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**Article:**

Fletcher, S.J., Johnson, B., Lowe, G.C. et al. (12 more authors) (2015) SLFN14 mutations underlie thrombocytopenia with excessive bleeding and platelet secretion defects. *Journal of Clinical Investigation*, 125 (9). 3600 - 3605. ISSN 0021-9738

<https://doi.org/10.1172/JCI80347>

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**Consecutive *SLFN14* mutations in 3 unrelated families with an inherited bleeding disorder, thrombocytopenia and secretion defects**

Sarah J. Fletcher,<sup>1</sup> Ben Johnson,<sup>1</sup> Gillian C. Lowe,<sup>1</sup> Danai Bem,<sup>1</sup> Sian Drake,<sup>1</sup> Marie Lordkipanidzé,<sup>1</sup> Isabel Sánchez Guiú,<sup>1,2</sup> Ban Dawood,<sup>1</sup> José Rivera,<sup>2</sup> Michael A. Simpson,<sup>3</sup> Martina E. Daly,<sup>4</sup> Jayashree Motwani,<sup>5</sup> Peter W. Collins,<sup>6</sup> Steve P. Watson<sup>1</sup> and Neil V. Morgan<sup>1</sup> on behalf of the UK GAPP Study Group

<sup>1</sup>Centre for Cardiovascular Sciences, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, UK

<sup>2</sup>Centro Regional de Hemodonación, Universidad de Murcia, IMIB-Arrixaca, Murcia, Spain

<sup>3</sup>Division of Genetics and Molecular Medicine, King's College, London, UK

<sup>4</sup>Department of Cardiovascular Science, University of Sheffield Medical School, University of Sheffield, UK

<sup>5</sup>Department of Haematology, Birmingham Children's Hospital, Birmingham, UK

<sup>6</sup>Arthur Bloom Haemophilia Centre, School of Medicine, Cardiff University, Cardiff, UK.

Address correspondence to:

Dr Neil V. Morgan, Centre for Cardiovascular Sciences, University of Birmingham, Institute of Biomedical Research, Edgbaston, Birmingham, B15 2TT, United Kingdom.

Tel: (+44) 121 414 6820 Fax: (+44) 121 415 8817

e-mail: n.v.morgan@bham.ac.uk

**Counts**

Text: 3496 words; Abstract: 239 words *Figures*: 3 Tables: 1 References: 18

## Abstract

Inherited thrombocytopenias are a rare heterogeneous group of disorders characterised by a low platelet count and sometimes associated with excessive bleeding, ranging from mild to severe. A cohort of 36 patients and 17 family members all displaying a reduced platelet count were recruited to the UK-GAPP (Genotyping and Phenotyping of Platelets) study. All patients had a history of excessive bleeding. Critically, all major known causes of inherited thrombocytopenia (e.g. MYH9-related disorder, Bernard Soulier syndrome and Wiskott-Aldrich syndrome), had been ruled out prior to enrolment. Platelet phenotyping and whole exome sequencing was performed on all patients, resulting in identification of mutations in the novel gene *SLFN14* in 12 patients from three unrelated families. All patients displayed an analogous phenotype of moderate thrombocytopenia, enlarged platelets, decreased ATP secretion ~~upon stimulation with platelet agonists~~ measured by lumiaggregometry and a dominant inheritance pattern. *SLFN14* codes for a protein of unknown function, Schlafen family member 14 (SLFN14). Three heterozygous missense mutations predicting p.K218E, p.K219N, p.V220D substitutions within an ATPase-AAA-4, GTP/ATP binding region were identified in affected but not unaffected family members. Platelets from all three families had a marked reduction in expression of endogenous SLFN14. This corresponded with expression studies in HEK293T cells which demonstrated a significant reduction in all three mutants relative to the wild type protein, suggesting instability. Electron microscopy studies demonstrated a significant reduction in the number of dense granules in ~~patient~~ platelets from affected patients relative to those from healthy volunteers ~~healthy control platelets~~, correlating with a decrease in measured ATP secretion using lumiaggregometry phenotype. Together these results identify

mutations in *SLFN14* as causative for an inherited thrombocytopenia and significant bleeding, outlining a fundamental role for *SLFN14* in platelet formation and megakaryopoiesis.

