Diagnosing platelet secretion disorders: examples cases

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<table>
<thead>
<tr>
<th>Disclosure Type</th>
<th>Statement</th>
</tr>
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<tbody>
<tr>
<td>Research Support/P.I.</td>
<td>No relevant conflicts of interest to declare</td>
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<td>No relevant conflicts of interest to declare</td>
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<td>No relevant conflicts of interest to declare</td>
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<td>No relevant conflicts of interest to declare</td>
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</table>
Platelet granule release

Agonists
(FIIa, Collagen, ADP)

Signals

Activation

Shape change

Membrane fusion

Release of granule contents
Platelet storage organelles

**α granules**
- Adhesive proteins
- Clotting factors and their inhibitors
- Fibrinolytic factors and their inhibitors
- Proteases and antiproteases
- Growth and mitogenic factors
- Chemokines, cytokines
- Anti-microbial proteins
- Membrane glycoproteins

**dense (δ) granules**
- ADP/ATP
- Serotonin
- Histamine
- Inorganic polyphosphate

**lysosomes**
- Enzymes including cathepsins
- Acid hydrolases
# Platelet α-granule contents

<table>
<thead>
<tr>
<th>Type</th>
<th>Prominent components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane glycoproteins</td>
<td>GPIb, αIIbβ3, GPVI</td>
</tr>
<tr>
<td>Clotting factors</td>
<td>VWF, FV, FXI, FII, Fibrinogen, HMWK, FXIII?</td>
</tr>
<tr>
<td>Clotting inhibitors</td>
<td>TFPI, protein S, protease nexin-2</td>
</tr>
<tr>
<td>Fibrinolysis components</td>
<td>PAI-1, TAFI, α2-antiplasmin, plasminogen, uPA</td>
</tr>
<tr>
<td>Other protease inhibitors</td>
<td>α1-antitrypsin, α2-macroglobulin</td>
</tr>
<tr>
<td>Inflammatory, pro-atherogenic, wound healing and antimicrobial proteins</td>
<td>P-selectin, thrombospondin, CD40L, chemokines and cytokines (e.g. PF4, β-TG, IL-8, IL-1β, TGFβ, MCP-1, RANTES), TLT-1, osteonectin, complement components (e.g. C3, C4, C1 inhibitor)</td>
</tr>
<tr>
<td>Pro-angiogenic growth factors</td>
<td>VEGF, PDGF, EGF, IGF, FGF, angiopoietin</td>
</tr>
<tr>
<td>Anti-angiogenic growth factors</td>
<td>Angiostatin, endostatin, inhibitors of matrix metalloproteinases, TIMPs, LAMP-2</td>
</tr>
<tr>
<td>Matrix metalloproteinase inducer</td>
<td>EMMPRIN (CD147)</td>
</tr>
<tr>
<td>Ligand for cell surface receptors</td>
<td>Semaphorin 7A</td>
</tr>
</tbody>
</table>

*Mumford et al, Thromb Haemost, 2015*
Platelet secretion disorders

Heterogeneous disorders characterised by defective release of $\alpha$-granule contents, $\delta$-granule contents or both during platelet activation

Estimated that PSDs constitute >90% of all inherited platelet disorders and may be more prevalent than VWD

Molecular and genetic defects largely unknown
Types of platelet secretion defects

- **α-granule defects**
- **δ-granule defects**

**Signals**
- Agonists
- Activation
- Shape change
- Membrane fusion
- Release of granule contents

**Receptor, signalling or granule trafficking defects**
Diagnosis of suspected inherited platelet secretion disorders

Tests are available for measuring content and release of platelet granules

Not widely or consistently used

ISTH 2014 survey found >50% of 202 labs who responded did not evaluate δ- or α-granule content or release in patients with suspected platelet function defects

Diverse and poorly standardised methods in general
Clinical assessment of the patient

1. Clinical Interview
   - Detailed clinical (bleeding and syndromic) history
   - Family history
   - Assessment of bleeding using standardised questionnaire
   - Exclude acquired causes

