ISTH Training Course
Genetic testing in thrombocytopenia
Dr Neil Morgan, University of Birmingham (UK)
Disclosures for Neil Morgan

In compliance with COI policy, ISTH requires the following disclosures to the session audience:

<table>
<thead>
<tr>
<th>Category</th>
<th>No relevant conflicts of interest to declare</th>
</tr>
</thead>
<tbody>
<tr>
<td>Research Support/P.I.</td>
<td></td>
</tr>
<tr>
<td>Employee</td>
<td></td>
</tr>
<tr>
<td>Consultant</td>
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<tr>
<td>Major Stockholder</td>
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<tr>
<td>Speakers Bureau</td>
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<tr>
<td>Honoraria</td>
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<tr>
<td>Scientific Advisory Board</td>
<td></td>
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</tbody>
</table>

Presentation includes discussion of the following off-label use of a drug or medical device:

<N/A>
Contents:

PART 1

- Introduction to thrombocytopenia
- Overview gene defects in known genes
- Exome/Targetted Next Generation Sequencing as a tool to identify genetic variants

PART 2

- Our experience – the GAPP Project
What is inherited thrombocytopenia?

- Normal platelet count is between $150-450 \times 10^9/l$
- Platelet count is maintained by a regulated harmony between megakaryopoiesis/thrombopoiesis, platelet senescence and platelet consumption and destruction
- Thrombocytopenia is classed platelet count $<150 \times 10^9/l$ and can lead to bleeding complications due to a hypocoagulability
- Thrombocytopenia can be acquired or inherited
- Average incidence of inherited thrombocytopenia = 2.7 per 10,000 live births (Balduini @ESHG2014)
### 30+ Thrombocytopenia associated genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Disease</th>
<th>Platelet size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG5</td>
<td>Thrombocytopenia associated with sitosterolaemia</td>
<td>Normal</td>
</tr>
<tr>
<td>ABCG8</td>
<td>Bleeding disorder, platelet-type 15</td>
<td>Normal</td>
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<tr>
<td>ACTN1</td>
<td>Thrombotic thrombocytopenia purpura, Upshaw-Schulman syndrome</td>
<td>Normal</td>
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<tr>
<td>ADAMTS13</td>
<td>ANKRD26-related thrombocytopenia (THC2)</td>
<td>Normal</td>
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<tr>
<td>CYCS</td>
<td>CYCS-related thrombocytopenia</td>
<td>Micro</td>
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<tr>
<td>ETV6</td>
<td>THC5</td>
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<tr>
<td>FLI1</td>
<td>Paris Trousseau type thrombocytopenia/Jacobsen (11q23 del)</td>
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</tr>
<tr>
<td>FLNA</td>
<td>FLNA related thrombocytopenia</td>
<td>Normal</td>
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<tr>
<td>FYB</td>
<td>Novel thrombocytopenia</td>
<td>Micro</td>
</tr>
<tr>
<td>GATA1</td>
<td>GATA1 related disease (XLT and XLTT)</td>
<td>Normal</td>
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<tr>
<td>GFI1B</td>
<td>Grey Platelet Syndrome + novel thrombocytopenia</td>
<td>Normal</td>
</tr>
<tr>
<td>GP1BA</td>
<td>Bernard Soulier Syndrome + Platelet type von-Willebrand disease</td>
<td>mono = normal</td>
</tr>
<tr>
<td>GP1BB</td>
<td></td>
<td>biallelic = macro</td>
</tr>
<tr>
<td>GP9</td>
<td>Amegakaryocytic thrombocytopenia with radio-ulnar synostosis</td>
<td>Normal</td>
</tr>
<tr>
<td>ITGA2B</td>
<td>ITGA2B/ITGB3-related thrombocytopenia</td>
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<tr>
<td>ITGB3</td>
<td>Congenital amegakaryocytic thrombocytopenia</td>
<td>Micro</td>
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<tr>
<td>MPL</td>
<td>MYH9 related disease</td>
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<tr>
<td>MYH9</td>
<td>Grey Platelet Syndrome</td>
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<tr>
<td>NBEAL2</td>
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<td>Normal</td>
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<tr>
<td>PRKACG</td>
<td>Bleeding disorder, platelet-type 19</td>
<td>Macro</td>
</tr>
<tr>
<td>RBM8A</td>
<td>Thrombocytopenia with absent radii</td>
<td>Micro</td>
</tr>
<tr>
<td>RUNX1</td>
<td>Familial platelet disorder and predisposition to AML</td>
<td>Normal</td>
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<tr>
<td>STIM1</td>
<td>Stormorken syndrome</td>
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<td>TPM4</td>
<td>Novel thrombocytopenia</td>
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<td>TUBB1</td>
<td>TUBB1-related macrothrombocytopenia</td>
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<td>vWF</td>
<td>Von-Willebrand type disease 2B</td>
<td>Macro</td>
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<tr>
<td>WAS</td>
<td>Wiskott-Aldrich syndrome, X-linked thrombocytopenia</td>
<td>Micro</td>
</tr>
</tbody>
</table>
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

