“Genetics of inherited platelet disorders - The GAPP study”

Dr Neil Morgan, University of Birmingham (UK)  
Chief Investigator GAPP study
2,426 patients registered with UKHCDO as having a platelet disorder (other than Glanzmann’s thrombasthenia and Bernard Soulier disease). Additional approx 258 registered as miscellaneous / unclassifiable.
Clinical Platelet Research

- **Bleeding phenotype** variable – more than one defect may exist in some patients
- **Symptoms vary** between individuals
- **Platelets testing** is complicated and not always reproducible
- “Gold standard” of **light transmission aggregometry** not always available in clinical environment
- **Rare disorders** requiring **multi-centre studies** for success
Genotyping And Phenotyping of Platelets (GAPP) study

- rare disorders need multiple centres: > 25 Haemophilia Centres in UK
- Recruitment based on clinical diagnosis of mild bleeding of unknown cause
- Platelet dysfunction symptoms: menorrhagia, epistaxis, bleeding following invasive procedures e.g. tooth extractions & surgery
- >1000 participants recruited to date
GAPP - Patient groups studied

Group 1 - Clinically diagnosed mild bleeding with a normal platelet count
• (Platelet count $>150 \times 10^9$/L in whole blood)

Group 2 - Thrombocytopenia (inherited)
• (Platelet count $<150 \times 10^9$/L in whole blood)

Group 3 - Menorrhagia
Clinical and Bleeding History

- Filling in and sign a consent form

- Bleeding Assessment Tool (BAT) - ISTH

<table>
<thead>
<tr>
<th>Type of bleeding</th>
<th>Age where the bleeding symptoms started</th>
</tr>
</thead>
<tbody>
<tr>
<td>✓ Epistaxis</td>
<td>✓ Before 1 year</td>
</tr>
<tr>
<td>✓ Cutaneous bleeding</td>
<td>✓ Between 1-5 years of age</td>
</tr>
<tr>
<td>✓ Bleeding after circumcision</td>
<td>✓ Between 6-12 years of age</td>
</tr>
<tr>
<td>✓ Bleeding after tooth extraction</td>
<td>✓ Between 13-25 years of age</td>
</tr>
<tr>
<td>✓ Bleeding after surgery</td>
<td>✓ After 25 years of age</td>
</tr>
<tr>
<td>✓ Prolonged menorrhagia</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of bleeding episodes</th>
<th>Medical intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>✓ Less than 1 per year</td>
<td>✓ No medical attention required</td>
</tr>
<tr>
<td>✓ 1-5 every six month</td>
<td>✓ Consultation only</td>
</tr>
<tr>
<td>✓ 1-3 every month</td>
<td>✓ Required treatment</td>
</tr>
<tr>
<td>✓ 1 every week</td>
<td>• Cauterisation/packing</td>
</tr>
<tr>
<td></td>
<td>• Antifibrinolytics/ iron therapy</td>
</tr>
<tr>
<td></td>
<td>• Plasma/ RBC replacement</td>
</tr>
</tbody>
</table>
Pre-screening of other bleeding disorders

- Screening for non platelet disorders
  - Coagulation screen
  - Factors assay
  - von Willebrand disease assay

- Screening for severe forms of Platelet abnormality
  - Glanzmann's Thrombasthenia
  - Bernard–Soulier syndrome
Platelet Defects

Abnormalities of platelet aggregation

Signalling pathway defects

Abnormalities of dense granule secretion

Cytoskeleton Abnormalities

Abnormalities of platelet procoagulation activity

Abnormalities of platelet adhesion

Abnormalities of alpha granule secretion
GAPP - Methodologies

Remote Platelet function test

Multiplate ®

Aggregation/Flow Cytometry

Platelet protein - Western Blotting

Buffy coat - genomic DNA

SYSMEX: Blood counter
GAPP – Platelet Phenotyping

Phenotyping based on >800 participants (100+ have low platelet count and bleeding)
Thrombocytopenia Gene mutation identification benefits

(i) aids early diagnosis
(ii) prevent unnecessary investigations/treatment e.g. splenectomies, steroids, immunosuppression, monitoring of blood malignancies

• Provide information on platelet regulation and function

• Guide new treatment strategies for
  (i) Patients with low platelet counts and bleeding
  (ii) Patients at risk of thrombosis
Defective megakaryocyte differentiation/maturation

