and factor assays were normal (**Supplementary Table 1**).

II:2 was investigated for a possible genetic cause using the R90 bleeding and platelet disorders targeted gene panel, which was performed at Oxford Genetics Laboratories (8). This detected two heterozygous variants in the *ANO6* gene: c.199C>T p.(Arg67*) and c.2007_2011del p.(Glu669Aspfs*41) (**Figure 1B**). Both variants were predicted to be pathogenic using strict ACMG guidelines (9) and predicted to lead to premature termination of translation and non-sense mediated RNA decay. The patient was negative for all genes on this panel except the ANO6 variants reported here. Extended genetic testing to his children (III:1 and III:2) with no bleeding symptoms revealed them both to be heterozygous for the p.Arg67* variant and therefore confirmed that the index case variants were trans-acting in line with autosomal recessive inheritance in Scott syndrome (**Figure 1A**). These two novel variants add to the growing collection of reported ANO6 variants to date (**Figure 1C**) and were predicted to destabilise the ANO6 structure using the AlphaFold software predicted software tool (**Figure 1Di & ii**) (10).

Citrated blood was collected from II:2 and a same-day healthy control for deep phenotyping of II:2 platelets, procoagulant capacity and thrombin generation. Platelet aggregation (light transmission aggregometry (**Supplementary Figure 1**), and platelet activation responses (P-selectin expression and integrin activation) (**Supplementary Figure 1**) were normal for all standard agonists tested (11). Washed platelets from II:2 also demonstrated normal spreading on fibrinogen and collagen compared to a same-day healthy control (**Supplementary Figure 2Ai & ii)** (12).

However, flow cytometry analysis of II:2's platelets showed reduced PS expression following stimulation with collagen-related peptide (CRP) and thrombin compared to controls (**Figure 2A**). We measured the platelet surface expression of ANO6 at rest and post-agonist stimulation and showed that this was reduced in both resting and stimulated patient platelets compared to healthy controls (**Figure 2B**) but normal expression of other platelet components (CD41, CD42b and GPVI) was observed (**Figure 2C**). A very small increase in ANO6 expression was observed in the patient following activation, which could represent low expression of the Glu669Aspfs*41 variant. However, PS expression is markedly decreased compared to controls indicating that ANO6 expression is either extremely low, or the protein is severely defective. In addition, platelets spread on collagen-coated surfaces also had greatly reduced PS expression (**Figure 2D**), despite similar levels of platelet adhesion. To assess thrombus formation, peripheral blood was recalcified, treated with 40 μM PPACK, and flowed over precoated collagen micro-spots in the Maastricht flow chamber at 1500 s^{-1}. Thrombi were imaged using annexin V-AF647. Surface area coverage and intensity of brightfield and annexin V staining were quantified using ImageJ (Fiji). This further illustrated the functional consequences of impaired platelet procoagulant activity (**Figure 2E**). Control thrombi displayed robust

annexin V binding, reflecting substantial PS exposure. In the patient, annexin V binding to thrombi was significantly reduced (P<0.0001, **Figure 2D i &ii**), confirming defective PS externalisation in the dynamic flow setting. The surface area coverage of thrombi was similar between II:2 and control (**Figure 2Diii**) again indicating no effect on platelet adhesion. Detailed visual observations using expansion microscopy (ExM (13)) of the platelet aggregates demonstrated that patient's thrombi were smaller and less well-defined (**Supplementary Figure 2Bi**) and P-selectin expression was also significantly diminished (P<0.001 - **Supplementary Figure 2Bii**), suggesting that impaired procoagulant capacity adversely affected granule secretion (possibly via reduced fusion capacity at the inner leaflet of the membrane) and subsequent thrombus formation**.**

We previously demonstrated reduced thrombin generation in a patient with Scott syndrome (14). This was also tested in II:2. Citrated whole blood was mixed with substrate ZGGR-AMC (417 µmol/L) and a solution containing either tissue factor (TF, 0.1 pM) alone, or TF with thrombomodulin (TM, 1650 nm). Fluorescence emission was recorded using a Fluoroskan Ascent microplate fluorometer (Thermo LabSystems) and Fluoroskan Ascent Software (λex = 355 nm, λem = 460 nm) and fluorescence was calibrated using a calibrator to correspond to 300 nmol/L thrombin activity. Thrombin generation was also measured in Platelet Rich Plasma (PRP) using calibrated automated thrombinography (CAT), PRP reagent, thrombin calibrator, and fluorescent substrate/$CaCl_2$ (Fluca). In healthy control whole blood, thrombin generation was normal and was partially inhibited by TM due to activation of the protein C pathway (**Figure 3Ai**). In contrast, this was markedly reduced in II:2 with TF alone, and the addition of TM further suppressed thrombin generation (**Figure 3Ai**). Thrombin generation in PRP was also markedly reduced in II:2 compared to control, demonstrating the role of platelet PS exposure in the process **(Figure 3Aii**). The lag time for thrombin generation in whole blood was also markedly increased in II:2 compared to controls (**Figure 3Bi**) with a small increase in lag time observed for PRP (**Figure 3Ci**). Endogenous thrombin potential (ETP) was diminished in whole blood with TF and TM with similar findings in PRP (**Figure 3Bii & Figure 3Cii**). A parallel trend was also seen with peak thrombin levels (**Figure 3Biii & 3Ciii)**. Notably, for II:2 all parameters were outside of the assay reference ranges (displayed as red lines on the plots) whilst all same-day healthy control values were within our normal healthy donor ranges for these assays. Together these data support the conclusion that platelet surface abnormalities in ANO6 and PS expression are highly likely to underlie the observed defect in thrombin generation and subsequent increased bleeding.