Proliferation

- HSC

Cytoplasmic maturation

- Progenitors

Preplatelets

- HSC

Platelet production

- Immature
  - Megakaryocytes

Other

- ABCG5/8
- ADAMTS13
- STIM1
- VWF

Senescence/consumption/death/other

Johnson et al (2016) Platelets
Inherited thrombocytopenia in 160 families (500 patients) in Pavia

DNA-based diagnosis in patients with thrombocytopenia

- Sanger sequencing of identified genes
e.g. Bernard Soullier syndrome (GP1b-IX-V complex genes)

- Whole exome sequencing and consanguinity
- Grey platelet syndrome ($NBEAL2$)

- Whole exome sequencing in non-consanguineous families
- penetrance (autosomal dominant)

- no penetrance (combination of mutations?)
DNA-based diagnosis in patients with thrombocytopenia

• Sanger sequencing of identified genes
e.g. Bernard Soullier syndrome (GP1b-IX-V complex genes)

• Whole exome sequencing and consanguinity
  - Grey platelet syndrome (NBEAL2)
    *Albers et al (2011) Nature Gen. 43, 735-737*

• Whole exome sequencing in non-consanguineous families
  - penetrance (autosomal dominant)

• no penetrance (combination of mutations?)
Next Generation/Whole exome sequencing

<table>
<thead>
<tr>
<th></th>
<th>HiSeq2000</th>
<th>HiSeq2500</th>
<th>MiSeq</th>
<th>MiSeq</th>
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<tbody>
<tr>
<td>Reads</td>
<td>2 x 100</td>
<td>2 x 100</td>
<td>2 x 250</td>
<td>2 x 100</td>
</tr>
<tr>
<td>Yield</td>
<td>600 Gb</td>
<td>120 Gb</td>
<td>1 Gb</td>
<td>1 Gb</td>
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<tr>
<td>Run time</td>
<td>10 days</td>
<td>1.5 days</td>
<td>&lt;2 days</td>
<td>&lt;1 day</td>
</tr>
</tbody>
</table>

Applications: Whole Genomes → Targeted Tests
Generating an Exome Profile – Sample Preparation

**DNA fragmentation**
- Genomic DNA
- Ultrasonic shearing
- Fragmented DNA ~150bp

**Library preparation**
- End repair and ‘A’ overhang
- Adaptor ligation

**Sequencing**
- 76bp Paired end sequencing
- Low cycle PCR amplification
- 3-7Gb Sequence

**Exome capture**
- Exome library
- 24 hour hybridisation
- Untargeted regions
- Biotinylated RNA oligos
Generating an exome profile – Analysis

**Sequence Read Alignment**
- Aligned reads
- Reference genome

**Target Coverage Reporting**
- Capture efficiency
- Coverage

**Variant Annotation**
- Chr1:37,373,998C>T
- c.328C>T
- p.Leu110Phe
- dbSNP
- 1000genomes
- SIFT
- PolyPhen

**Variant Calling**
Data Analysis Pipeline

Reference Genome

hg18 fasta

Novoindex

Novoalign

SAM file

SAM tools QC

BAM file

BEDtools

Coverage report

Capture report

Sequence Reads

fastq file

fastq file

Genes

Exon coordinates

Transcript ranges

Variants

dbSNP

1000 Genomes

Collaborator’s variants

‘in house’ database

Genome annotations

Homozygous variants

var QC

Variant pileup

Heterozygous variants

Variant Classifier

Fully Annotated List of variants

‘novel’ variants
IGV viewer – Identification of a *RUNX1* variant


Total count: 21 reads
A : 0
C : 12 (57%, 1+, 11-) WT
G : 0
T : 9 (43%, 1+, 8-) MUT
N : 0
1) Consanguineous families

2) Dominant families with >2 affected members

3) Isolated/Sporadic individuals – well phenotyped!
Consanguineous family with inherited thrombocytopenia

