

Expanded repertoire of *RASGRP2* variants responsible for platelet dysfunction and severe bleeding

Sarah K Westbury,^{1,2*} Matthias Canault,^{3,4*} Daniel Greene,^{2,5,6} Emilse Bermejo,⁷ Katharine Hanlon,⁸ Michele P Lambert,^{2,9,10} Carolyn M Millar,^{2,11,12} Paquita Nurden,^{2,13} Samya G Obaji,¹⁴ Shoshana Revel-Vilk,^{2,15} Chris Van Geet,^{2,16} Kate Downes,^{2,5,17} Sofia Papadia,^{2,5,17} Salih Tuna,^{2,5,17} Christopher Watt,^{2,5,17} NIHR BioResource-Rare Diseases,² Kathleen Freson,^{2,16} Michael A Laffan,^{2,11,12} Willem H Ouweland,^{2,5,17,18} Marie-Christine Alessi,^{3,4} Ernest Turro,^{2,5,6,17#} Andrew D Mumford.^{1,2,19#}

* SKW and MC contributed equally to this study

ET and ADM contributed equally to this study

¹School of Clinical Sciences, University of Bristol, Bristol, UK, ²NIHR BioResource - Rare Diseases, Cambridge University Hospitals, Cambridge, UK, ³Inserm, UMR 1062, Inra, UMR 1260, Aix Marseille Université, 13005 Marseille, France, ⁴Centre de référence sur les pathologies plaquettaires (CRPP), CHU Timone, Marseille, France, ⁵Department of Haematology, University of Cambridge, Cambridge, UK, ⁶Medical Research Council Biostatistics Unit, Cambridge Institute of Public Health, Cambridge, UK, ⁷Hematological Research Institute, National Academy of Medicine, Buenos Aires, Argentina, ⁸Department of Haematology, Glasgow Royal Infirmary, Glasgow, UK, ⁹Division of Hematology, Children's Hospital of Philadelphia, Philadelphia, USA, ¹⁰Department of Pediatrics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, USA, ¹¹Centre for Haematology, Hammersmith Campus, Imperial College Academic Health Sciences Centre, Imperial

College London, London, UK, ¹²Imperial College Healthcare NHS Trust, DuCane Road, London, United Kingdom, ¹³Institut Hospitalo-Universitaire LIRYC, PTIB, Hôpital Xavier Arnoz, Pessac, France, ¹⁴Department of Haematology, University Hospital of Wales and School of Medicine, Cardiff University, Cardiff, UK, ¹⁵Pediatric Hematology/Oncology Department, Hadassah Hebrew-University Hospital, Jerusalem, Israel, ¹⁶Department of Cardiovascular Sciences, Center for Molecular and Vascular Biology, University of Leuven, Belgium ¹⁷NHS Blood and Transplant, Cambridge, UK, ¹⁸Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK, ¹⁹School of Cellular and Molecular Medicine, University of Bristol, Bristol, UK.

Scientific category: Platelets and thrombopoiesis

Running title: *RASGRP2* platelet function disorder

Word count: main text 1199 abstract 195

Figures: 2

References: 22

Corresponding author:

Andrew D Mumford, University of Bristol, Research Floor Level 7, Bristol Royal Infirmary, Upper Maudlin Street, Bristol, UK, BS2 8HW.

Tel. +44 117 34 23152

E-mail a.mumford@bristol.ac.uk

KEY POINTS

- Eleven pedigrees were identified with biallelic pathogenic variants in *RASGPR2*, which encodes platelet CalDAG-GEFI
- CalDAG-GEFI deficiency is a severe, recessive, non-syndromic platelet function disorder with defective aggregation to multiple agonists