In summary, the convergence of these functional findings with the presence of two pathogenic *ANO6* variants provides strong evidence that these variants are causally related to Scott syndrome. This correlation underscores the diagnostic value of integrating thrombin generation assays with genomic analysis in patients presenting with unexplained bleeding symptoms. The data also raises some interesting questions on the biology of ANO6 in platelets. Taken together, this study provides sufficient support for the

case of *ANO6* to be defined as a definitive clinically relevant gene in patients with a suspected bleeding disorder, who are normal on routine coagulation testing and have normal platelet count.

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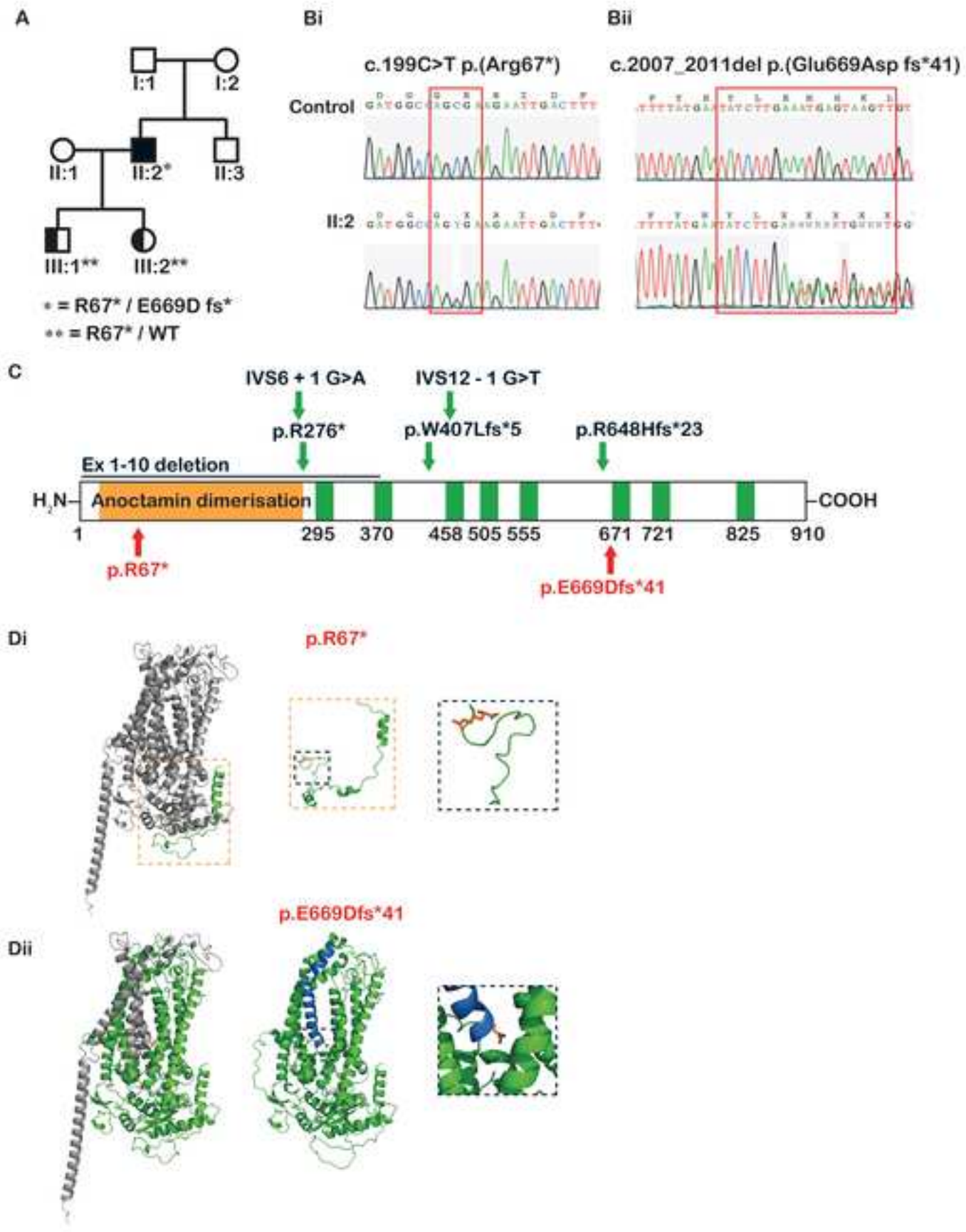
Figure legends