## Introduction

Inherited thrombocytopenias are a group of heterogeneous disorders associated with bleeding of varying severity depending both on the reduction in platelet count and whether there is associated altered qualitative platelet function (1). The normal range of platelet count in humans is wide ( $150-400 \times 10^9/L$ ), but platelet count is normally maintained within a narrow range for each individual. This homeostasis requires a constant balance between thrombopoiesis, which is primarily controlled by the cytokine thrombopoietin (TPO), and platelet senescence and consumption. Heritable forms of thrombocytopenia are usually caused by mutations in genes involved in platelet production and megakaryocytic differentiation. Just over 20 forms of inherited thrombocytopenia have been described to date in OMIM (<http://www.ncbi.nlm.nih.gov/omim>), however in approximately 50% of patients with an inherited thrombocytopenia a causative gene remains to be identified (2, 3). Identification of such genes is fundamental to providing information on proteins involved in normal platelet physiology and is critical for developing our understanding of disease pathogenesis.

To date the UK-GAPP Study (4) has investigated over 500+ patients ~~displaying platelet dysfunction associated~~ with excessive bleeding of unknown aetiology, and has identified a platelet defect in approximately 60% of participants. A key criterion for recruitment is that known genetic causes of platelet dysfunction have been ruled out on the basis of functional studies and targeted gene sequencing. Platelets from patients recruited to this study undergo extensive phenotypic analysis, including  ~~lumiaggregometry ation~~ to a variety of platelet agonists and detailed analysis of platelet number and morphology. This phenotypic analysis is then followed by Whole Exome Sequencing (WES) which has greatly enhanced the

probability and speed of identifying novel causative gene mutations in such conditions (5-7). In this study, we demonstrate how combined phenotyping and genotyping has enabled us to identify three single nucleotide variations in a novel gene *SLFN14*, predicting substitutions of three consecutive amino acids in *SLFN14*, in three unrelated families displaying a moderate form of congenital thrombocytopenia and a strong bleeding history. In all individuals in which these mutations were found the bleeding history was more severe than would have been predicted on platelet count alone and was associated with a similar reduction in platelet count, reduced aggregation and [ATP](#) secretion to several platelet agonists including ADP, collagen and ~~a~~ PAR-1-activating peptide. This phenotyping and genotyping approach has identified *SLFN14* as causative gene for a new form of [platelet](#) thrombocytopenia.

## Results and Discussion

There are approximately 3,000 patients with platelet function disorders associated with excessive bleeding within the UK Comprehensive Care Haemophilia Centres, of which approximately 10% have a reduced platelet count. A candidate gene mutation has not been identified in approximately 50% of these patients. Over the course of the last 6 years, we have recruited [over 500+](#) patients from 25 ~~(~2/3rds)~~ UK Haemophilia Care Centres with [excessive bleeding and](#) suspected platelet function disorders to the UK-GAPP Study. 60 (13%) of these patients have been classified as having a low platelet count of unknown cause. [The combination of a strong family history of low platelet count and significant bleeding ~~platelet function defect~~ allowed us to prioritise these for gene identification studies.](#) WES was performed and analysis was undertaken in 53 patients (36 index cases) who had reduced platelet

counts ranging from 15-140 x 10<sup>9</sup>/l. Family A had 9 affected members over three generations with a strong history of bleeding and moderate thrombocytopenia with platelet counts ranging from 74-140 x 10<sup>9</sup>/l (Figure 1A and Table 1). The proband, IV:4, was aged 31 years and had a platelet count of 100x10<sup>9</sup>/l when entered into the study. She had a history of frequent and severe cutaneous bruising, prolonged bleeding from minor wounds, menorrhagia, postpartum haemorrhage and spontaneous muscle haematoma. Several other members of family A also had significant bleeding histories and moderate thrombocytopenia (Table 1).

The gold-standard test for platelet function testing is Born aggregometry, but increased information can be obtained by real-time measurement of secretion of ATP (lumiaggregometry) (8). We tested affected members of family A (III:2, III:3, IV:2, IV:4 and IV:5) and observed reduced aggregation to ADP (10 and 30µM), collagen (3µg/ml and 10µg/ml) and PAR-1 activating peptide (100 µM) with reduced ATP secretion (Figure 2, A-B). The similar platelet phenotype in the affected individuals of family A was consistent with a dominant mode of inheritance.