- Age 7
  - Platelet count is 15–20x10^9/l
  - Weekly HLA matched platelet transfusions

- Age 3
  - Intraventricular haemorrhages at birth
  - Platelet count is 10x10^9/l
  - Multiple HLA matched platelet transfusions
Exome sequencing – 2 affected patients

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total variants</td>
<td>24,601</td>
<td>25,020</td>
</tr>
<tr>
<td>Homozygous variants</td>
<td>675</td>
<td>695</td>
</tr>
<tr>
<td>Homozygous and novel *</td>
<td>71</td>
<td>80</td>
</tr>
<tr>
<td>Homozygous, non-synomonous new novel variants shared</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

* novel = not in dbSNP 134, in house >250 exomes, 1000 genomes project
<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position (bp)</th>
<th>Human gene</th>
<th>Variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>36,217,280</td>
<td>GNE</td>
<td>p.G416R</td>
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<tr>
<td>9</td>
<td>37,727,217</td>
<td>FRMPD1</td>
<td>p.A509V</td>
</tr>
<tr>
<td>9</td>
<td>37,730,726</td>
<td>FRMPD1</td>
<td>p.P734L</td>
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<td>FRMPD1</td>
<td>p.A1025T</td>
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<td>p.N56H</td>
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<td>p.R18P</td>
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<td>EXOSC3</td>
<td>p.R18C</td>
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<tr>
<td>9</td>
<td>38,567,995</td>
<td>ANKRD18A</td>
<td>p.Glu801del</td>
</tr>
<tr>
<td>Chromosome</td>
<td>Position (bp)</td>
<td>Human gene</td>
<td>Variant</td>
</tr>
<tr>
<td>------------</td>
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<td>38,567,995</td>
<td>ANKRD18A</td>
<td>p.Glu801del</td>
</tr>
</tbody>
</table>
Exome candidates segregation analysis in all family members

- **GNE** c.G1246A; p.G416R
- **FRMPD1** c.C1526T; p.A509V
- **ANKRD18A** c.2401_2403delGAA; p.Glu801del
3 Candidate variants

- GNE  p.G416R
- UDP-N-acetylglucosamine 2-epimerase
- heterozygous mutations cause a rare inborn error of metabolism, sialuria.
- homozygous GNE mutations cause an autosomal recessive Inclusion Body Myopathy-2 and Nonaka Myopathy.

- FRMPD1  p.A509V
- \textit{FRMPD1} encodes a FERM and PDZ domain containing 1: however, the Alanine 509 residue is not conserved throughout different species.

- ANKRD18A  p.Glu801del
- Ankyrin Repeat domain protein 18A – novel protein of unknown function
DNA-based diagnosis in patients with thrombocytopenia

- Sanger sequencing of identified genes candidates genes on the basis of lumi-aggregation e.g. ADP, Thromboxane receptor defects

- Whole exome sequencing and consanguinity
  - Grey platelet syndrome (NBEAL2)
    

- Whole exome sequencing in non-consanguineous families
  - penetrance (autosomal dominant)

- no penetrance (combination of mutations?)
Inheritance of platelet function disorder in family 1 with dense granule secretion defect

- **Bleeding symptoms & alopecia**
- *Eczema, recurrent viral infections*
- +Psoriasis

### Graphical Representation

- **II-1** with a red circle indicates **Bleeding symptoms & alopecia**.
- **II-2** with a red plus sign indicates **Psoriasis**.
- **III-3** with a red cross indicates **Eczema, recurrent viral infections**.

### Quantitative Data

**ATP (nmol)**

- **Control**
- **Mother**
- **Daughter**

**Reduced Secretion**
- 100 µM PAR1 peptide
Inheritance of platelet function disorder in family 2 with dense granule secretion defect

Collagen (5mg/ml)

ATP (nmol)

1.0  1.5  2.0

Father  Control  Son

1 min

Bleeding, alopecia, mild thrombocytopenia

* Infective endocarditis

+ Eczema & colitis
Sequencing of 260 Platelet Genes

Family 1
- 2153 SNVs
- 691 SNVs
- 36 SNVs
- 4 SNVs

Family 2
- 2283 SNVs
- 670 SNVs
- 8 SNVs
- 2 SNVs

Coverage ≥ 10

Novel SNVs

Exonic SNV

Present in both Family members

1 SNV in FLI1
c.1009 C>T; p.R337W

4 SNVs including FLI1 SNV
c.1028 A>G; p.Y343C

Stockley et al (2013) Blood
The R337W and Y343C substitutions occur in the FLI-1 Ets domain.