Figure 1. Two novel pathogenic variants in the gene encoding Anoctamin-6 (*ANO6)* with predicted truncations in a Scott syndrome patient. **A)** Pedigree of family showing affected individuals (filled). The index patient II:2 is a compound heterozygote for the two variants. **B)** Sanger sequencing results of two novel variants in the gene encoding *ANO6.* **Bi)** Variant 1 is a nonsense variant with a C to T substitution at nucleotide position 199 of *ANO6* which introduces a premature stop codon at amino acid position 67, predicted to result in truncated ANO6 protein. B**ii)** Variant 2 is a frameshift deletion which removes five nucleotides (2007–2011), alters the reading frame beginning at glutamic acid 669, and introduces a premature stop codon 41 residues downstream, predicted to produce a truncated and non-functional protein. **C)** Schematic of the ANO6 protein showing position of the novel p.R67* and p.Glu669Asp fs* variants as red arrows. Previously identified variants are shown as green arrows and dark green boxes represent transmembrane domains. **Di)** AlphaFold prediction of the R67* mutated protein. Green sequence shows the extent of the truncated protein, with the non-expressed regions in grey. Yellow box shows the predicted structure of the truncated protein showing complete loss of the major ANO6 domains. The black box shows the mutated amino acid. **Dii)** AlphaFold prediction of the E669D fs*41 mutated protein. Green sequence shows the extent of the truncated protein, with the non-expressed regions in grey. The blue sequence shows the extent of the altered amino acid sequence and the black box, the mutated amino acid.

Figure 2. Reduced ability of patient platelets to express phosphatidylserine (PS) on their surface.
A) The ability of washed platelets to express PS on surface, as measured by Annexin V, in response to stimulation with 10 µg/mL CRP and 1 IU /mL thrombin (act) in the patient compared to the same-day control was assessed using flow cytometry (Rest = Resting platelets). Histogram of patient and control samples presented, and data plotted as changes in median fluorescence intensity (MFI). **B)** ANO6 protein levels on the surface of platelets at rest and after activation with 10 µg/mL CRP and 1 IU/mL thrombin was measured using flow cytometry (ANO6 extracellular antibody-FITC, Thermofisher Scientific). **Bi)** Histograms of changes in ANO6 levels of the patient compared to the same-day control (2° = Secondary only control, Rest = resting platelets & Act = activated platelets) and **Bii)** bar charts of median fluorescence intensity (MFI) of ANO6 on resting and activated platelets from patient and same-day control. Secondary only control values were subtracted from the MFI before plotting. Red lines indicate normal healthy donor control range (median ± 95% CI of 18 healthy controls). **C)** Levels of receptors on the surface of patient and control platelets (CD42b; GPIbα, CD41; GPIIb/IIIa and GPVI) as determined by flow cytometry. **Di)** Washed platelets ($2x10^7$/mL) from patient and control were spread on 10 µg/mL collagen (see also **Supplementary Figure 2**) and annexin V measured on the surface. Representative images of annexin V from the patient and same-day healthy control. Scale bar = 50 µm. **Dii)** Quantification of percentage PS-positive platelets. Bars are mean ±SD. 10 fields of view were analysed for each condition, with a total of 952 platelets for control and 1216 platelets for patient. **Ei)** Platelet aggregate formation over collagen (100 µg /mL) micro-spots at 1500 s^{-1} was measured using the Maastricht flow chamber. Representative images of annexin V from the

patient and same-day healthy control. Scale = 50 μm. Quantification of **Eii)** Surface area coverage (SAC) of Annexin V-AF-647 and **Eiii)** brightfield signal from 13 fields of view per individual are plotted with the bar representing mean ± SD. Unpaired T-test, **** = P <0.0001.