Sequencing of the exomes of DNA from IV:2, IV:4 and III:3 in family A revealed 22867, 23334 and 23153 sequence variations, respectively. Comparisons with dbSNP build 135, the 1000 Genomes project database and our in-house database (composed of >600 exomes) identified 124, 137 and 128 heterozygous novel variants, respectively. Of these only 8 variants were shared by the 3 affected patients of which 4 were significant including 3 non-synonymous variants and 1 frameshift deletion. Sanger sequencing of these remaining 4 variants in all individuals in family A left two remaining candidate variants in *NEMF* (p.H962Y) and *SLFN14* (p.V220D) that segregated with disease (Supplementary Table 1). The exomes of the further 35 index patients with thrombocytopenia and/or secretion

defects who had been recruited to the UK-GAPP study were scrutinised for novel variants in either the *NEMF* or *SLFN14* genes. Two further heterozygous missense variants were identified in *SLFN14* in affected members of family B (p.K219N) and family C (p.K218E) (Table 1 and Figure 1, A-B). Strikingly, all three missense mutations were predicted to result in substitutions in a three amino acid stretch of the ATPase-AAA-4 domain of the protein encoded by *SLFN14*, schlafen family member 14 (Figure 1B). Furthermore the three *SLFN14* variants are not present in the Exome Aggregation Consortium dataset of 61,486 unrelated individuals sequenced as part of various disease-specific and population genetic studies (<http://exac.broadinstitute.org/gene/ENSG00000236320>) and the latest version of dbSNP138.

Affected members of families B and C had remarkably similar platelet counts and platelet function defects. Family B included three patients, two of whom were recruited to the study (I:2, II:3). The proband in family B (II:3) was 35 years old when recruited to the study and had a platelet count of  $68 \times 10^9/l$  with a history of spontaneous epistaxis starting in childhood. Her mother (I:2) had a platelet count in whole blood of  $83 \times 10^9/L$  with a less severe bleeding history. The platelet count in II:3 was too low for lumiaggregometry (8) and so platelet function was analysed by flow cytometry for P-selectin expression, revealing reduced responses to CRP and PAR-1 peptide (Table 1). In line with this, lumiaggregometry on I:2 revealed deaggregation to ADP, collagen and PAR-1 and reduced ATP secretion (Table 1). These results are similar to those seen in affected members of family A (Figure 2A-B).

The index case in family C, II:2 was aged 3 years at the time of enrolment. His platelet count in whole blood was  $89 \times 10^9/l$  and he was noted to bruise easily from a

young age. The lumiaggregometry findings in this patient are similar to those of other study participants from families A and B with a selective loss of response to ADP, collagen and PAR-1 and normal responses to arachidonic acid (Table 1, Figure 2, Supplementary figure 1).

In humans and mice *SLFN14* is located in a SLFN cluster with other schlafen paralogues (9). Members of the SLFN gene family are highly conserved amongst mammalian species and lower vertebrates, and even in viruses. SLFN family proteins contain a unique motif of unknown function, the “SLFN box” and an AAA domain. The AAA+ domain consists of a P-loop NTPase which is implicated in ATP/GTP binding and hydrolysis (10). The SLFN family members are divided into three groups. SLFN 5, 8, 9, 10 and 14 all belong to group 3, although SLFN14 is unique in containing a putative nuclear localisation RKRRR motif in its C-terminus extension (10). The SLFN family of proteins have been suggested to be critical for a variety of processes including cell-cycle regulation, proliferation and differentiation (10-14), however there is no published data about the function of SLFN14.

Field Code Changed

Rowley et al., 2011 (15) previously described *SLFN14* mRNA expression in human and murine platelets. We also identified expression of *SLFN14* mRNA and protein in immature and mature megakaryocytes derived from CD34<sup>+</sup> hematopoietic progenitor cells isolated from cord blood (Supplementary Figure 2). Expression of SLFN14 protein was confirmed by Western blotting of platelets from 11 healthy individuals, normalized for GAPDH loading control (Supplementary Figure 3). Levels of SLFN14 in healthy volunteers ranged from 1.0 to 2.3 arbitrary units (mean 1.5, 95% CI 1.2 to 1.8).