ABSTRACT

Heritable platelet function disorders (PFDs) are genetically heterogeneous and poorly characterised. Pathogenic variants in *RASGRP2*, which encodes calcium and diacylglycerol-regulated guanine exchange factor I (CaLDAG-GEFI), have been reported previously in three pedigrees with bleeding and reduced platelet aggregation responses. To better define the phenotype associated with pathogenic *RASGRP2* variants, we compared high-throughput sequencing and phenotype data from 2,042 cases in pedigrees with unexplained bleeding or platelet disorders to data from 5,422 controls. Eleven cases harboured 11 different, previously unreported *RASGRP2* variants that were biallelic and likely pathogenic. The variants included five high-impact variants predicted to prevent CaLDAG-GEFI expression and six missense variants affecting the CaLDAG-GEFI CDC25 domain, which mediates Rap1 activation during platelet inside-out $\alpha\text{IIb}\beta\text{3}$ signalling. Cases with biallelic *RASGRP2* variants had abnormal mucocutaneous, surgical and dental bleeding from childhood, requiring at least one blood or platelet transfusion in 78% of cases. Platelets displayed reduced aggregation in response to ADP and epinephrine, but variable aggregation defects with other agonists. There were no other consistent clinical or laboratory features. These data enable definition of human CaLDAG-GEFI deficiency as a non-syndromic, recessive PFD associated with a moderate or severe bleeding phenotype and complex defects in platelet aggregation.

INTRODUCTION

Heritable platelet function disorders (PFD) are genetically heterogeneous rare diseases characterised by mucocutaneous, surgical and traumatic bleeding^{1,2} and reduced platelet responses to activating agonists.^{3,4} Human PFD have been associated with causal variants in more than 30 genes, often associated with distinctive clinical or laboratory phenotypes that inform selection of candidate genes for diagnosis.⁵ Several new PFD with less distinctive phenotypes have recently been identified using high-throughput sequencing.⁶⁻⁸ However, to date these have only been reported in small numbers of pedigrees and are incompletely characterised.

One such example is the PFD associated with loss-of-function variants in *RASGRP2*, which encodes calcium and diacylglycerol-regulated guanine exchange factor I (CalDAG-GEFI). This PFD was first identified in a consanguineous pedigree with a homozygous p.G248W variant associated with bleeding and reduced platelet light transmission aggregation (LTA) responses to multiple agonists.⁹ CalDAG-GEFI deficiency has been reported subsequently in only three pedigrees worldwide.^{10,11}

In order to better define the phenotype and mutational spectrum of CalDAG-GEFI deficiency, we analysed data from a collection of 2,042 cases with unexplained bleeding or platelet disorders (BPD) to identify 11 new affected pedigrees.

METHODS

RASGRP2 was investigated in two study collections in which all participants were enrolled to the National Institute for Health Research BioResource - Rare Diseases between 2012 and 2016 after providing informed written consent (UK Research

Ethics Committee approval 13/EE/0325). The first collection comprised 1,472 cases or pedigree members with unexplained BPD and 5,422 cases with other rare inherited disorders who underwent whole exome or genome sequencing.¹² The second collection comprised 570 other BPD cases analysed using the ThromboGenomics high-throughput platform¹³, which captures variants in 81 tier one genes for BPD, designated by the ISTH Genomics Scientific and Standardisation Subcommittee.

Collection of clinical and laboratory phenotypes and coding with Human Phenotype Ontology (HPO) terms were performed as previously described.¹² Variants from high-throughput sequencing were obtained using Isaac (Illumina, Inc.; San Diego, CA) or as described previously.¹² Variants were not considered to be potentially pathogenic if the Variant Effect Predictor¹⁴ identified them as synonymous relative to canonical transcript ENST00000354024, or if the allele frequency was greater than 1/1000 in reference databases.^{12,15} Index cases in the NIHR BioResource were classified as having an indicator phenotype of CalDAG-GEFI deficiency if they had HPO terms indicating abnormal bleeding and reduced LTA responses to at least three agonists. Associations between rare and non-synonymous *RASGRP2* variants in the NIHR BioResource and the presence or absence of the indicator phenotype were then assessed using the BeviMed method.¹⁶