ANKRD26
ETV6
FLI1
FYB
GATA1
GFI1B
HOXA11
MPL
NBEAL2
RBM8A
RUNX1
THPO

Defective proplatelet formation/release

ACTN1
CYCS
DIAPH1
FLNA
GP1BA/GP1BB/GP9
ITGA2B/ITGB3
MKL1
MYH9
PRKACG
TUBB1
WAS

Defective proplatelet formation/release

HSC

proliferation

HSC

HSC

Defective proplatelet formation/release

ACTN1
CYCS
DIAPH1
FLNA
GP1BA/GP1BB/GP9
ITGA2B/ITGB3
MKL1
MYH9
PRKACG
TUBB1
WAS

Johnson et al (2016) Platelets
Whole Exome Sequencing

Alignment to reference human genome sequence

Novelty determined by comparison of variants against ExAC, EVS, 1000, GAPP database (>1200 whole exomes)

358 genes known/associated with platelet count / function, coagulation or endothelial cell function

Exclude MAF ≥ 0.01, Synonymous changes, Splice site variants > 4 base pairs, not shared in additional affected family members

Remove all non-novel variants

Removal Synonymous variants, Splicing > 4 base pairs

Compare to exomes from relatives

Exclude all non-shared variants

Predict Pathogenicity

PolyPhen2, Provean, SIFT, Mutation Taster, mRNA expression levels

Candidate Gene Defects
Whole Exome Sequencing on 69 patients

• 119 fold average coverage across all patients, 92% average over 20x coverage

• Between 24,000 and 26,000 variants per patient

• Average of 2,531 variants per patient with MAF <0.01
  • (excluding synonymous variants)

• Average of 201 novel variants per patient

• Average of 186 copy number variants per exome (n=44, range= 63-421)

66% (31/47) index cases with proposed genetic aetiology in IT related genes

4 variants in novel candidate genes (ANKRD18A, MKL1, PF4 and SLFN14)
High prevalence of variants in RUNT-related transcription factor 1 (RUNX1)

- **13 variants** found within RUNX1 in 10 index cases

- Majority of patients (10/13) have a secondary qualitative defect in secretion

- No haematological malignancies have been reported to date in any patients
The discovery of variants in 4 novel candidate genes

- **Seven novel variants** were found in **four novel genes**; ANKRD18A, MKL1, PF4, and **SLFN14**

- All variants segregate within all affected family members

- All variants are predicted to be disease causing and are expressed in cells of the platelet/megakaryocyte lineage

<table>
<thead>
<tr>
<th>Family</th>
<th>Gene</th>
<th>Variant</th>
<th>PhyloP</th>
<th>Mutation taster</th>
<th>SIFT</th>
<th>Provean</th>
<th>PolyPhen-2</th>
<th>Pathogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>ANKRD18A</td>
<td>c.2396_2398del homo</td>
<td>0.772</td>
<td>p</td>
<td>NA</td>
<td>-9.4</td>
<td>NA</td>
<td>+/-</td>
</tr>
<tr>
<td>10</td>
<td>MKL1</td>
<td>c.G1723A</td>
<td>3.358</td>
<td>d</td>
<td>0.003</td>
<td>-2.2</td>
<td>d</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>MKL1</td>
<td>c.C554T</td>
<td>3.534</td>
<td>d</td>
<td>0.033</td>
<td>-3.96</td>
<td>b</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>PF4</td>
<td>c.33delC</td>
<td>-0.324</td>
<td>d</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>+/-</td>
</tr>
<tr>
<td>23</td>
<td>SLFN14</td>
<td>c.A652G</td>
<td>2.338</td>
<td>p</td>
<td>0.567</td>
<td>-0.65</td>
<td>d</td>
<td>+/-</td>
</tr>
<tr>
<td>24</td>
<td>SLFN14</td>
<td>c.A657T</td>
<td>0.852</td>
<td>p</td>
<td>0.158</td>
<td>-0.28</td>
<td>d</td>
<td>+/-</td>
</tr>
<tr>
<td>25</td>
<td>SLFN14</td>
<td>c.T659A</td>
<td>2.336</td>
<td>p</td>
<td>0.001</td>
<td>-3.49</td>
<td>pd</td>
<td>+/-</td>
</tr>
</tbody>
</table>
Large family with inherited thrombocytopenia

Multiple affected individuals.

Reduced platelet count in multiple family members.