R337A disrupts DNA binding capacity of FLI1 (Hu et al, Mol Cell Biol, 2005)
DNA-based diagnosis in patients with thrombocytopenia

- Sanger sequencing of identified genes candidates genes on the basis of lumi-aggregation e.g. ADP, Thromboxxane receptor defects

- Whole exome sequencing and consanguinity
  - Grey platelet syndrome (NBEAL2)

- Whole exome sequencing in non-consanguineous families
  - penetrance (autosomal dominant)

- no penetrance (combination of mutations?)
Next Generation Sequencing

Targeted sequencing panels

vs

Whole exome/genome sequencing
Aim: to sequence 100,000 whole genomes

- England was set up by the Department of Health

- create a lasting legacy for patients, the NHS and the UK economy

- Initially focus on rare disease, cancer and infectious disease.

- Pilot phase completed by end of 2017

- Genomics England has four main aims:
  1) bring benefit to patients
  2) create an ethical and transparent programme based on consent
  3) enable new scientific discovery and medical insights
  4) kickstart the development of a UK genomics industry
Conclusions

• Investigation of genetics in patients with inherited bleeding disorders helps in clinical management of patients

• Provides novel information on the regulation and role of platelet proteins and may even identify new targets for prevention of thrombosis
‘Our experience’ – The GAPP study
Genotyping And Platelet Phenotyping: - The GAPP study

- rare disorders need multiple centres: > 25 Haemophilia Centres
- recruiting subjects with lifelong bleeding indicative of platelet dysfunction
- > 800 participants recruited and phenotyped (108 patients have suspected inherited thrombocytopenia)
GAPP: Genotyping and Phenotyping of Platelets

Patients

Whole blood counts (Sysmex)

Normal platelet count >1x10^8/ml (PRP)

Thrombocytopenia <1x10^8/ml (PRP)

Whole exome sequencing

Candidate genomic variations

Confirmation and functional studies

Platelet function analysis
Platelet count (GAPP cohort)

n=58 patients, 40 healthy controls
Platelet volume (GAPP cohort)

n=51 patients, 40 healthy controls
Immature platelet fraction (GAPP cohort)

n=58 patients, 40 healthy controls
GAPP: Genotyping and Phenotyping of Platelets

Patients

Whole blood counts (Sysmex)

Whole exome sequencing

Platelet function analysis

Normal platelet count >1x10⁸/ml (PRP)

Thrombocytopenia <1x10⁸/ml (PRP)

Candidate genomic variations

Confirmation and functional studies
Exome sequencing - Data Analysis Pipeline

Whole exome sequencing

Alignment of exome sequence data to the human genome reference sequence, hg19

Filter for novelty by comparison with:
- dbSNP
- 1000 genomes
- EVS
- 'in house' database

Removal of variants with MAF≥0.01

Unique variants and variants with a MAF<0.01

Predictions of pathogenicity using Polyphen / SIFT / Mutation Taster / Provean

Candidate gene defects
The UK-GAPP study
Patients with thrombocytopenia

• Whole exome sequencing has been performed and analysed on 55 patients (37 index cases)

• Average platelet count in whole blood of $81 \times 10^9/l$ in the 54 affected patients (10-186x10^9/l)

• Mean platelet volumes were between 7.1 and 15fL, average = 10fL (n=49) (normal range – 7.83-12.39fL)

• Immature platelet fractions ranged from 1.8-87% of total platelet count, n=9 (normal range – 1.3-10.8%)

• 73% (37/51) have a secondary qualitative defect in addition to the reduction in platelet count
Whole Exome Sequencing (WES) of 54 patients

- **111** fold average coverage across all patients, 91% average over 20x coverage