RESULTS AND DISCUSSION

The CalDAG-GEFI deficiency indicator phenotype of bleeding and reduced LTA responses to at least three agonists was present in 119 (9.7%) of 1,229 BPD index cases from the NIHR BioResource. Analysis of all the rare, non-synonymous

variants in the BPD and non-BPD index cases revealed a strong statistical association between the indicator phenotype and the presence of at least two *RASGRP2* alleles, obtaining a posterior probability of association with recessive inheritance of 1 by inference using the BeviMed method (**Figure 1A**). The association was driven by eight different variants observed in eight BPD index cases as homozygous or compound heterozygous alleles (**Figure 1B**; pedigrees A-H in **Figure 2A**). There were a further 35 *RASGRP2* variants which were not associated with the indicator phenotype or which were present as a single allele (**Figure 1A**). In the ThromboGenomics collection, there were four rare, non-synonymous *RASGRP2* variants that were biallelic (**Figure 1B**) in 3 index cases with bleeding and reduced LTA responses to at least three agonists (pedigrees I-K in **Figure 2A**). Since this analysis, pedigree G has also been reported elsewhere.¹⁷

Of the 11 likely pathogenic *RASGRP2* variants, four resulted in frameshift or a stop codon when annotated against the *RASGRP2* canonical transcript (**Figure 1B, 1C**). One further variant (11:64507638 G>C: pedigree K) was at the -3 position in the intron 5 splice region, adjacent to the splice acceptor site. The Alamut[®] Visual splicing module (Interactive Biosoftware, Rouen, France) predicted this to result in loss of the native splice acceptor site and co-option of a cryptic acceptor site in exon 6, leading to deletion of nine codons and a stop gain at codon 125 (**Figure 1C**). Thus, a total of five variants were predicted to prevent full length CaIDAG-GEFI expression.

All six missense variants predicted substitutions of amino acids that were conserved in CaIDAG-GEFI orthologs in nine distantly related species, except for human

residue A345 that is a T residue in the *Xenopus* ortholog (**Figure 1B** and **Supplementary table S1**). All had high CADD pathogenicity scores (>20; within the top 1% of deleterious variants genome-wide)¹⁸ and were either absent or at very low allele frequency in the ExAC population database (**Figure 1B**).¹⁵ All the predicted substitutions were in the CDC25 domain of CalDAG-GEFI that is essential for guanine nucleotide exchange activity, and directly interacts with Rap1, the major platelet target for CalDAG-GEFI in the inside-out $\alpha\text{IIb}\beta\text{3}$ signalling pathway.¹⁹ This suggests that the missense variants reduce platelet function by altering CalDAG-GEFI function, although we cannot exclude reduced protein expression, as observed previously for the *RASGRP2* p.Ser381Phe variant.¹¹ There were no consistent phenotype differences between the cases with missense and high-impact variants.

The 11 likely pathogenic *RASGRP2* variants occurred in a total of 11 unrelated cases (7 males) as homozygous (7 cases) or compound heterozygous (4 cases) alleles, indicating autosomal recessive inheritance. One pedigree (J) contained a sibling with biallelic *RASGRP2* variants (**Figure 2A**). All the index cases had a first diagnosis of a bleeding disorder during childhood (age 1-14 years; **Supplementary table S2**). Abnormal bleeding was predominantly mucocutaneous or followed surgery or dental extraction. Gastrointestinal bleeding occurred in two cases (**Figure 2B**), but there was no intracranial bleeding. Of the nine index cases with available data, seven had required red cell or platelet transfusion on at least one occasion suggesting severe bleeding. There were no other consistently reported phenotypes. Platelet number and size were normal (**Supplementary table S2**).

All 11 index cases had reduced platelet LTA responses with ADP and epinephrine, and in some cases also with collagen, arachidonic acid and TRAP-6 (**Figure 2C, 2D**). Detailed analysis of A-II3 and B-II1 showed additionally that the velocity of aggregation to all tested doses of ADP, epinephrine and TRAP-6 was reduced compared with controls (**Figure 2D**). The normal platelet ultrastructure, nucleotide content and release of alpha (P-selectin exposure) and dense (CD63 exposure) granules in response to high dose TRAP14 indicated no defect in platelet granule biosynthesis. However, dense granule release with ADP was diminished in most tested cases, mirroring the functional defects observed with LTA (**Supplementary table S2**), similar to previous reports.^{9,10,11}