Dominant inheritance pattern

Normal platelet count: 150-450x10^9/l

Isabel Sánchez Guiú/Ben Johnson
Clinical features of index case

Index Case : Family A IV:4

31 year old woman
Platelet count 100x10^9/l
Cutaneous bruising
Prolonged bleeding (minor wounds and after tooth extraction)
Menorrhagia
Postpartum hemorrhage
Spontaneous muscle haematoma

Platelet transfusion
Antifibrinolytics
Uterine packing after postpartum haemorrhage
Red blood cell transfusions
Iron Therapy

Normal platelet count: 150-450x10^9/l
Clinical features of index case

Index Case : Family A IV:4

31 year old woman
Platelet count 100x10^9/l
Cutaneous bruising
Prolonged bleeding (minor wounds and after tooth extraction)
Menorrhagia
Postpartum hemorrhage
Spontaneous muscle haematoma

Bleeding disproportionate for platelet count
Significant family history with several similarly affected individuals
Whole Exome Sequencing – 3 affected patients

II:1

III:2 140x10^9/l

IV:2 110x10^9/l

III:3 74x10^9/l

IV:4 100x10^9/l

IV:5 116x10^9/l

Isabel Sánchez Guiú/Ben Johnson
## Genetic variants identified in large family

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total variants</td>
<td>22,867</td>
<td>23,334</td>
<td>23,153</td>
</tr>
<tr>
<td>*Novel or Rare variants</td>
<td>124</td>
<td>137</td>
<td>128</td>
</tr>
<tr>
<td>Overlapping variants</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>minus synonymous</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>variants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Segregation analysis</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>(5 patients and 1 unaffected)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* novel = not in dbSNP 134, 1000 genomes project, in-house 600 exomes
SLFN14 mutations cause bleeding and platelet function defects

3 unrelated families each with a heterozygous SLFN14 variant

Fletcher et al 2015 JCI
All patients show platelet dysfunction in addition to thrombocytopenia.
SLFN14 protein and mutations

3 consecutive mutations identified in the AAA-4 ATPase domain of SLFN14 in affected individuals from 3 unrelated families.
Patient platelets have significantly decreased SLFN14 protein levels
4th SLFN14 mutation identified

SLFN14-related thrombocytopenia: identification within a large series of patients with inherited thrombocytopenia

Caterina Marconi1; Christian A. Di Buduo2; Serena Barozzi3; Flavia Palombo1; Simonetta Pardini4; Carlo Zaninetti3; Tommaso Pippucci1; Patrizia Noris3; Alessandra Balduini2,5; Marco Seri1; Alessandro Pecchi3

1Department of Medical and Surgical Science, Policlinico Sant’Orsola Malpighi and University of Bologna, Bologna, Italy; 2Department of Molecular Medicine, University of Pavia, Pavia, Italy; 3Department of Internal Medicine, IRCCS Policlinico San Matteo Foundation and University of Pavia, Pavia, Italy; 4Hematology Institute, University Hospital of Sassari, Sassari, Italy; 5Department of Biomedical Engineering, Tufts University, Bedford, Massachusetts, USA

p.Arg223Trp

<table>
<thead>
<tr>
<th>Patient</th>
<th>Amino Acid Residue</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homo sapiens</td>
<td>208</td>
<td>THVEFKRFTTKKVLPRIKEMLPHYSAFANTQGGYVLIGVDDKSKSEVGC</td>
</tr>
<tr>
<td>Pan troglodytes</td>
<td>208</td>
<td>THVEFKRFTTKKVLPRIKEMLPHYSAFANTQGGYVLIGVDDKSKSEVGC</td>
</tr>
<tr>
<td>Macaca mulatta</td>
<td>208</td>
<td>THVEFKRFTTKKVLPRIKEMLPHYSAFANTQGGYVLIGVDDKSKSEVGC</td>
</tr>
<tr>
<td>Canis lupus</td>
<td>208</td>
<td>THVEFKRFTTKKVLPRIKETLASHYSAFANTQGGYILLGVDDKSKSEVGC</td>
</tr>
<tr>
<td>Boa taurus</td>
<td>206</td>
<td>THVEFKRFTKLLPRTKEMLPHYVSFAINTQGGYLLILGVDDKSKSEVGC</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>197</td>
<td>THVEFKRFTKIKLYPIKETLAHYVSFAINTQGGYIIIIGVDDKSKSEVGC</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>299</td>
<td>THVEFKRFTKIKLYPIKETLAHYVSFAINTQGGYIIIIGVDDKSKSEVGC</td>
</tr>
</tbody>
</table>