The effect of the *SLFN14* variants on protein expression in platelets from affected family members was investigated by Western blotting (Figure 3A). Platelet

lysates from carriers of the three *SLFN14* variants showed a 65-80% reduction in SLFN14 protein expression when compared to control platelets (Figure 3 A-B). Patient III:2 from family A expressing the p.V220D mutation demonstrated a reduction in SLFN14 expression to ~24% of control ( $P \leq 0.001$ ), both patients I:2 and II:3 from family B expressing the K219N mutation showed a reduction to 33% ( $P \leq 0.005$ ) and 34% ( $P \leq 0.001$ ) respectively, and patient II:2 from family C expressing the K218E mutation showed a decrease to 19% ( $P \leq 0.001$ ). This reduction in SLFN14 levels in all three patients is over 50% despite the heterozygosity suggesting that the mutant gene/protein influences the translation of mRNA or stability of the wild type protein (e.g. due to protein dimerization). Significantly, the reduction in SLFN14 levels in platelets from carriers of the *SLFN14* variants was confirmed in overexpression studies, where despite there being no significant difference in transfection efficiency, average field of view intensity measurements and western blot analysis demonstrated a significant reduction in expression of all SLFN14 variant constructs compared to the wild type construct i.e. SLFN14(K218E)-myc, SLFN14(K219N)-myc and SLFN14(V220D)-myc expression was reduced to 5%, 8% and 52% of SLFN14(WT)-myc expression respectively (Supplementary Figure 4). The much larger reduction in both SLFN14(K218E)-myc and SLFN14(K219N)-myc expression may be a result of increased instability in these mutations in comparison to the SLFN14(V220D)-myc.

Expression of SLFN14(WT)-myc in HEK293T cells revealed a punctate structure localized throughout the cytoplasm, with low level nuclear punctate staining also observed. No significant difference in protein localization was observed between overexpression of all SLFN14 mutants and the wild type construct (Supplementary figure 5).

The morphology of platelets ~~derived~~ from patients carrying heterozygous *SLFN14* mutations was examined by transmission electron microscopy (TEM). Compared with ~~healthy control~~ platelets from healthy volunteers the number of alpha-granules and other organelles present per platelet section showed a small increase but this was not statistically significant (Supplementary Figure 6). A small increase in platelet area was also observed corresponding with data shown in Table 1, however a statistically significant increase was only observed in patient I:2 of Family B ( $P \leq 0.005$ ). When alpha granule number was normalized to account for surface area, no significant difference was observed (Figure 3C and D).

Whole mount electron microscopy was utilized in order to quantify electron dense granules (dense granules) within platelets of both affected patients and healthy ~~control~~ platelets volunteers. A significant reduction in the number of dense-granules was observed in patient platelets from families A and B, patients IV:4 and II:3 respectively (Figure 3E and F). ~~Decreased platelet dense granule content~~ This observation correlates with both the reduced ATP secretion measured by lumiaggregometry (Figure 2). Spreading of platelets on fibrinogen was not significantly different in platelets between affected individuals and healthy controls (Supplementary figure 7).

Finally, we examined megakaryocytes derived from CD34+ cells isolated from the peripheral blood of patients or healthy donors. We saw no significant difference in the level of ploidy between megakaryocytes derived from healthy donors or patients from Family A (patient IV:4) or Family B (patient II:3) (Supplementary figure 8). Proplatelet formation was also examined and a small but significant decrease in the number of megakaryocytes bearing tubulin positive proplatelet-like extensions was reduced from ~20% in control megakaryocytes to ~11% in patient A IV:4, a non-

significant reduction to ~16% was observed in megakaryocytes derived from patient II:3 from family B. This data may be indicative of decreased proplatelet formation in patients carrying heterozygous *SLFN14* mutations.

To further investigate the effect of *SLFN14* on ploidy levels, DAMI cells were treated with siRNAs against *SLFN14* (*SLFN14*-s50927 and *SLFN14*-s50928) or with a non-silencing control (control-siRNA). *SLFN14* mRNA levels were reduced by 50% in DAMI cells for both siRNAs (Supplementary figure 9). However knockdown of siRNA by *SLFN14* did not effect ploidy levels after 72 hours when compared with control cells (control-siRNA transfection), corresponding with findings in patient derived megakaryocytes.