This report of 11 new pedigrees significantly expands the reported cases with human CalDAG-GEFI deficiency and enables description of this disorder as an autosomal recessive, non-syndromic PFD that is associated with moderate or severe bleeding, similar to other disorders of $\alpha\text{IIb}\beta\text{3}$ integrin signalling^{20,21} and some types of Glanzmann thrombasthenia in which there is reduced expression of functional $\alpha\text{IIb}\beta\text{3}$ integrin.²² We show further that there is a consistent laboratory phenotype of reduced aggregation responses to ADP and epinephrine in all reported cases, but no defect in dense granule secretion, distinguishing this disorder from δ -storage pool disease. This distinctive phenotype is likely to assist genetic diagnosis of further pedigrees.

ACKNOWLEDGMENTS

The NIHR BioResource-Rare Diseases is funded by the National Institute for Health Research of England (NIHR, www.nihr.ac.uk; award number RG65966). SKW is supported by a Medical Research Council Clinical Research Training Fellowship (MR/K023489/1). CMM and MAL are supported by the NIHR Imperial College Biomedical Research Centre. KF and CVG are supported by the Fund for Scientific Research-Flanders (FWO-Vlaanderen, Belgium, G.0B17.13N) and Research Council of the University of Leuven (BOF KU Leuven, Belgium, OT/14/098). WHO is supported by the British Heart Foundation, European Commission, Medical Research Council, NIHR, Wellcome Trust, and National Health Service Blood and Transplant. ADM is supported by the NIHR Biomedical Research Centre at the University Hospitals Bristol NHS Foundation Trust and the University of Bristol. The views expressed in this publication are those of the authors and not necessarily those of the NHS, the National Institute for Health Research or the Department of Health. We thank Professor J-F Gris (Nimes), Dr Ségolène Claeysens (Toulouse) and Dr Yeon Ahn (Miami) for providing the genomic DNA for index cases A, B and G, and Dr Alan Nurden (Bordeaux) for contributing to the initial characterisation of the platelet function abnormality of the index cases in pedigrees A, B, G and K.

AUTHOR CONTRIBUTIONS

SKW and MC wrote the paper with assistance from M-CA, ET and ADM. MC, DG, ET and SKW analysed data. EB, KH, MPL, CMM, PN, SGO, SR-V and CvG provided samples and clinical data. KD managed the ThromboGenomics programme. SP co-ordinated the NIHR BioResource – Rare Diseases BPD project

including ethics and governance. ST and CW provided sample logistics, QC and WGS oversight. KF, MAL and WHO contributed to the study design.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors report no relevant conflicts of interest.

REFERENCES

1. Lentaigne C, Freson K, Laffan MA, et al. Inherited platelet disorders: toward DNA-based diagnosis. *Blood*. 2016;127(23):2814-2823.
2. Nurden AT, Nurden P. Inherited disorders of platelet function: selected updates. *J Thromb Haemost*. 2015;13 Suppl 1:S2-9.
3. Gresele P, Subcommittee on Platelet Physiology of the International Society on T, Hemostasis. Diagnosis of inherited platelet function disorders: guidance from the SSC of the ISTH. *J Thromb Haemost*. 2015;13(2):314-322.
4. Cattaneo M, Hayward CP, Moffat KA, Pugliano MT, Liu Y, Michelson AD. Results of a worldwide survey on the assessment of platelet function by light transmission aggregometry: a report from the platelet physiology subcommittee of the SSC of the ISTH. *J Thromb Haemost*. 2009;7(6):1029.
5. Gresele P, Harrison P, Bury L, et al. Diagnosis of suspected inherited platelet function disorders: results of a worldwide survey. *J Thromb Haemost*. 2014;12(9):1562-1569.
6. Johnson B, Lowe GC, Futterer J, et al. Whole exome sequencing identifies genetic variants in inherited thrombocytopenia with secondary qualitative function defects. *Haematologica*. 2016;101(10):1170-1179.