Figure 3. Reduced thrombin generation in patient platelets. A) Thrombin generation curves showing reduced thrombin generation in II:2 in **Ai)** whole blood (WB), after stimulation with tissue factor (TF; 0.1 pM) and thrombomodulin (TM; 1650 nM) and **Aii)** PRP after stimulation with TF (670 nM). Control = same-day healthy control. **B & C)** Specific thrombin generation parameters tested in whole blood and PRP (lag time (**Bi & Ci**), endogenous thrombin potential (ETP, **Bii & Cii**) and peak thrombin generation (**Biii & Ciii**)). All parameters were altered in II:2 samples compared to the same-day control as well as being outside our normal healthy donor ranges for these assays (indicated by red lines = mean ± 2SD of 40 controls).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I:2
II:1
II:2*
II:3
III:1**
III:2**
* = R67* / E669D fs*
** = R67* / WT
Bi
c.199C>T p.(Arg67*)
Control
II:2
Bii
c.2007_2011del p.(Glu669Asp fs*41)
C
IVS6 + 1 G>A
p.R276*
IVS12 - 1 G>T
p.W407Lfs*5
p.R648Hfs*23
Ex 1-10 deletion
H2N
Anoctamin dimerisation
COOH
1
295
370
458
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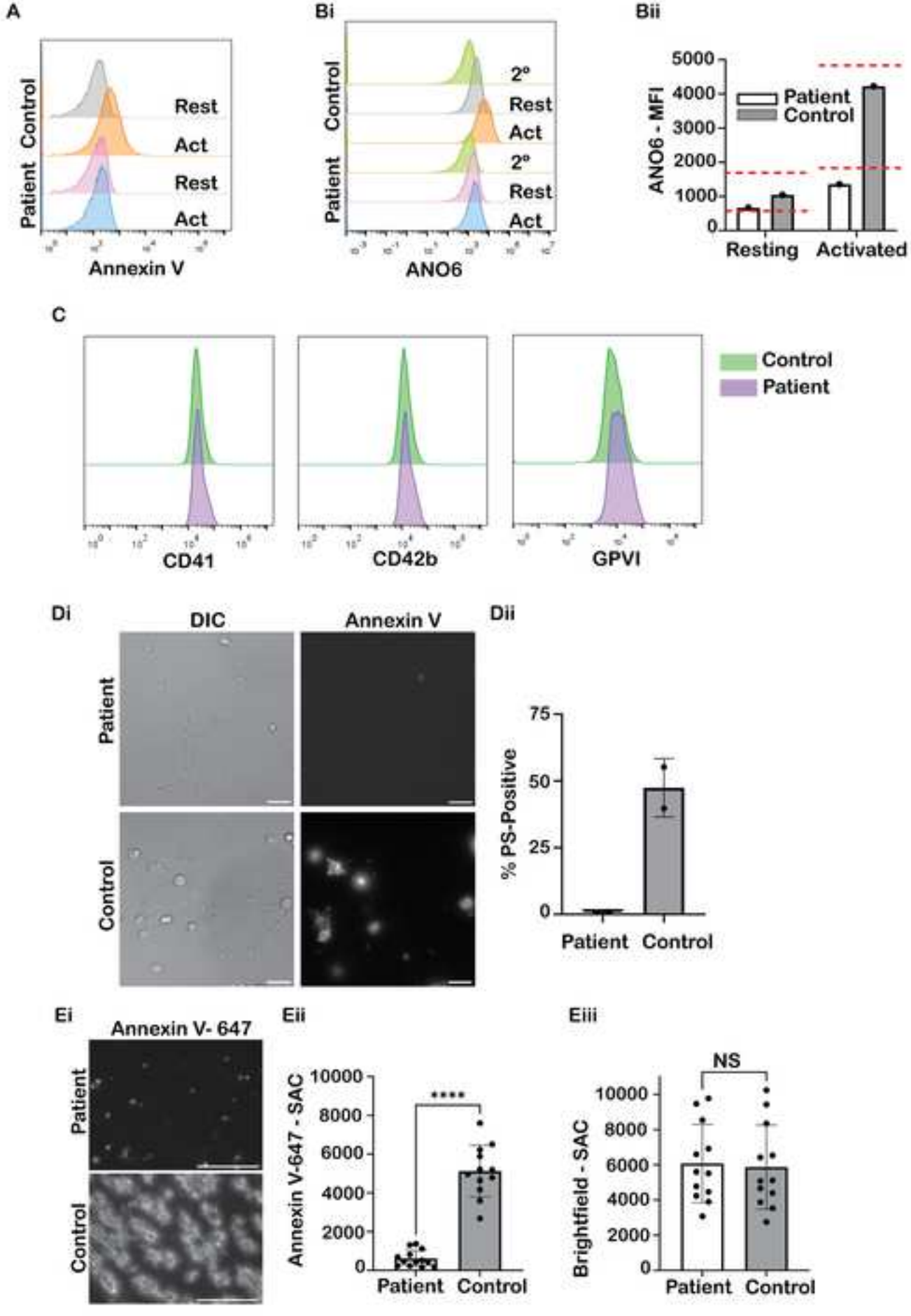
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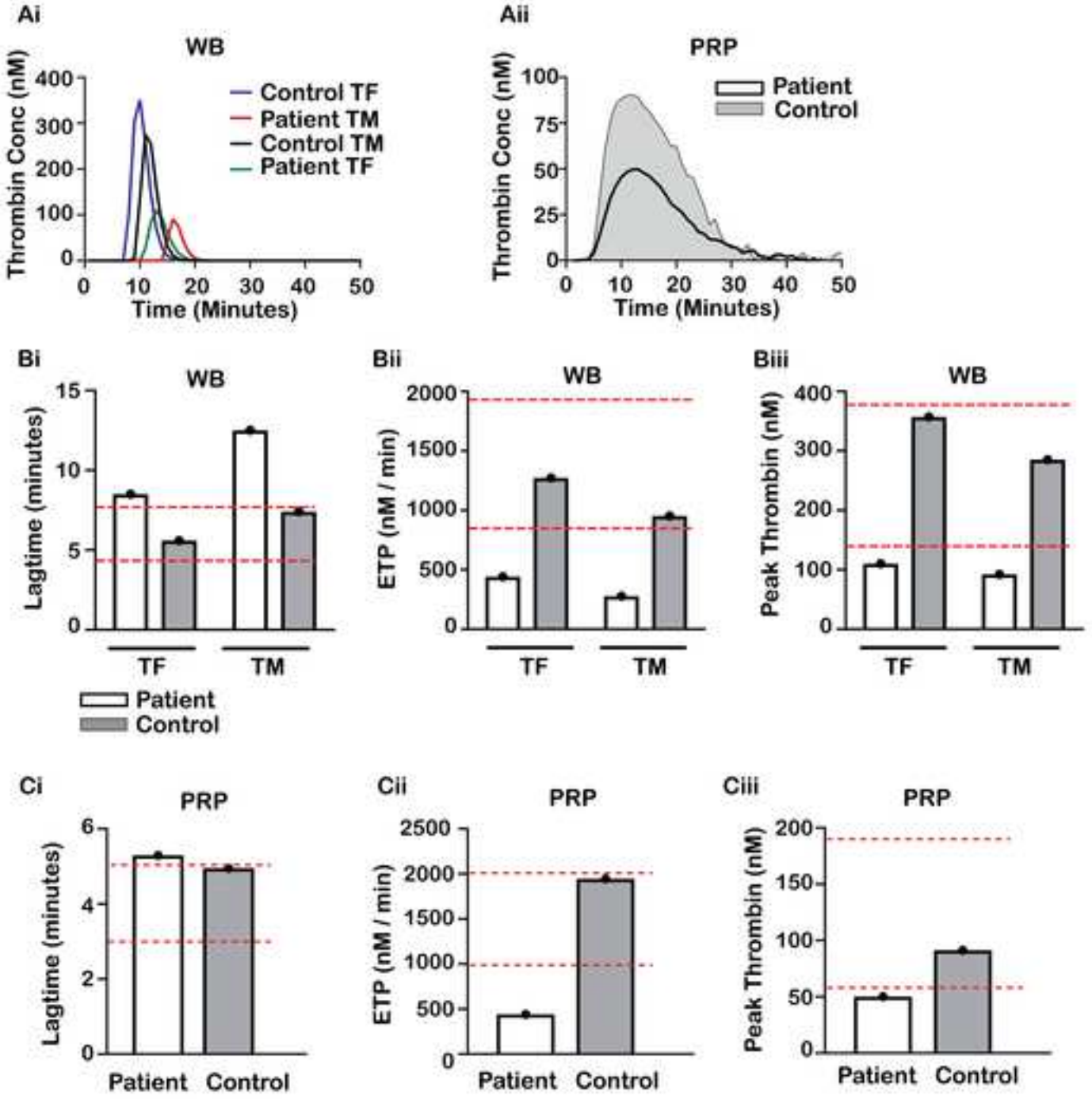
Figure 3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Thrombin Conc (nM)
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25
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0 10 20 30 40 50
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Lagtime (minutes)
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ETP (nM / min)
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Biii
WB
Peak Thrombin (nM)
400
300
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Lagtime (minutes)
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Peak Thrombin (nM)
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Patient
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Scott syndrome with novel compound heterozygous pathogenic variants in *ANO6* and absent thrombin generation