2. Initial tests
   - Platelet count
   - Blood smear
   - Assays to exclude VWD, and other coagulation disorders

3. Further tests of platelet function
Platelet secretion disorders

Up to 23% of patients have normal LTA responses in PRP to standard agonist doses

Platelet count is usually normal but can be reduced

PFA-100 should be interpreted with caution; Abnormal results more frequently seen with C-EPI cartridge than the C-ADP cartridge

Measurement of δ-granule release is a necessary investigation
Assessment of δ-granules

Measurement of ATP secreted during the secondary aggregation phase in LTA using luciferin/luciferase reagent
Aggregation and ATP secretion in patient with a dense granule defect

A

![Graph showing aggregation and ATP secretion with different stimuli](image)

B

![Bar chart showing ATP secretion in different conditions](image)

Dawood et al, Blood, 2013
Distinguishing between storage and release defects

Requires measurement of total ADP and ATP content in platelet lysates before and after degranulation

Platelets have two nucleotide pools – metabolic (40%) and granular (60%)

<table>
<thead>
<tr>
<th>Normal ranges</th>
<th>ADP nmol/10^9 platelets</th>
<th>ATP nmol/10^9 platelets</th>
<th>ATP/ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>19 - 38</td>
<td>41 - 61</td>
<td>1.24 - 2.56</td>
</tr>
<tr>
<td>Released</td>
<td>18 - 28</td>
<td>8 - 20</td>
<td>0.43 - 0.79</td>
</tr>
</tbody>
</table>

Storage defects
- reduced stored ADP
- reduced released ADP
- increased ATP/ADP ratio

Release defects
- normal ADP levels
- normal ATP/ADP ratio
- reduced released ADP
Assessment of α-granule content and release

ELISAs to detect α-granule proteins released during platelet activation, e.g. βTG and PF-4

Flow cytometry to detect α-granule proteins that fuse with the membrane after granule release

Detection of P-selectin on resting and activated platelets

Hartwell et al, 1998
Transmission electron microscopy of platelets

Gunay-Aygun et al, Blood, 2010
Super-resolution microscopy of platelets

Red – tubulin
Green – CD63 (dense granule marker)

Westmoreland et al, JTH, 2016
Hermansky-Pudlak Syndrome

Recessive disorder
Normal platelet count
Clinical features include oculocutaneous albinism
Mild bleeding due to absence of platelet δ-granules

Due to defects in HPS1, APCB1, HPS3, HPS4, HPS5, HPS6, DTNBP1, BLOC1S3, BLOC1S6 that encode subunits of the Biogenesis of Lysosome-related Organelle Complexes (BLOC-1, -2, -3) or Adaptor Protein-3 complex, AP3

Abnormal biogenesis of lysosome related organelles, including platelet δ-granules, melanosomes of melanocytes, and granules of cytotoxic T and NK lymphocytes

Cullinane et al, Am J Hum Genet, 2011
Gray Platelet syndrome

Gray platelets | Control platelets

Autosomal recessive

Mild to moderate bleeding that can be life threatening

Severe and specific deficiency of $\alpha$-granules and their contents

Macrothrombocytopenia

Clinical features include early onset myelofibrosis and splenomegaly

Due to mutations in *NBEAL2* that encodes Neurobeachin-like 2 a BEACH-domain containing protein involved in granule biogenesis in megakaryocytes