7. Fletcher SJ, Johnson B, Lowe GC, et al. SLFN14 mutations underlie thrombocytopenia with excessive bleeding and platelet secretion defects. *J Clin Invest*. 2015;125(9):3600-3605.
8. Albers CA, Cvejic A, Favier R, et al. Exome sequencing identifies NBEAL2 as the causative gene for gray platelet syndrome. *Nat Genet*. 2011;43(8):735-737.
9. Canault M, Ghalloussi D, Grosdidier C, et al. Human CalDAG-GEFI gene (RASGRP2) mutation affects platelet function and causes severe bleeding. *J Exp Med*. 2014;211(7):1349-1362.
10. Kato H, Nakazawa Y, Kurokawa Y, et al. Human CalDAG-GEFI deficiency increases bleeding and delays alphaIIb beta3 activation. *Blood*. 2016;128(23):2729-2733.
11. Lozano ML, Cook A, Bastida JM, et al. Novel mutations in RASGRP2, which encodes CalDAG-GEFI, abrogate Rap1 activation, causing platelet dysfunction. *Blood*. 2016;128(9):1282-1289.
12. Westbury SK, Turro E, Greene D, et al. Human phenotype ontology annotation and cluster analysis to unravel genetic defects in 707 cases with unexplained bleeding and platelet disorders. *Genome Med*. 2015;7(1):36.
13. Simeoni I, Stephens JC, Hu F, et al. A high-throughput sequencing test for diagnosing inherited bleeding, thrombotic, and platelet disorders. *Blood*. 2016;127(23):2791-2803.
14. McLaren W, Gil L, Hunt SE, et al. The Ensembl Variant Effect Predictor. *Genome Biol*. 2016;17(1):122.
15. Lek M, Karczewski KJ, Minikel EV, et al. Analysis of protein-coding genetic variation in 60,706 humans. *Nature*. 2016;536(7616):285-291.

16. Greene D, NIHR BioResource, Richardson S, Turro E. A fast association test for identifying pathogenic variants involved in rare diseases. *Am J Hum Genet.* 2017 DOI:10.1016/j.ajhg.2017.05.015.
17. Desai A, Bergmeier W, Canault M, et al. Phenotype analysis and clinical management in a large family with a novel truncating mutation in RASGRP2, the CalDAG-GEFI encoding gene. *RPTH.* 2017 in press.
18. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet.* 2014;46(3):310-315.
19. Stefanini L, Bergmeier W. RAP1-GTPase signaling and platelet function. *J Mol Med (Berl).* 2016;94(1):13-19.
20. Kuijpers TW, van de Vijver E, Weterman MA, et al. LAD-1/variant syndrome is caused by mutations in FERMT3. *Blood.* 2009;113(19):4740-4746.
21. Svensson L, Howarth K, McDowall A, et al. Leukocyte adhesion deficiency-III is caused by mutations in KINDLIN3 affecting integrin activation. *Nat Med.* 2009;15(3):306-312.
22. Nurden AT, Pillois X, Fiore M, et al. Expanding the mutation spectrum affecting alphaIIb beta3 integrin in Glanzmann thrombasthenia: screening of the ITGA2B and ITGB3 genes in a large international cohort. *Hum Mutat.* 2015;36(5):548-561.

