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

Scott syndrome is a recessively inherited, mild to moderate bleeding disorder attributed to variants in the gene encoding Anoctamin-6 (*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 personalized 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, a calcium-activated chloride channel responsible for phospholipid scrambling and apoptotic membrane dynamics.[4] Thrombin generation is a central event in hemostasis 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 hematologic investigations in these patients are usually normal, with platelet count, morphology, and functional responses such as aggregation, within reference ranges. Therefore, diagnosis requires more specialized 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 (International Society on Thrombosis and Haemostasis-Bleeding Assessment Tool [ISTH-BAT] score of 8) (*Online Supplementary Table S1*), which included excessive bleeding after surgery. Interestingly, neither parent of II:2 had any known history of excessive bleeding (Figure 1A). Following informed consent, II:2 was recruited to the UK Genotyping and Phenotyping of Platelets (GAPP) study for genetic studies and platelet function testing.[7] This study was approved by the UK National Research Ethics Service by the Research Ethics Committee of West Midlands (06/MRE07/36). Initial investigations including platelet count, coagulation screen and factor assays were normal (*Online Supplementary Table S1*).

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 American College of Medical Genetics and Genomics (ACMG) guidelines[9] and predicted to lead to premature termination of translation and nonsense-mediated RNA decay. The patient was negative for all genes on this panel except the *ANO6* variants reported here. Extended genetic testing to his two children (III:1 and III:2), neither of whom had any 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 known to date (Figure 1C), and were predicted to destabilize the ANO6 structure using the AlphaFold 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; *Online Supplementary Figure S1*) and platelet activation responses

(P-selectin expression and integrin activation; *Online Supplementary Figure S1*) 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 (*Online Supplementary Figure S2Ai, ii*).[12]