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Supplementary Data

Comprising:

Supplementary Table 1: Haematological Parameters for index patient II:2

Parameter	II:2	Reference Ranges*
Age (y)	60	-
ISTH BAT score**	8	0 – 3 (Males)
Sex	Male	-
White Blood Cells (x10^9/L)	8.22	3.26 – 11.2
Red Blood Cells (x10^9/L)	5.20	3.9 – 5.77
Haemoglobin (g/L)	148	130 – 171
Mean corpuscular volume (fL)	93.8	83 – 103
Platelets (x10^9/L)	363	150 – 400
Mean platelet volume (fL)	9	9-12
Lymphocytes (x10^9/L)	2.87	0.5 – 4.0
Monocytes (x10^9/L)	0.53	0.3 – 0.9
Neutrophils (x10^9L)	4.18	1.5 – 7.0
Prothrombin time (s)	14.9	10-14
Activated partial thromboplastin time ratio	1	0.8-1.2
von Willebrand Factor Antigen (vWF:Ag)	166	48 – 175
von Willebrand factor activity level (Ristocetin Co-Factor (vWF:RCo) (IU/dL)	205	47 – 154
von Willebrand Factor Antigen (vWF:Ag)	166	48 – 175
von Willebrand Factor Collagen binding activity (vWF:CBA)	155	57 – 164
FVIII:C (IU/dL) * **	>150	>40
FIX (IU/dL)	160	>40

* Reference ranges taken from document HAE.N002 Version 21.04 available at https://qehbpathology.uk/images/files/PUB_040.pdf

** International Society for Thrombosis and Haemostasis – Bleeding Assessment Tool (ISTH BAT) score comprised of Epistaxis (3), Surgery (3), Haematuria (2)

*** - reference range for FXIII:C was from UHCW – https://www.uhcw.nhs.uk/download/clientfiles/files/CWPS%20Handbook%20V17%20November%202025.pdf

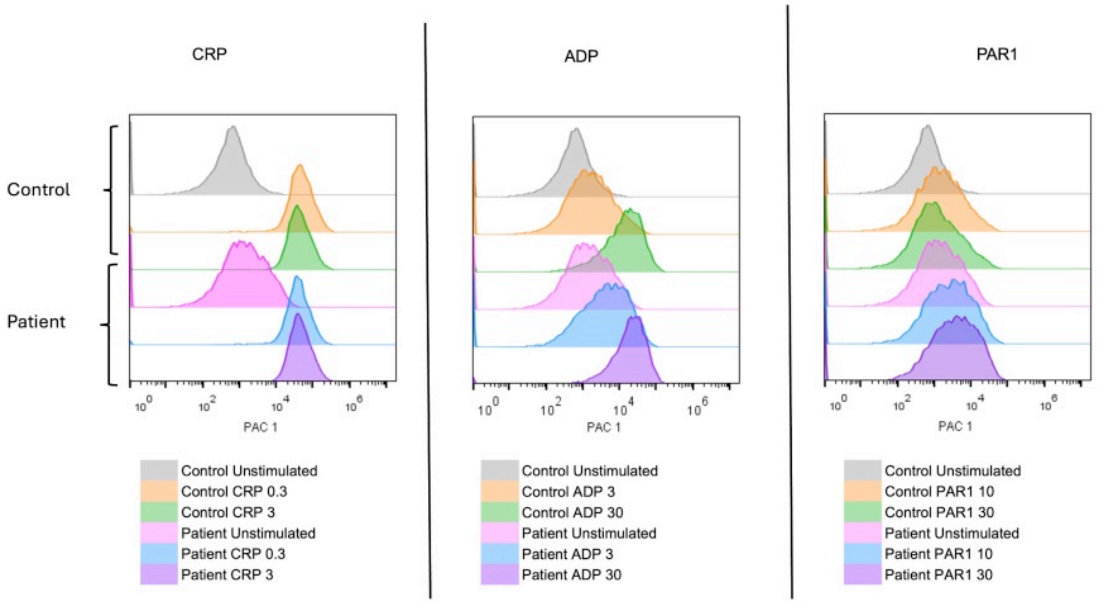
A

Platelet Aggregation

Agonist	Control Normal Ranges *				Patient ***	Same Day Control ***
	Mean	*SD*	*Lower **	*Upper **		
ADP 10	85	15	55	114	92	81
Adren 10	73	22	29	116	102	91
PAR1 30	*nd*	*nd*	*nd*	*nd*	94	90
Col 1	61	30	0.1	121	42	81
Col 3	66	21	23	109	110	90
CRP 3	*nd*	*nd*	*nd*	*nd*	112	85
Risto 1.5	85	17	51	118	107	120