FIGURE LEGENDS

Figure 1. Identification and characteristics of pathogenic variants in *RASGRP2*

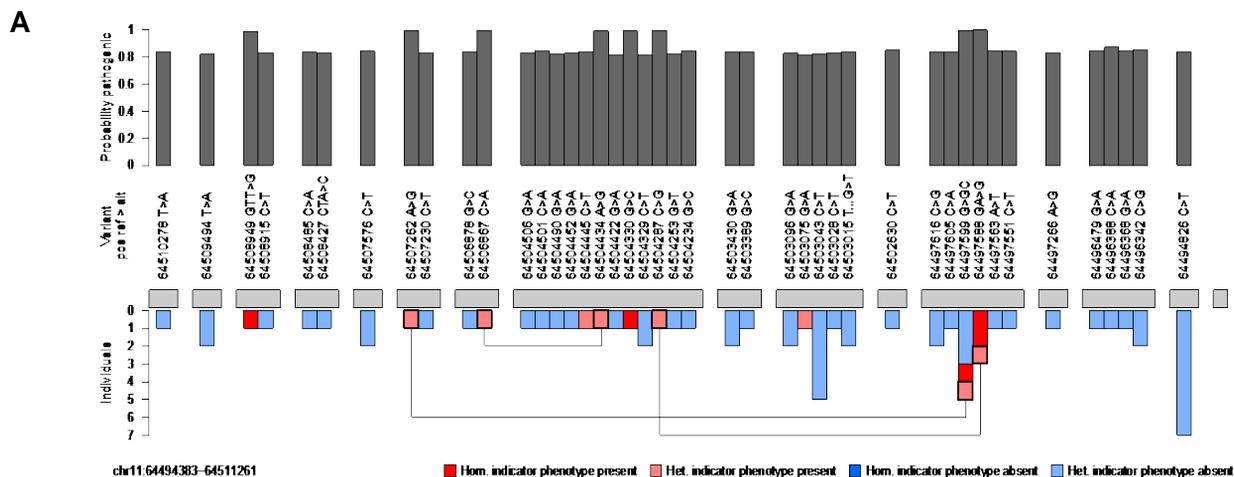
A. BeviMed inference analysis applied to rare, non-synonymous *RASGRP2* variants observed in all index cases from the NIHR BioResource. Cases were designated as having the indicator phenotype of CalDAG-GEFI deficiency if they had HPO terms indicating bleeding and reduced light transmission aggregation responses to at least three activating agonists. The indicator phenotype was present in 119 index cases and was absent in 5,982 index cases. The posterior probability of the association model was 1 (prior was 0.1) and the posterior probability of recessive inheritance was 1 (prior was 0.5). The *RASGRP2* exons are represented by grey blocks. The bar chart above shows the marginal posterior probabilities of pathogenicity for individual variants observed in all NIHR BioResource index cases, conditional on an association under a recessive mode of inheritance. The bar chart beneath indicates whether the variant was observed in an index case with (pink) or without (blue) the indicator phenotype and whether present as a heterozygous and homozygous allele. Variants observed in index cases as compound heterozygous alleles are linked. **B.** The characteristics of the 11 likely pathogenic *RASGRP2* variants across the NIHR BioResource (NBR) and ThromboGenomics (TG) collections. Variants were annotated against the canonical transcript ENST00000354024 using the Variant Effect Predictor (VEP).¹⁴ Population allelic frequencies are derived from the Exome Aggregation Consortium (ExAC)¹⁵. The likely pathogenicity of the variants is expressed as the Combined Annotation Dependent Depletion (CADD) score¹⁸ and the percentage conservation as the proportion of CalDAG-GEFI orthologs in nine species that have the same amino acid as human CalDAG-GEFI. **C.** Localization

and predicted consequence of the likely pathogenic *RASGRP2* variants identified in NIH BioResource and ThromboGenomics collections (below protein diagram) and the previously reported variants⁹⁻¹¹ (above protein diagram). The exploded view shows the predicted consequence of the 11:64507638 G>C variant identified as a homozygous allele in case in ThromboGenomics case K II.1. Bioinformatic analysis of the variant sequence predicts preferential use of an alternative exonic splice acceptor, resulting in codon deletion, frameshift and a stop gain at codon 125.

Figure 2: Characteristics of the 11 index cases with likely pathogenic biallelic *RASGRP2* variants.

A. Pedigrees of the index cases (*) with likely pathogenic *RASGRP2* variants indicating the genotype of the index cases. The black symbols indicate cases with abnormal bleeding and reduced platelet aggregation responses. The white symbols indicate pedigree members without bleeding symptoms and the grey symbols, pedigree members unavailable for evaluation. +/V: heterozygous, V/V: compound heterozygous or homozygous for the variant allele. **B.** Annotation of 11 index cases with HPO terms for bleeding symptoms. **C.** Summary light transmission aggregation for all 11 index cases. Data are expressed as the proportion of index cases with any reported defect in aggregation responses to ADP, epinephrine (Epi), collagen (Coll), arachidonic acid (AA), TRAP-6 and ristocetin (Risto). **D.** Detailed light transmission aggregation response in cases AII-3 and BII-1 in response to the stated agonist concentrations. Data are presented as the maximum aggregation and initial velocity of the aggregation responses.