Fabbro et al, Blood, 2011
# Inherited platelet α-granule defects

<table>
<thead>
<tr>
<th></th>
<th>Gray platelet syndrome</th>
<th>ARC syndrome</th>
<th>GFI1b-related syndrome</th>
<th>GATA-1 related syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inheritance</strong></td>
<td>AR</td>
<td>AR</td>
<td>AD</td>
<td>X-linked</td>
</tr>
<tr>
<td><strong>Bleeding</strong></td>
<td>Mild to moderate</td>
<td>Can be severe</td>
<td>None to moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td><strong>Associated phenotype</strong></td>
<td>Myelofibrosis, splenomegaly, possible autoimmune defects</td>
<td>Multisystem; arthrogryposis, renal dysfunction, cholestasis</td>
<td>Little known as yet</td>
<td>Deregulation of blood cell lineages, anaemia; can be linked to trisomy 21</td>
</tr>
<tr>
<td><strong>Platelet count/morphology</strong></td>
<td>MTP. Severe α-granule deficiency</td>
<td>MTP. Virtual complete absence of α-granules</td>
<td>MTP, highly variable; Normal and α-granule deficient platelets present</td>
<td>MTP. Defect in α-granule production and/or distribution. Not all platelets affected</td>
</tr>
<tr>
<td><strong>Platelet aggregation</strong></td>
<td>Variable platelet function defects</td>
<td>Defective platelet aggregation to ADP</td>
<td>Highly variable; collagen appears particularly affected</td>
<td>Variable but reportedly more reduced for collagen</td>
</tr>
<tr>
<td><strong>Other blood lineages affected</strong></td>
<td>Possible neutrophil secretory defects</td>
<td>No reports</td>
<td>Variable red cell size in one family</td>
<td>Dyserythropoiesis, β-thalassemia-like</td>
</tr>
<tr>
<td><strong>Gene</strong></td>
<td>NBEAL2</td>
<td>VPS33B or VIPAS39</td>
<td>GFI1B</td>
<td>GATA1</td>
</tr>
</tbody>
</table>

MTP=macrothrombocytopenia

*Nurden and Nurden, Am J Hematol, 2016*
FLI1 gene defects in two families with platelet secretion disorders

Family 1

![Family 1 Pedigree]

- c.1009 C>T; p.R337W

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

Family 2

![Family 2 Pedigree]

- c.1028 A>G; p.Y343C

- Bleeding, alopecia, mild thrombocytopenia
- Infective endocarditis
- Eczema & colitis
Failure in ATP secretion from platelets in family 1

100 μM PAR1 peptide

[Graph showing ATP secretion (nmol) compared to Controls, Control, Mother, and Daughter]
Failure in ATP secretion from platelets in family 2

Collagen (5μg/ml)

Thrombin (1U/ml)
Role of FLI1 in megakaryocytogenesis

Endomitosis → Maturation → Proplatelet formation → Platelets

*ITGA2B*, *MPL*, *FLI1*, *GP9*, *GP1BA*, *GP6*

ETS family member that regulates genes expressed during megakaryocytogenesis

Paris Trousseau Syndrome caused by 11q23.3-24 deletion that includes *FLI1*

Patients have increased tendency to bleed, thrombocytopenia, and enlarged platelets with giant α-granules
Assessment of ability of recombinant FLI1 variants to promote gene expression

Measurement of luciferase activity

Co-transfection in HEK 293 cells

Lysis of cells
The R337W and Y343C FLI1 variants fail to transactivate $GP6$

$N=3$, **$p<0.01$, *** $p<0.001$
EM of platelets from patients with *FLI1* defects

Reduction in δ-granules in *FLI1* platelets
Mean 2.1 granules per platelet (n=93)
(Normal platelets: 3.95 - 6.75 δ-granules per platelet)

Giant α-granules observed in 15% of *FLI1* platelets examined
*FLI1* platelets 0.8 µm (0.5 -1.3 µm, n=35)
(Normal α-granules 0.23µm (0.15 - 0.3 µm))
The Y343C and R337W FLI1 variants display reduced nuclear accumulation

**One-way ANOVA p<0.0001**
The Y343C and R337W FLI1 variants display reduced DNA binding.
PTS is phenocopied by autosomal recessive inheritance of DNA-binding domain mutation in FLI1

p.R324W
Whole exome sequencing

Alignment of exome sequence to the human genome reference sequence,

Variants in 329 genes known or predicted to be associated with platelet count and/or function

Filter for novelty against EVS, 1000 genomes, dbSNP129, dbSNP132 and ‘in house’ database