- Over 99% sensitivity with ~3% false discover rate (FDR) was observed

- Between 23,000 and 25,000 variants per patient

- Average of **144 novel variants** per patient

- Average of **137 copy number variants** per exome (n=32, range= 63-421)
<table>
<thead>
<tr>
<th>Family</th>
<th>Patient</th>
<th>Platelet count (x10^9/l)</th>
<th>MPV (fl)</th>
<th>IPF (%)</th>
<th>2ndry defect?</th>
<th>Gene(s)</th>
<th>Genomic variation</th>
<th>Protein effect</th>
<th>Mutation type</th>
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<tbody>
<tr>
<td>2</td>
<td>I</td>
<td>15</td>
<td>10.4</td>
<td>87</td>
<td>Yes (F)</td>
<td>ANKR18A</td>
<td>c.2395_2397del</td>
<td>p.E799del</td>
<td>Missense</td>
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<tr>
<td>II</td>
<td>I</td>
<td>15</td>
<td>15</td>
<td></td>
<td>Yes (S and other)</td>
<td>ANKR18A</td>
<td>c.2395_2397del</td>
<td>p.E799del</td>
<td>Missense</td>
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<td>3</td>
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<tr>
<td>II</td>
<td>I</td>
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<td>CYCS</td>
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<td>I</td>
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<tr>
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<tr>
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<td>I</td>
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<td>Yes (S)</td>
<td>FLI1</td>
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<td>100</td>
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<td>Yes (S)</td>
<td>FLI1</td>
<td>c.A1028G</td>
<td>p.Y343C</td>
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<tr>
<td>7</td>
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<td>110</td>
<td>8.9</td>
<td></td>
<td>Yes (S)</td>
<td>GFI1B</td>
<td>c.676+1G&gt;A</td>
<td>p.A610T</td>
<td>Splicing</td>
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<tr>
<td>II</td>
<td>I</td>
<td>100</td>
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<td></td>
<td>Yes (S)</td>
<td>GFI1B</td>
<td>c.676+1G&gt;A</td>
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<tr>
<td>8</td>
<td>I</td>
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<td></td>
<td>Yes (S)</td>
<td>GP1BA</td>
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<tr>
<td>9</td>
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<td>70</td>
<td>10.7</td>
<td></td>
<td>No</td>
<td>GP1BA</td>
<td>c.G413T</td>
<td>p.G138V</td>
<td>Missense</td>
</tr>
<tr>
<td>II</td>
<td>I</td>
<td>70</td>
<td>10.7</td>
<td></td>
<td>No</td>
<td>GP1BA</td>
<td>c.G413T</td>
<td>p.G138V</td>
<td>Missense</td>
</tr>
<tr>
<td>10</td>
<td>I</td>
<td>130</td>
<td>9.7</td>
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<td>MKL1</td>
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<td>p.V575M</td>
<td>Missense</td>
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73% (27/36) patients with proposed genetic aetiology

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

- 7 variants found within RUNX1

- All variants, excluding a c.G16A missense variant, are located within the RUNT homology domain

- 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 and have been confirmed by Sanger sequencing

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

<table>
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<th>Family</th>
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<th>Variant</th>
<th>PhyloP</th>
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Two unrelated individuals presented with novel variants within \textit{MKL1}; c.1723G>A (p.V575M) and c.554C>T (p.P185L)

An additional frameshift variant in \textit{TUBB1} is also observed in the more severe patient

Disease causing?
- Selected from cross comparing WES variants to a database of 357 platelet related genes

- **MKL1** is increasingly expressed during megakaryocyte and is required for cytoskeleton organisation within platelets through the binding of SRF

- **MKL1** is associated with the reciprocal t(1;22)(p13;q13) translocation that causes AMKL

- Both variants occur at highly conserved sites and are predicted disease causing

- p.P185L occurs within the domain associated with actin binding

![Diagram of MKL1 protein with domains and variants](image-url)
A novel frameshift variant within platelet factor 4 (PF4)

- Index case is a 35 year old female with a history of severe menorrhagia

- Patient suffers from a mild thrombocytopenia (104x10^9/l), with an increased MPV (13.3fL) and a significantly high IPF (17%)

- No secondary qualitative defect was observed

- A novel frameshift variant within PF4; c.33delC (p.R11fs*31)

- Pathogenic?
Conclusions

• Combination of extensive phenotyping coupled with genotyping gives us a novel and unique approach to diagnosis

• WES has allowed us to successfully suggest the possible genetic aetiology in 73% of our 36 index cases

• Found 26 variants in known thrombocytopenia causing genes including genetic variants missed by prior testing

• A high prevalence of *RUNX1* variants in several kindreds

• Discovered 7 variants in four novel genes that have a putative roles in disease
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Dr Andrew Mumford

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Dr Tina Bliss
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Dr Peter Collins
Dr Nicola Curry
Dr Jayashree Motwani
Dr Sue Pavord
Dr Katherine Talks
Dr Jecko Thacil
Dr Jonathon Wilde
Dr Mike Williams

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