Current and Emerging Approaches for Evaluating Platelet Disorders

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MILANO – ITALY
Defects of Primary Hemostasis

- **Thrombocytopenia**
  - Congenital
  - Acquired

- **Platelet Function Disorders**
  - Congenital
  - Acquired

- **Von Willebrand Disease**
  - Congenital
  - Acquired
ADP

TxA

2

Epinephrine

5HT

FIIa, ....

FVa

FII

FXa

FV

ADP

G proteins; PLC; PLA2; COX-1;...

α

δ

Subendothelium

Adhesive protein

Adhesive protein

Adhesive protein

1 – RECEPTORS FOR ADHESIVE PROTEINS

2 – RECEPTORS FOR SOLUBLE AGONISTS

3 – SIGNAL TRANSDUCTION

4 – GRANULES

5 – PHOSPHOLIPIDS

Adhesive protein

Modified from Cattaneo M, in PLATELETS (Michelson AD ed., 3rd ed, 2013)
Classification of Congenital Platelet Function Disorders

1. Abnormalities of the platelet receptors for adhesive proteins
   - GPIb-IX-V complex: Bernard-Soulier syndrome, platelet-type von Willebrand disease
   - GPIIb-IIIa (αIIbβ3): Glanzmann thrombasthenia
   - GPla-IIa (α2β1)
   - GPVI

2. Abnormalities of the platelet receptors for soluble agonists
   - P2Y12 receptor
   - Thromboxane A2 receptor
   - α2-adrenergic receptor
   - PAR-1 defect

3. Abnormalities of the platelet granules
   - δ-granules: non-syndromic δ-storage pool deficiency, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, MRP4 deficiency, thrombocytopenia with absent radii syndrome, Wiskott-Aldrich syndrome
   - α-granules: gray platelet syndrome, Quebec platelet disorder, 11q terminal deletion disorder, White platelet syndrome, Medich platelet disorder, X-linked macrothrombocytopenia with thalassemia, arthrogryposis renal dysfunction and cholestasis (ARC) syndrome
   - α- and δ- granules: α,δ-storage pool deficiency

4. Defects of signal transduction
   - abnormalities of the arachidonic acid/thromboxane A2 pathway: defects in phospholipase A2, cyclooxygenase, thromboxane synthetase
   - abnormalities of GTP binding proteins: Gαq deficiency, Gα11 defect, hyperresponsiveness of platelet Gsα
   - defects in phospholipase C activation: partial selective PLC-β2 isozyme deficiency
   - abnormalities in transcription factors
   - abnormality in GPVI/FcRc signaling
   - Leukocyte adhesion deficiency III (LAD-III)
   - Ca2+DAG/GEF1 defect

5. Abnormalities of membrane phospholipids
   - Scott syndrome
   - Stormorken syndrome

6. Miscellaneous abnormalities of platelet function
   - Primary secretion defects
   - Other (osteogenesis imperfecta, Ehlers-Danlos syndrome, Marfan syndrome, hexokinase deficiency, glucose-6-phosphate deficiency)
Prevalence of Congenital Platelet Function Disorders
Prevalence of hemostasis defects in 74 women with unexplained menorrhagia

<table>
<thead>
<tr>
<th>Defect</th>
<th>N (%)</th>
</tr>
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<tbody>
<tr>
<td>Platelet aggregation</td>
<td>35 (47.3%)</td>
</tr>
<tr>
<td>Platelet secretion</td>
<td>43 (58.1%)</td>
</tr>
<tr>
<td>VWF:Ag and/or VWF:Rco &lt;60 U/dL</td>
<td>10 (13.5%)</td>
</tr>
<tr>
<td>VWD*</td>
<td>5 (6.7%)</td>
</tr>
<tr>
<td>Factor XI</td>
<td>2 (0.3%)</td>
</tr>
<tr>
<td>Factor V</td>
<td>1 (0.15%)</td>
</tr>
<tr>
<td>Factor VIII (HA carrier)</td>
<td>1 (0.15%)</td>
</tr>
</tbody>
</table>

* Using race- and blood group-specific ranges
Frequency of diagnosis and demographic data of 280 patients with congenital mucocutaneous bleedings