Remove variants with MAF $\geq 0.01$

Unique variants and variants with a MAF < 0.01

Predictions of pathogenicity using PolyPhen / SIFT / Mutation Taster / HSF / SplicePort / ASSP

Candidate gene defects

Mean no. variants/exome

25, 295

339

27

9
Candidate gene defects in cases with secretion abnormalities

<table>
<thead>
<tr>
<th>Variant type</th>
<th>Secretion abnormality (n=6)</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Hetero</td>
<td>Homo</td>
<td>Total</td>
<td>Novel</td>
</tr>
<tr>
<td>Missense</td>
<td>34</td>
<td>2</td>
<td>36</td>
<td>15</td>
</tr>
<tr>
<td>Indel/frameshift</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Indel/inframe</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Splicing</td>
<td>8</td>
<td>1</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>TOTAL</td>
<td>45</td>
<td>8</td>
<td>53</td>
<td>25</td>
</tr>
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</table>
Functional annotation analysis of genes harbouring candidate defects in cases with secretion abnormalities

<table>
<thead>
<tr>
<th>Annotation term</th>
<th>$P$ value</th>
<th>No. of genes</th>
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</thead>
<tbody>
<tr>
<td>Establishment of protein localization</td>
<td>$3.2 \times 10^{-4}$</td>
<td>13</td>
</tr>
<tr>
<td>Protein transport</td>
<td>$5.9 \times 10^{-4}$</td>
<td>13</td>
</tr>
<tr>
<td>Protein localization</td>
<td>$4.1 \times 10^{-4}$</td>
<td>13</td>
</tr>
<tr>
<td>Vesicle-mediated transport</td>
<td>$8.3 \times 10^{-4}$</td>
<td>11</td>
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</table>

$P<0.05$ denotes significance
<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of defects</th>
<th>Mutation type</th>
<th>Patient ID</th>
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</thead>
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<tr>
<td>SCFD1</td>
<td>1</td>
<td>Missense</td>
<td>F18.1</td>
</tr>
<tr>
<td>STX2</td>
<td>1</td>
<td>Missense</td>
<td>F18.1</td>
</tr>
<tr>
<td>STX7</td>
<td>1</td>
<td>Missense</td>
<td>F18.1</td>
</tr>
<tr>
<td>STXBP1</td>
<td>2</td>
<td>Splicing</td>
<td>F13.1, F15.1</td>
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<tr>
<td>STXBP2</td>
<td>1</td>
<td>Splicing</td>
<td>F17.1</td>
</tr>
<tr>
<td>STXBP4</td>
<td>1</td>
<td>Splicing</td>
<td>F18.1</td>
</tr>
<tr>
<td>STXBP5L</td>
<td>1</td>
<td>Missense</td>
<td>F13.1</td>
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<tr>
<td>SYTL3</td>
<td>2</td>
<td>Missense</td>
<td>F17.1</td>
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<tr>
<td>HOOK3</td>
<td>1</td>
<td>Splicing</td>
<td>F13.1</td>
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<tr>
<td>LYST</td>
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<td>VPS4B</td>
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<td>F17.1</td>
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<td>VPS16</td>
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<tr>
<td>VPS41</td>
<td>1</td>
<td>Missense</td>
<td>F16.1</td>
</tr>
<tr>
<td>VAV1</td>
<td>1</td>
<td>Missense</td>
<td>F14.1</td>
</tr>
</tbody>
</table>

Genes showing enriched annotation by functional annotation analysis in cases with secretion abnormalities.
When diagnosing platelet secretion disorders…

A detailed clinical history is essential

LTA alone is not appropriate for diagnosis of platelet secretion disorders

Measurement of $\delta$-granule release is essential

$\alpha$-granule release can be assessed by flow cytometry to detect P-selectin

Both $\alpha$- and $\delta$-granule disorders display considerable heterogeneity

Whole exome/genome approaches are increasingly being used to assist with diagnosis
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[British Heart Foundation logo]