In summary we report 3 unrelated families with a dominantly inherited moderate thrombocytopenia with more severe bleeding than would have been predicted from their platelet count alone. We have used a combination of extensive platelet phenotyping and WES to identify three mutations in a novel gene, *SLFN14* that underlies a moderate thrombocytopenia and platelet secretion defects. The patients have a distinct platelet phenotype with loss of responses to ADP, collagen and PAR1. Together the reduced aggregation to ADP, collagen and PAR-1 may points towards a defect in the G<sub>i</sub> signalling pathway. A reduction in dense granule secretion may also result in a loss of positive feedback during platelet activation. Patient platelets from affected patients display a relatively normal ultra-structure howeverbut show a significant reduction in dense granule number, correlating with secretorylumiaggregometry data showing decreased ATP secretionand storage. Finally analysis of megakaryocytes isolated from patient peripheral blood show decreased proplatelet formation in comparison to control megakaryocytes. This

study is the first description of a major role for *SLFN14*, in human disease and suggests that it has a critical function in platelet formation and megakaryopoiesis.

## **Methods**

Extensive methods are detailed in supplementary methods.

## **Study approval**

The GAPP study was approved by the National Research Ethics Service Committee West Midlands – Edgbaston (REC reference: 06/MRE07/36) and participants gave written informed consent in accordance with the Declaration of Helsinki. This study was registered at [www.isrctn.org](http://www.isrctn.org) as #ISRCTN 77951167. The GAPP study is included in the National Institute of Health Research Non-Malignant Haematology study portfolio, ref 9858.

## **Acknowledgements**

We thank the families for providing samples and our clinical and laboratory colleagues for their help. This work was supported by the British Heart Foundation (RG/PG/13/36/30275; RG/09/007) and a Wellcome Trust Combined Training Programme Fellowship (093994) (GCL). The authors also acknowledge support from the Department of Health via the National Institute for Health Research (NIHR). We thank the NIHR Non Malignant Haematology Specialty Group for their help in recruiting to the study, and all our clinical investigators and collaborators.

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**Table 1**

Family /Patient ID	<i>SLFN14</i> nucleotide alteration ¥	Effect on <i>SLFN14</i> Protein	Platelet count (x10 <sup>9</sup> /L) <sup>^</sup>	Mean Platelet Volume (fL) (7.83-10.5) <sup>^</sup>	ISTH BAT score $\psi$	Lumiaggregometry/ Flow cytometry defects	ATP Secretion nmol/1 x10 <sup>8</sup> platelets*
Family A; II:1	c.659 T>A	p.Val220Asp	NA	NA	NA	NA	NA
Family A; III:2	c.659 T>A	p.Val220Asp	140	9.1	5	ADP (10, 30, 100 $\mu$ M) collagen (1, 3 $\mu$ g/ml) PAR-1 (100 $\mu$ M)	0.81
Family A; III:3	c.659 T>A	p.Val220Asp	74	10.4	10	ADP (10 $\mu$ M) collagen (3 $\mu$ g/ml) PAR-1 (30 $\mu$ M)	0.42
Family A; IV:2	c.659 T>A	p.Val220Asp	110	9.3	13	ADP (10, 30 $\mu$ M) collagen (1, 3 $\mu$ g/ml) PAR-1 (100 $\mu$ M)	ND
<b>Family A; IV:4</b>	c.659 T>A	p.Val220Asp	100	11.1	22	ADP (30 $\mu$ M), collagen (3 $\mu$ g/ml) PAR-1 (100 $\mu$ M)	0.28
Family A; IV:5	c.659 T>A	p.Val220Asp	116	11.2	21	ADP (10 $\mu$ M) collagen (3 $\mu$ g/ml) PAR-1 (30 $\mu$ M)	0.48
<b>Family B; II:3</b>	c.657 A>T	p.Lys219Asn	68	11.9	20	Flow cytometry reduced responses to high conc. CRP and PAR-1	NA
Family B; I:2	c.657 A>T	p.Lys219Asn	83	11.9	13	ADP (10, 30 $\mu$ M), collagen (3, 10 $\mu$ g/ml) PAR-1 (100 $\mu$ M)	0.63
<b>Family C; II:2</b>	c.652 A>G	p.Lys218Glu	89	13.0	NA	ADP (10, 100 $\mu$ M) collagen (1, 3 $\mu$ g/ml) PAR-1 100 $\mu$ M	0.12