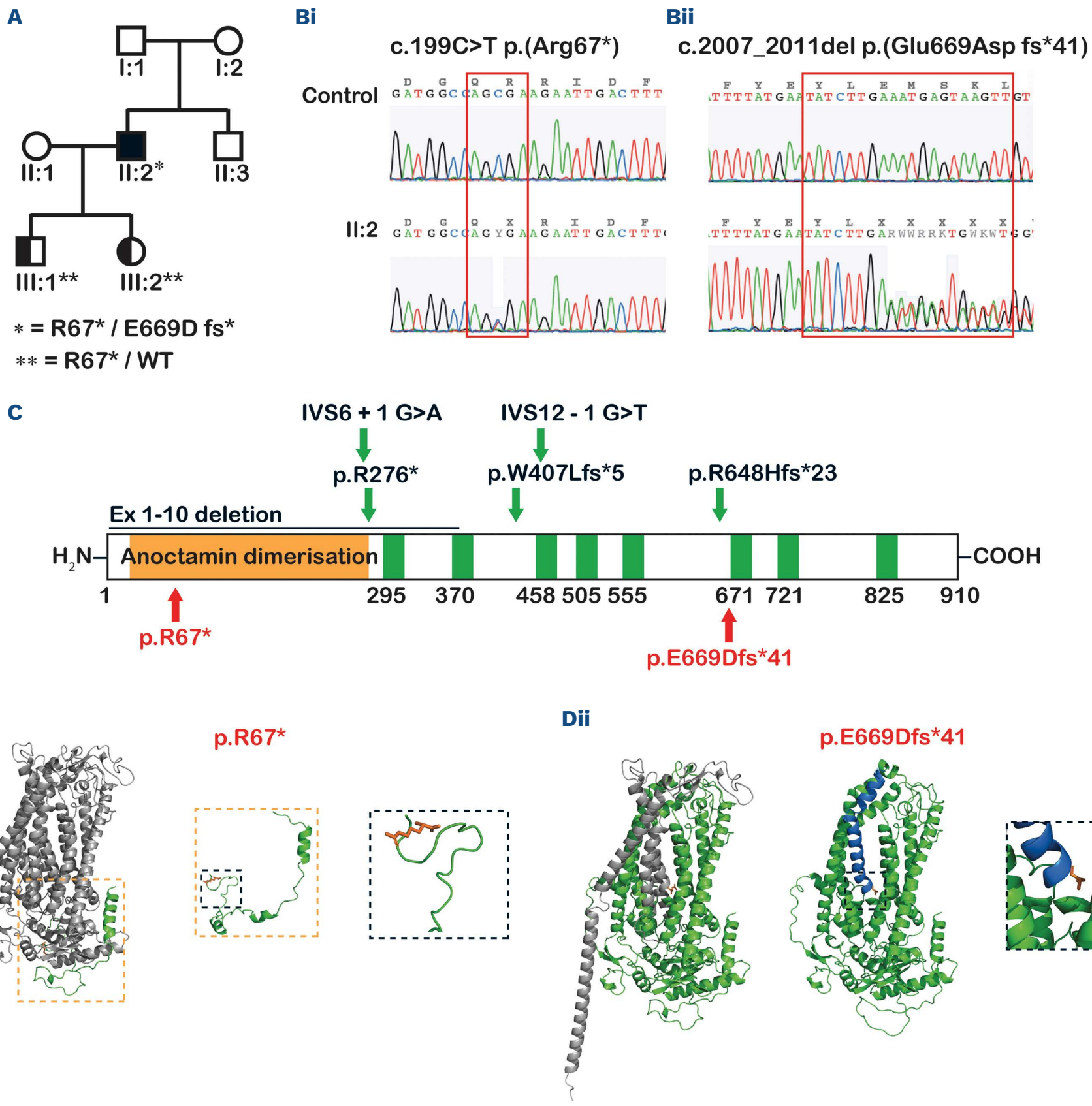


Figure 1. Two novel pathogenic variants in the gene encoding Anoctamin-6 with predicted truncations in a Scott syndrome patient. (A) Pedigree of family showing affected individuals (filled). The index patient II:2 is a compound heterozygote for the two variants. (B) Sanger sequencing results of two novel variants in the gene encoding Anoctamin-6 (*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 a truncated ANO6 protein. (Bii) 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. Position of the novel p.R67* and p.Glu669Asp fs* variants shown as red arrows, previously identified variants as green arrows, and transmembrane domains as dark green boxes. (Di) AlphaFold prediction of the R67* mutated protein. Green sequence shows the extent of the truncated protein, with the non-expressed regions in gray. Yellow box indicates 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 gray. The blue sequence shows the extent of the altered amino acid sequence, and the black box shows the mutated amino acid.