Figure 1



B

Variant position	VEP consequence	Protein effect	Study collection	ExAC allele frequency	CADD score	% conservation*
11:64508949 GTT>G	Frameshift	p.N67Lfs*24	NBR	0	26.4	-
11:64507638 G>C	Splice region	p.P125*	TG	0	3.8	-
11:64507262 A>G	Missense	p.F181S	NBR	0	29.5	100
11:64506867 C>A	Stop gained	p.E260*	NBR	0	38	-
11:64504454 T>C	Missense	p.Y289C	TG	0	25.7	100
11:64504434 A>G	Missense	p.C296R	NBR	0	25.5	100
11:64504406 C>T	Missense	p.G305D	TG	0	29.3	100
11:64504330 G>C	Missense	p.N330K	NBR	0	25.3	100
11:64504287 C>G	Missense	p.A345P	NBR	0	22.4	89
11:64497599 G>GC	Frameshift	p.R494Afs*54	NBR	8.525×10^{-4}	35	-
11:64497588 GA>G	Frameshift	p.F497Sfs*22	NBR + TG	0	35	-

C

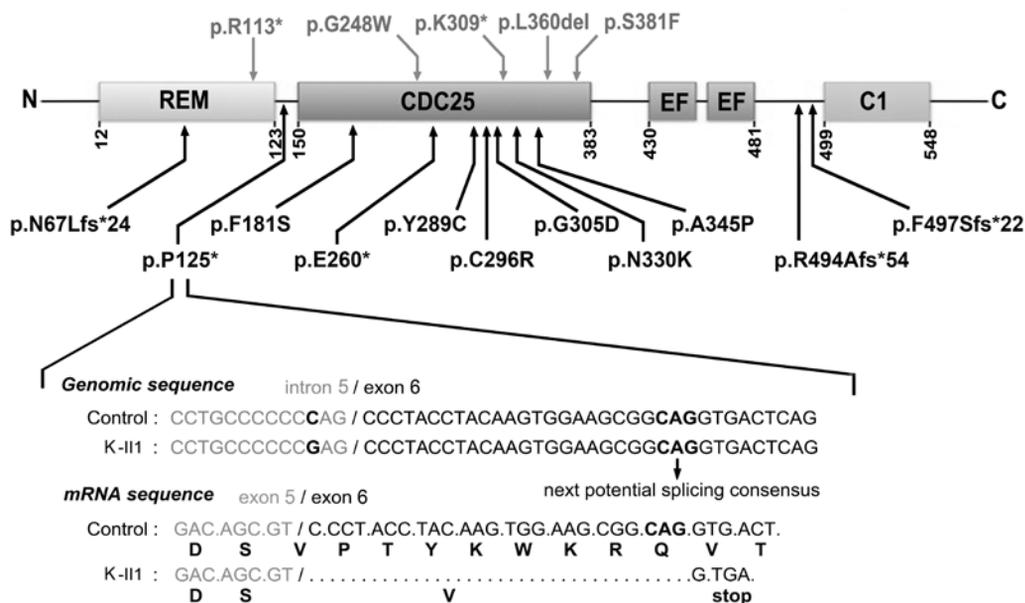
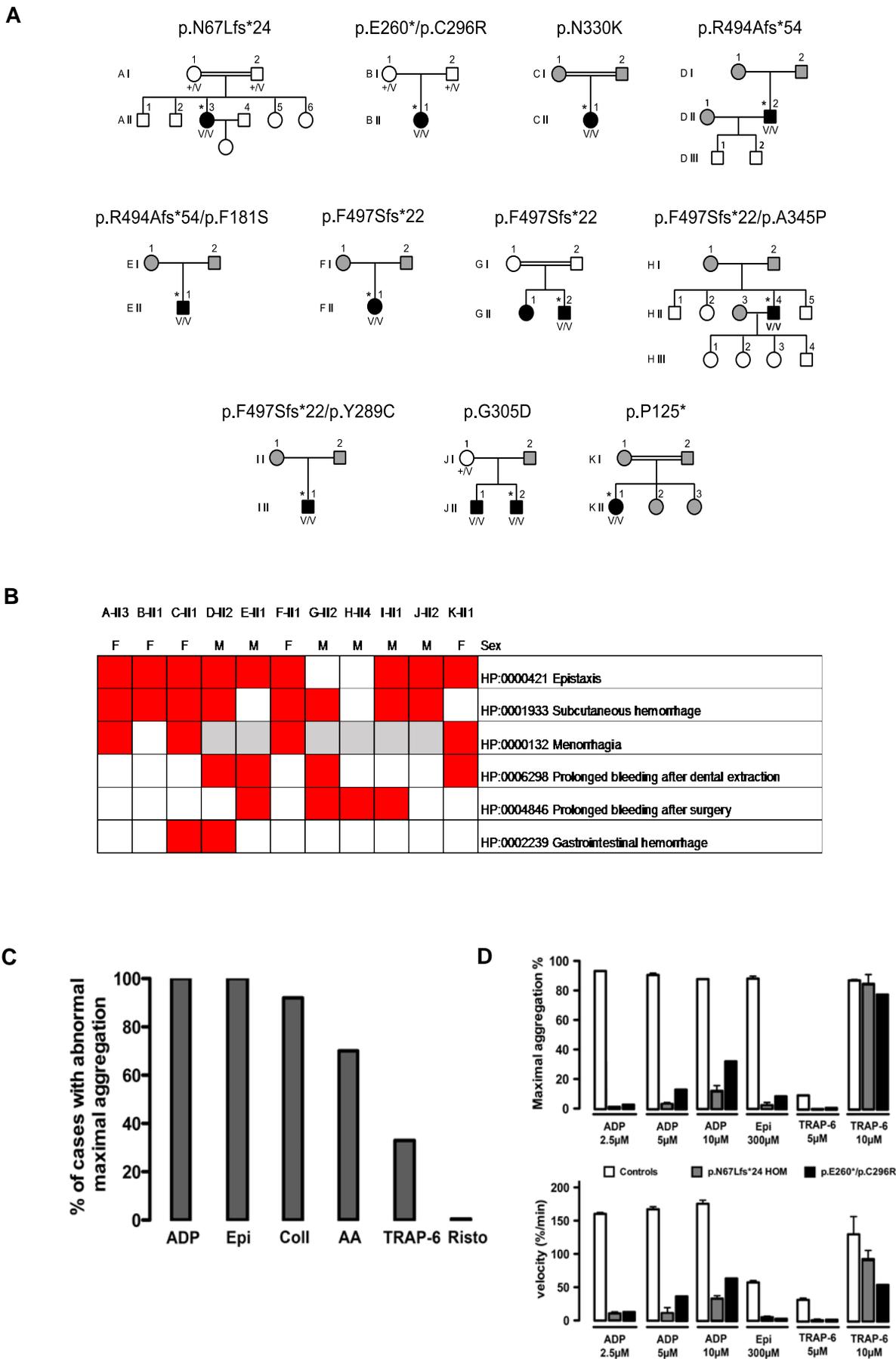


Figure 2





blood[®]

Prepublished online June 21, 2017;
doi:10.1182/blood-2017-03-776773

Expanded repertoire of *RASGRP2* variants responsible for platelet dysfunction and severe bleeding

Sarah K Westbury, Matthias Canault, Daniel Greene, Emilse Bermejo, Katharine Hanlon, Michele P. Lambert, Carolyn M. Millar, Paquita Nurden, Samya G. Obaji, Shoshana Revel-Vilk, Chris Van Geet, Kate Downes, Sofia Papadia, Salih Tuna, Christopher Watt, NIHR BioResource-Rare Diseases Consortium, Kathleen Freson, Michael A. Laffan, Willem H. Ouwehand, Marie-Christine Alessi, Ernest Turro and Andrew D. Mumford

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
<http://www.bloodjournal.org/site/misc/rights.xhtml#reprints>

Information about subscriptions and ASH membership may be found online at:
<http://www.bloodjournal.org/site/subscriptions/index.xhtml>

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include digital object identifier (DOIs) and date of initial publication.