<table>
<thead>
<tr>
<th>Table 2. Frequency of diagnosis and demographic data of patients and controls.</th>
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<tbody>
<tr>
<td><strong>DIAGNOSIS</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>VWD*</td>
</tr>
<tr>
<td>VWD* + PFD</td>
</tr>
<tr>
<td>VWD* + CFD</td>
</tr>
<tr>
<td>PFD</td>
</tr>
<tr>
<td>PFD+CFD</td>
</tr>
<tr>
<td>CFD</td>
</tr>
<tr>
<td>BUC</td>
</tr>
<tr>
<td>TOTAL</td>
</tr>
<tr>
<td>CONTROLS</td>
</tr>
</tbody>
</table>

VWD: von Willebrand’s disease; PFD: platelet function disorder; CFD: coagulation factor deficiency; BUC: bleeding of unknown cause; BT: bleeding time. *36 patients had type 1 VWD, one had type 2A, and two had type 2B VWD. +seven patients had type 1 VWD, one had type 2A, and one had type 2B VWD. **the two patients had type 1 VWD. *differences in blood type O: p=0.01 (controls vs. all the patients). The significance rose to p < 0.002 when controls were compared with the 50 patients with VWD. (Fisher’s exact test). **the proportion of patients with VWD or PFD with prolonged BT was significantly higher than that of patients with BUC (p=0.005 and 0.003, respectively). (Fisher’s exact test).
# Frequency of diagnosis and demographic data of 280 patients with congenital mucocutaneous bleedings

<table>
<thead>
<tr>
<th>DIAGNOSIS</th>
<th>Nº (%)</th>
<th>Age (years) mean±SD (range)</th>
<th>Females %</th>
<th>Blood type O Nº (%)</th>
<th>Abnormal BT Nº (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWD*</td>
<td>39 (14.0)</td>
<td>16.2±11.2 (4-47)</td>
<td>74.4</td>
<td>35 (90)</td>
<td>16 (41.0)**</td>
</tr>
<tr>
<td>VWD+ PFD</td>
<td>9 (3.2)</td>
<td>15.9±11.1 (7-40)</td>
<td>77.8</td>
<td>6 (67)</td>
<td>5 (56)</td>
</tr>
<tr>
<td>VWD+ CFD</td>
<td>2 (0.7)</td>
<td>13.5±0.7 (13-14)</td>
<td>50.0</td>
<td>2 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PFD</td>
<td>54 (19.3)</td>
<td>14.5±8.1 (4-41)</td>
<td>51.9</td>
<td>31 (57)</td>
<td>21 (39)**</td>
</tr>
<tr>
<td>PFD+CFD</td>
<td>2 (0.7)</td>
<td>12.5±2.1 (11-14)</td>
<td>0</td>
<td>1 (50)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>CFD</td>
<td>7 (2.5)</td>
<td>10.4±3.2 (7-16)</td>
<td>14.3</td>
<td>4 (57)</td>
<td>2 (28.6)</td>
</tr>
<tr>
<td>BUC</td>
<td>167 (59.6)</td>
<td>14.2±9.8 (4-48)</td>
<td>59.3</td>
<td>110 (66)</td>
<td>31 (18.6)**</td>
</tr>
<tr>
<td>TOTAL</td>
<td>280</td>
<td>14.5±9.6 (4-48)</td>
<td>58.9</td>
<td>189 (67)¹</td>
<td>75 (26.8)</td>
</tr>
<tr>
<td>CONTROLS</td>
<td>299</td>
<td>12.2±6.5 (4-44)</td>
<td>54.5</td>
<td>170 (57)¹</td>
<td>22 (7.4)</td>
</tr>
</tbody>
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VWD: von Willebrand’s disease; PFD: platelet function disorder; CFD: coagulation factor deficiency; BUC: bleeding of unknown cause; BT: bleeding time. *36 patients had type 1 VWD, one had type 2A, and two had type 2B VWD. Seven patients had type 1 VWD, one had type 2A, and one had type 2B VWD. The two patients had type 1 VWD. **differences in blood type O: p=0.01 (controls vs. all the patients). The significance rose to p < 0.002 when controls were compared with the 50 patients with VWD. (Fisher’s exact test). **the proportion of patients with VWD or PFD with prolonged BT was significantly higher than that of patients with BUC (p=0.005 and 0.003, respectively). (Fisher’s exact test).
Patients screened for Platelet Function Disorders at the A. Bianchi Bonomi Hemophilia and Thrombosis Ctr, University of Milano. 1990 - 2002