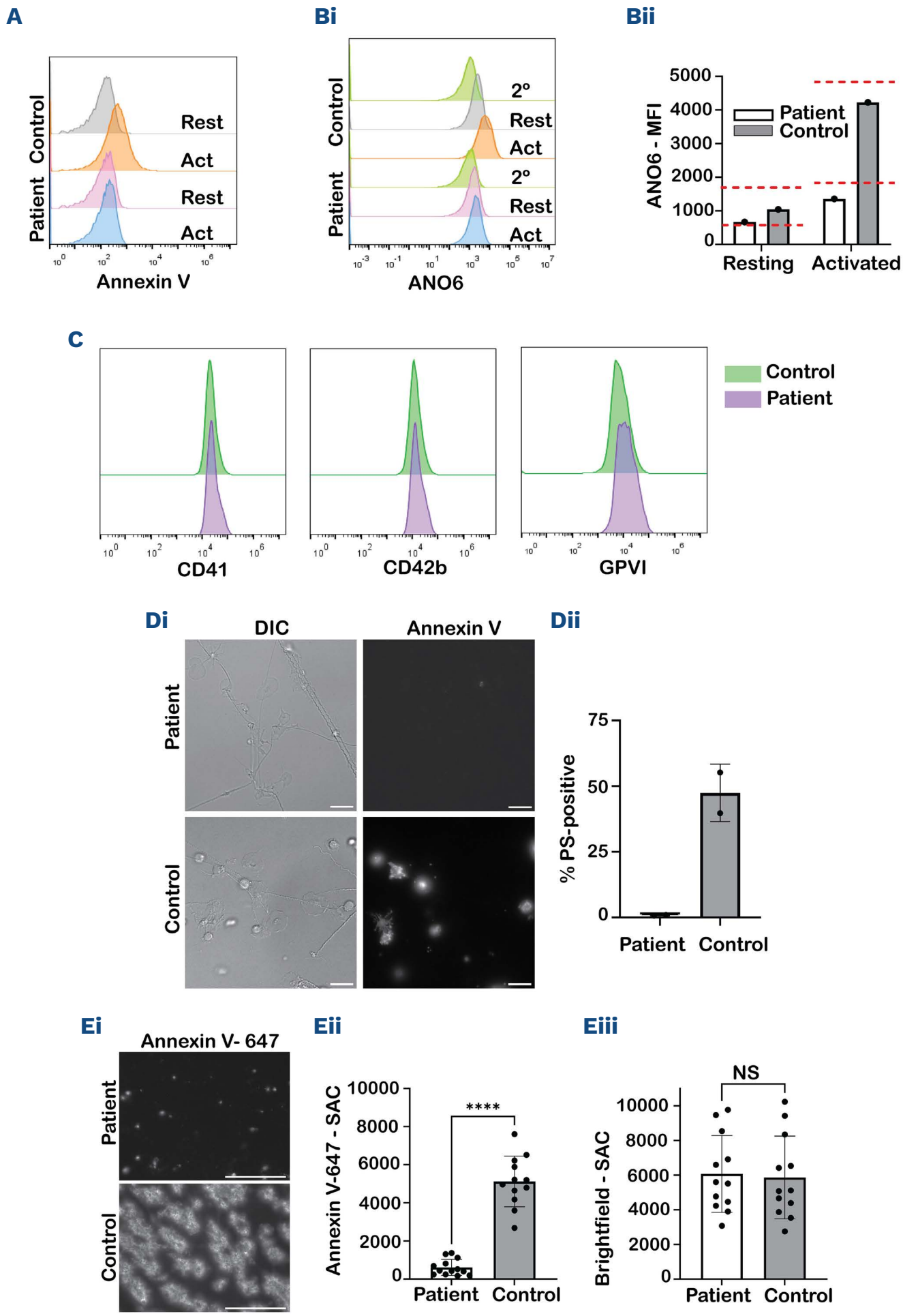


Figure 2. Reduced ability of patient platelets to express phosphatidylserine on their surface. (A) Flow cytometry was used to assess the ability of washed platelets to express phosphatidylserine (PS) on their surface, as measured by annexin V, in response to stimulation with 10 μg/mL collagen-related peptide (CRP) and 1 IU/mL thrombin in the patient compared to the same-day control. 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 (Rest) and after activation (Act) 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 and (Bii) bar charts of MFI of ANO6 on resting and activated platelets from patient and same-day control. Secondary only control values (2°) 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 *Online Supplementary Figure S2*) and annexin V measured on the surface. Representative images of annexin V from the patient and same-day healthy control. Scale

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bar = 50 μm. (Dii) Quantification of percentage PS-positive platelets. Bars are mean ± standard deviation (SD). 10 fields of view were analyzed for each condition, with a total of 952 platelets for control and 1,216 platelets for the patient. (Ei) Platelet aggregate formation over collagen (100 μg /mL) micro-spots at 1,500 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. conc: concentration; ETP: endogenous thrombin potential; NS: not significant.

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). However, 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, pe-