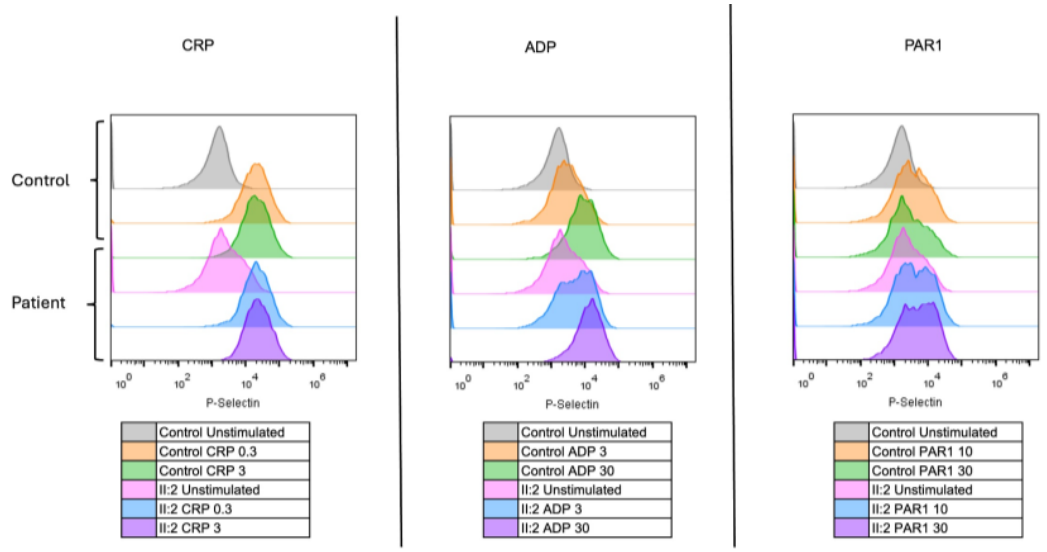
B

Platelet Activation

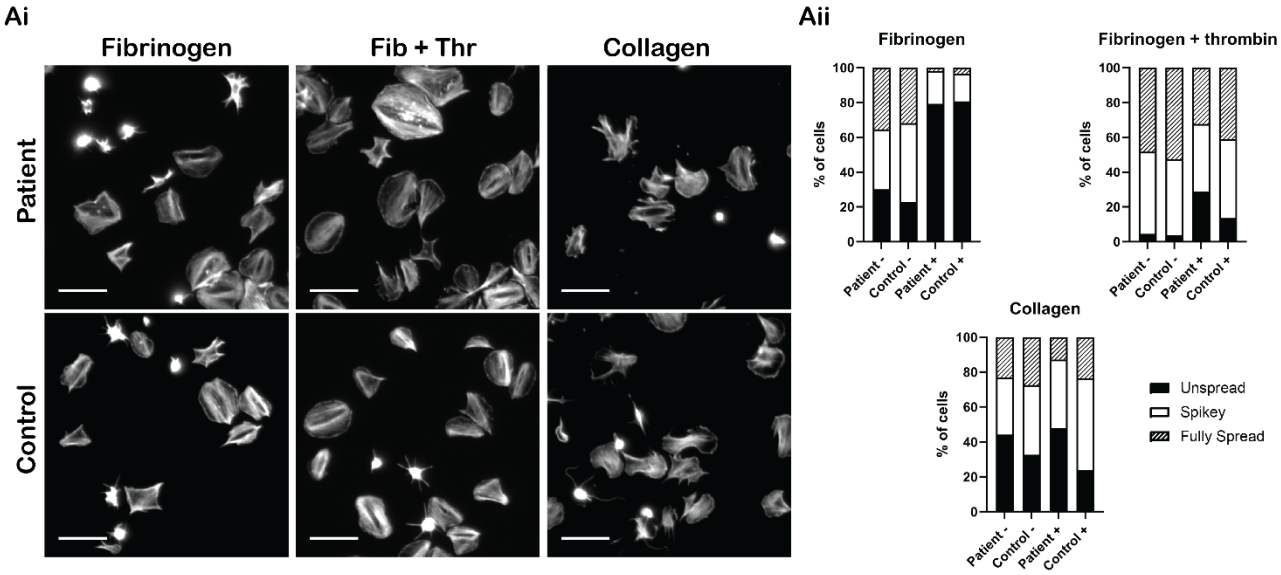


C

Platelet Secretion



Supplementary Figure 1. Platelet function analysis of the Scott patient compared to same-day healthy control. A) Percentage aggregation for index patient II:2 and same day control. PRP from patient II:2 and same-day healthy control, patient was stimulated with a range of platelet agonists for 7 min and aggregation and measured by light transmission aggregometry (LTA, Chrono-log model 700). ADP; Adenosine diphosphate (µM), Adren; Adrenaline (µM), PAR1; PAR1 peptide (TRAP6, µM), Coll; collagen (µg/mL), CRP; collagen-related peptide (µg/mL), Risto; ristocetin (mg/mL). * Control ranges taken from 33 healthy volunteer controls. ** Lower and upper values are ± 2 x SD, *** Green indicates value is within control normal range, red that it is outside. *nd* – Agonist/concentration not routinely performed with healthy control panels**. B)** Histograms of median fluorescence intensity (MFI) of PAC1-FITC and **C)** CD62P-APC (P-selectin) positive (platelets after stimulation with 0.3, 3 µg/mL CRP, 3, 30 µM ADP and 10, 30 µM PAR1 measured using flow cytometry.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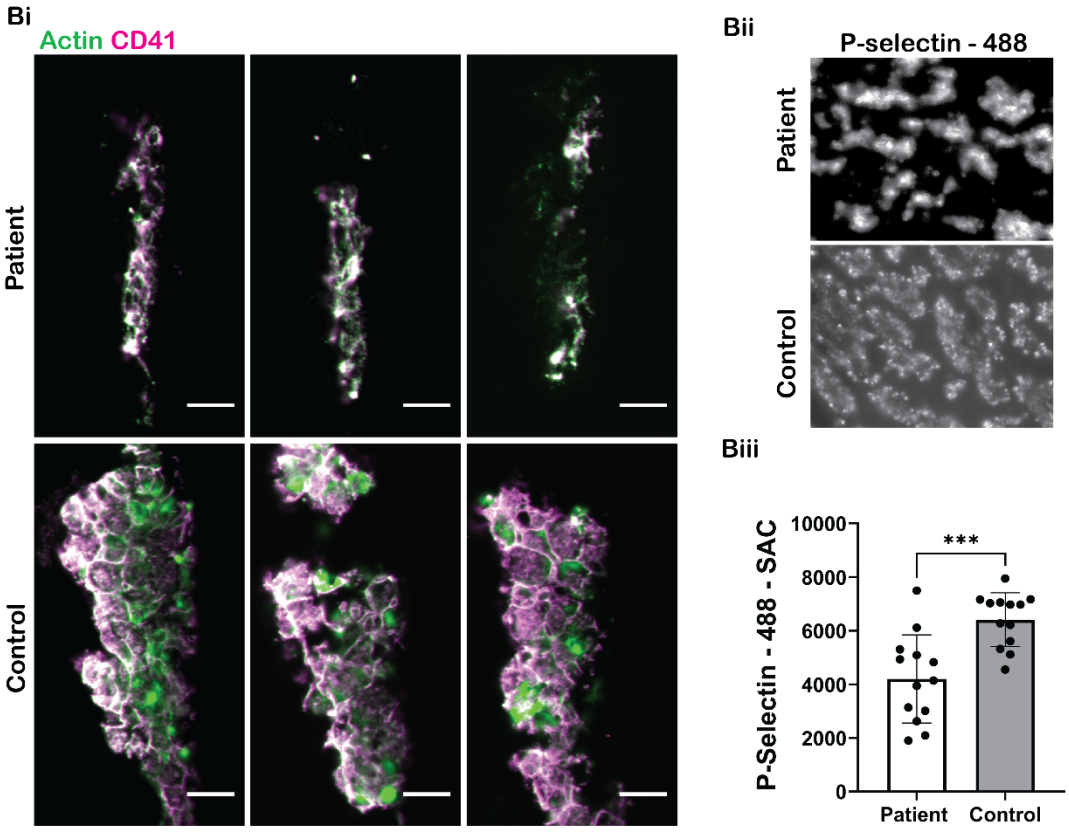