Heterozygous nucleotide changes present in *SLFN14* and their predicted effects on the resulting protein are shown. Index cases are indicated in bold font; ¥ Alterations are numbered according to positions in the NM\_001129820; <sup>^</sup>Mean platelet counts are shown, normal reference range is 150-400 x 10<sup>9</sup> platelets/L, thrombocytopenia is defined as platelet count <150 x 10<sup>9</sup> platelets/L;  $\psi$  ISTH

bleeding assessment tool score, 95<sup>th</sup> percentile (score of 4) calculated from healthy volunteers (16);  
\*ATP secreted in response to 100  $\mu$ M PAR-1 receptor specific peptide SFLLRN, 5th centile in healthy  
volunteers is 0.82 nmol /  $1 \times 10^8$  platelets. ND= not detectable. NA= not available

## Figures and legends

### Figure 1

**Figure 1. Identification of *SLFN14* mutations in 3 unrelated families with a dominant form of thrombocytopenia.** (A) Pedigrees from 3 families with moderate thrombocytopenia. Affected individuals are shaded, in some individuals the platelet count is not known (?). Asterisks indicate those patients whose exomes were sequenced. Representative Sanger sequencing electropherograms confirming the presence of the *SLFN14* mutations in patients are shown below the relevant families. Black arrows indicate the nucleotide change. *SLFN14* mutation status is shown for individuals that were genotyped as +/- for heterozygous state or -/- for wild type state (B) Linear domain organisation of *SLFN14* protein showing the amino acid position of each of the 3 different missense *SLFN14* mutations (K218E, K219N, V220D) located in the ATPase-AAA-4 domain and conservation of the protein in higher order species.



## Figure 2

**Figure 2. Platelet phenotyping of patients from the 3 families with *SLFN14* mutations** (A) Impaired aggregation in PRP from patients represented from of each of the 3 families (Family A; IV:4, Family B; III:3, Family C; II:2 and representative control) following lumiaggregometry performed on native undiluted PRP to assess percentage aggregation after stimulation with ADP (10 $\mu$ M), collagen (3 $\mu$ g/ml) and PAR1-peptide (100 $\mu$ M). (B) Reduced ATP secretion from dense granules in representative patients from each of the 3 families using Chronolume<sup>®</sup> after stimulation with PAR1-peptide (100 $\mu$ M). (C) Flow cytometric assessment of platelet function in PRP from patient III.2 from family A. Responses to different agonists are determined using anti-CD62P. Data for healthy volunteers shown as mean  $\pm$  SD (n=9). Isotype control = IgGk1. Data for healthy volunteers 1 in 3 dilution (with PBS) shown to demonstrate expected effect of moderate thrombocytopenia alone on this assay.

### Figure 3

**Figure 3. Functional characterisation of SLFN14 in patient platelets.** (A) Western blot analysis of both healthy control and patient platelet lysates, demonstrating significantly decreased SLFN14 protein levels in platelets from carriers of the *SLFN14* variants when compared with platelets from healthy individuals. (B) Quantification of 4 repeats of the experiments shown in A, \*\* $P \leq 0.005$  and \*\*\* $P \leq 0.001$  when compared to control. (C) Transmission electron micrographs of patient platelets showing normal morphology in comparison to healthy control platelets. (D) Quantification of (C) demonstrating no significant difference in alpha granule number per  $\mu\text{m}^2$  between patient and healthy control platelets. (E) [Quantification of whole mount EM images of patient/healthy donor platelets](#) (F) [showing a reduction in the number of dense granules in patient-platelets from affected patients in comparison to those from healthy volunteer controls, \\* \$P \leq 0.05\$  and \\*\\*\\* \$P \leq 0.001\$  when compared to control.](#) At least 40 platelets analysed per patient/healthy ~~volunteerecontrol~~. All values are mean  $\pm$  SD.

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