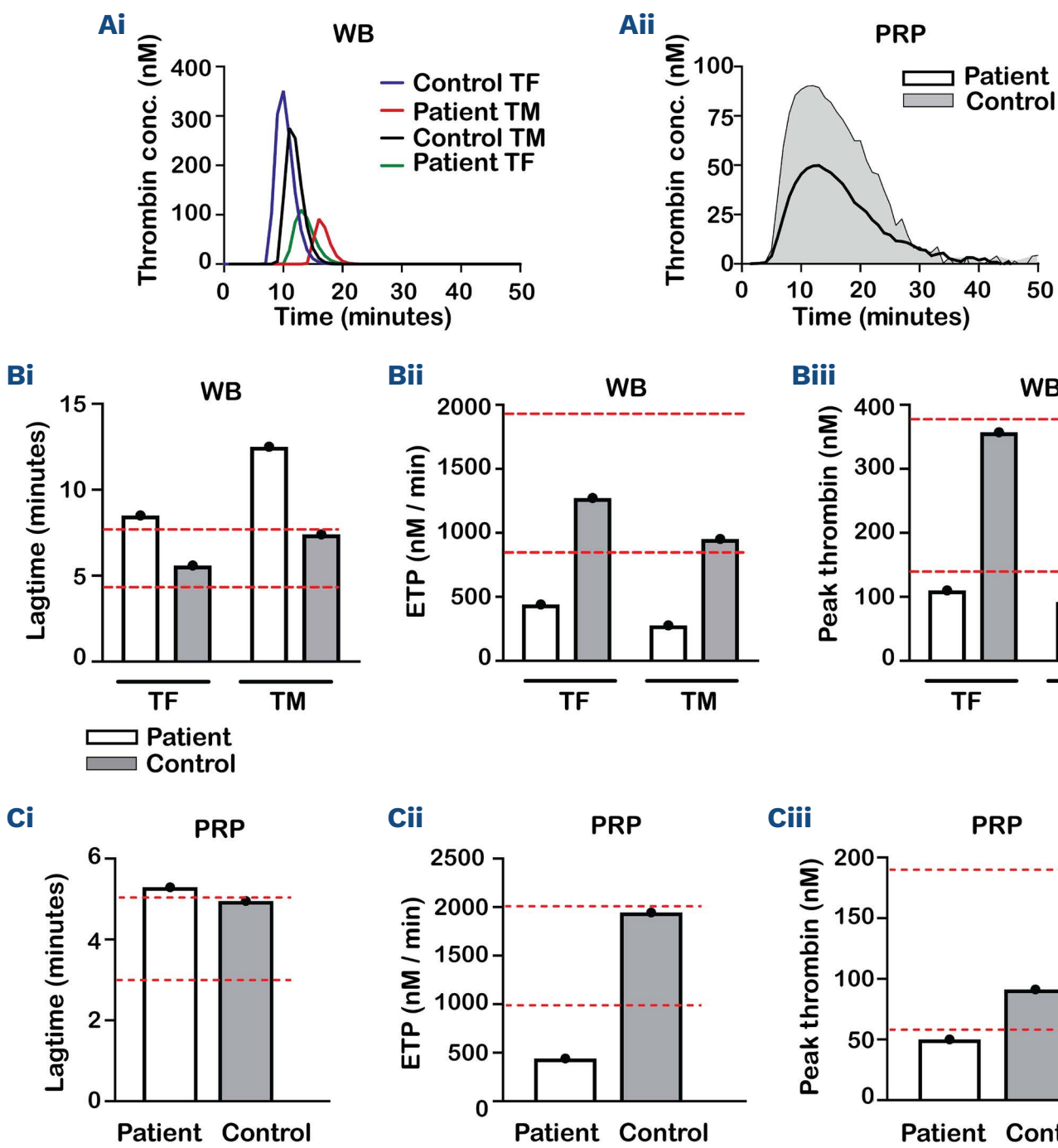


Figure 3. Reduced thrombin generation in patient platelets. (A) Thrombin generation curves showing reduced thrombin generation in II:2 in (i) whole blood (WB), after stimulation with either tissue factor (TF) (0.1 pM) alone, or TF and thrombomodulin (TM) (1650 nM) and (ii) platelet rich plasma (PRP) after stimulation with TF (670 nM). Control: same-day healthy control. (B and C) Specific thrombin generation parameters tested in whole blood and PRP (lagtime (Bi and Ci), endogenous thrombin potential (ETP) (Bii and Cii), and peak thrombin generation (Biii and Ciii)). All parameters were altered in II:2 samples compared to the control, as well as being outside our normal healthy donor ranges for these assays (lines are mean ± 2 standard deviation of 40 controls).

ripheral blood was recalcified, treated with 40 μM PPACK, and flowed over pre-coated 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 2Di, ii), confirming defective PS externalization 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 (ExM13) of the platelet aggregates demonstrated that the patient's thrombi were smaller and less well-defined (*Online Supplementary Figure S2Bi*) and P-selectin expression was also significantly diminished (P<0.001) (*Online Supplementary Figure S2Bii*). This suggests 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 the control, demonstrating the role of platelet PS exposure in the process (Figure 3Aii). The lagtime for thrombin generation in whole blood was also markedly increased in II:2 compared to controls (Figure 3Bi), with a small increase in lagtime 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, Cii). A parallel trend was also seen with peak thrombin levels (Figure 3Biii, Ciii). Notably, for II:2, all parameters were outside of the assay reference ranges (displayed as red lines on the plots), while 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 raise 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, for whom routine coagulation test results are normal and who have a normal platelet count.

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https://doi.org/10.3324/haematol.2025.300111

Received: October 27, 2025.
Accepted: February 17, 2026.
Early view: February 26, 2026.

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Disclosures

DH and BdL are employees of Synapse Research Institute, part of Diagnostica Stago S.A.S. All of the other authors have no conflicts of interest to disclose.

Contributions

NVM designed the study, extracted and generated clinical and

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

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.

Funding

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.

Data-sharing statement

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

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