Unspread
Spikey
Fully Spread


Bi
Actin CD41
Patient
Control
Bii
P-selectin - 488
Patient
Control
Biii
P-Selectin - 488 - SAC
***
Patient
Control

Supplementary Figure 2. Platelet spreading and thrombus formation of patient compared to same-day control. A) Washed platelets (2×10^7 /mL) were spread on fibrinogen (100 µg/mL), fibrinogen and thrombin (Fib + Thr, 100 µg/mL and 0.1 UI/mL respectively) and collagen (10 µg/mL). **Ai)** Representative images for patient (II:2) and same-day control. **Aii)** distribution of spread platelet phenotype for platelets on fibrinogen, fibrinogen+ thrombin and collagen in the absence (−) and presence (+) of apyrase (2 U/mL) and indomethacin (10 µM). **B)** Thrombus formation on collagen (100 µg/mL) microspots in the Maastricht flow chamber at 1500 s^{-1}. **Bi)** Expansion microscopy images of selected regions of interest of thrombi using selective plane illumination microscopy (SPIM) stained for CD41 and actin**.** Scale bar = 20 µM. **Bii)** Representative images of patient thrombi and same-day healthy control stained for P-selectin. Scale = 50 µm. **Biii)** Quantitation of surface area coverage (SAC) of P-selectin images (13 fields of view per individual, mean ± SD). Unpaired T-test, *** = P <0.005