** END **
Characterization of Novel Ribosome-Associated Endoribonuclease SLFN14 from Rabbit Reticulocytes

Vera P. Pisareva,*† Ilham A. Muslimov,‡ Andrew Tcherepanov,‡ and Andrey V. Pisarev*†

†Department of Cell Biology and ‡Department of Physiology and Pharmacology, SUNY Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, New York 11203, United States

Pisareva et al 2015 Biochemistry
SLFN14 mediates degradation of ribosomal RNA (2)

New pathway for platelet production/megakaryocyte development

Fletcher et al 2018 RNA
CRISPR - Investigation of patient mutations

GAPP

in silico design of guide RNA and Donor Template

Assembly of Donor Vectors and guide Constructs

Transfection and Single Colony Isolations

Screening Clones for Off-Target Effects

Clonal Population Carrying Patient Mutations for Investigation

Generate Endogenously Expressed Fusion Protein of Interest

Introduce Patient Genotypes to Cells Expressing Fluorescent Protein

Khan et al 2017
“No defects” patient group

- No Defect: 59%
- Gi Defect: 7%
- P2Y12 Defect: 6%
- Secretion Defect: 15%
- Cox Defect: 9%
- Other Defects: 4%
‘No defect’ GAPP patients

- Rare coagulation genes
- Endothelial defects
- Platelet defects missed

p.Cys537Stop truncation mutation in the carboxyl-terminal transmembrane helix
Thrombomodulin (TM) is shed from the endothelial surface into plasma

Mother and son recruited *
Normal platelet counts
Both have lifelong bleeding tendency especially post-traumatic bleeding

Maclachlan et al 2017
Dargaud et al. 2015,
Langdown et al. 2014
Conclusion and Future studies

• Combination of extensive phenotyping coupled with genotyping gives us a novel and unique approach to diagnosis
• Discovery both **known** and **novel** genetic variants
• 100,000K genome project (Genomics England)
• Important to interrogate other genetic databases e.g. BRIDGE, thrombogenomics
• Development gene editing/CRISPR and iPSC pipeline
Acknowledgements

UNIVERSITY OF BIRMINGHAM
Dr Sarah Fletcher
Mr Ben Johnson
Mr Abdullah Khan
Annabel Maclachlan
Mr Rashid Al Ghaithi
Mr Ibrahim Almazni
Prof Steve Watson
Dr Steve Thomas
Dr Paul Harrison
Dr Gillian Lowe
Jane Futterer
Isabel Sánchez Guiu
Sian Drake
David MacDonald
Dr Danai Bem
Dr Marie Lordipanidzé
Ban Dawood

The University Of Sheffield.
Dr Martina Daly
Prof Michael Makris
Dr Vincenzo Leo
Dr Jeanette Payne

University of BRISTOL
Dr Stuart Mundell
Dr Andrew Mumford

Dr Michael Simpson
Prof Paul Gissen
Jose Riviera

Dr David Allsup
Dr Tina Bliss
Dr Paula Bolton-Maggs
Dr Peter Collins
Dr Nicola Curry
Dr Charlotte Grimley
Dr Beki James
Dr Jayashree Motwani
Dr Sue Pavord
Dr Katherine Talks
Dr Jecko Thacil
Dr Jonathan Wilde
Dr Mike Williams
Dr Bethan Myers
Dr Angela Thomas
Dr Will Lester
Dr Justin Clark
Dr Richard Gooding
Dr Gerry Dolan
Dr Simone Stokley
Dr Emma Astwood
Dr Cherry Chang
Dr Charlie Hay
Dr Gillian Evans
Inherited thrombocytopenias: the Italian experience
Joint seminar with the Centre for Rare Diseases

Professor Anna Savoia
University of Triste, Italy

Hosts: Dr Neil Morgan
N.V.Morgan@bham.ac.uk

Thursday 24th May
16.00 – 17.00pm
IBR Seminar Room N143
SLFN14 mediates degradation of ribosomal RNA

**SLFN14(WT)-Myc**

**5.8S**

Solid white line shows cells not transfected

Dashed white line indicates cells expressing SLFN14
Colocalisation of SLFN14 & ribosomal subunits

**SLFN14(WT)-anti-myc**

**anti-ribosomal RNA 5.8s**

**TO-PRO-3 Iodide**

**co-localisation**