- Total number of screened patients: 318
- Diagnosis:

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>No abnormalities</td>
<td>187</td>
<td>59%</td>
</tr>
<tr>
<td>Primary Secretion Defects</td>
<td>63</td>
<td>20%</td>
</tr>
<tr>
<td>(\delta)-Storage Pool Deficiency</td>
<td>38</td>
<td>12%</td>
</tr>
<tr>
<td>Glanzmann Thrombasthenia</td>
<td>5</td>
<td>2%</td>
</tr>
<tr>
<td>“Aspirin-like” Defects</td>
<td>4</td>
<td>1.3%</td>
</tr>
<tr>
<td>Bernard-Soulier syndrome</td>
<td>3</td>
<td>1%</td>
</tr>
<tr>
<td>(P2Y_{12}) defect</td>
<td>2</td>
<td>0.6%</td>
</tr>
<tr>
<td>Incomplete</td>
<td>15</td>
<td>5%</td>
</tr>
</tbody>
</table>
Conclusion

- Although their actual prevalence is unknown, congenital Platelet Function Disorders seem to be common than generally believed, probably more common than VWD.

- We are unable to diagnose the majority of non VWF-related defects of primary hemostasis (which are likely associated with defects of platelet function).
Diagnosis of Platelet Function Disorders

Are *Bleeding Assessment Tools* useful?
Bleeding manifestations of defects of primary hemostasis

• Cutaneous purpura
• Mucosal bleedings (gum bleeding, GI bleeding, menorrhagia,…)
• Excessive post-traumatic or post-surgical bleeding (early-onset)
• Other types of bleeding may be observed, but are usually rare
Performance of the ISTH Bleeding Assessment Tool Score in patients with suspected Platelet Function Defects

Lowe et al, JTH 2013
Global tests of primary hemostasis

- Bleeding time
- PFA-100
- IMPACT
- Placor PRT system

Global systems of Platelets and Coagulation

- Thromboelastography
- Sonoclot - Coagulation and Platelet Function Analyzer
- Hemostasis Analysis System – platelet contractile force
- Haemostatus Device - platelet procoagulant activity
- Endogenous Thrombin Potential in PRP
Platelet function tests - 2

*Platelet aggregation-based tests*
- LTA (lumiaggregometry)
- Impedance aggregometry (lumiaggregometry)
- Multiplate
- PlateletWorks – platelet counting with and without activation
- Thrombovision T guide
- 96 well plate assays (Kenny & Warner)
- VerifyNow

*Flow cytometry*
- Measurement of activation markers
- Measure Responsiveness to agonists
- VASP phosphorylation

*Thromboxane A₂ metabolites*
- Serum TxB₂
- Urinary Tx metabolites
Diagnosis of Platelet Function Disorders

Are Global Tests of Hemostasis useful?
Global tests of primary hemostasis

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- Endogenous Thrombin Potential in PRP
Bleeding Time

Great variability in results
Operator-dependency
Unreliable
Invasive
**Insensitive**
No guide in diagnostic work-up
Unpopular with patients
Diagnosis of patients with mucocutaneous bleeding

Patient with mucocutaneous bleeding

Bleeding time

Prolonged
- Screening for VWD or Platelet Function Disorders

Normal
- Screening for VWD or Platelet Function Disorders
Diagnosis of patients with mucocutaneous bleeding

Patient with mucocutaneous bleeding

Screening for VWD or Platelet Function Disorders
In Vivo

- Capillary aperture 150µm
- Epinephrine or ADP
- Platelet plug
- Flow

PFA-100

- 40 mbar
- Aperture 150µm
- Membrane
- Collagen
- Platelet plug
- Capillary
Percent of patients with prolonged test (>90\textsuperscript{th} centile)

Podda et al, JTH 2007
Patient with mucocutaneous bleeding

PFA-100® C-ADP closure time

Prolonged

Screening for VWD

VWD

No VWD

Screening for PFD

PFD

No PFD

Normal

Screening for PFD

Screening for VWD
Patient with mucocutaneous bleeding

PFA-100® C-ADP closure time

?? To be validated!!

Screening for VWD
- VWD
- No VWD

Screening for PFD
- PFD
- No PFD

Screening for PFD
Screening for VWD
Platelet function tests

**Platelet aggregation-based tests**
- LTA (lumiaggregometry)
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**Thromboxane A₂ metabolites**
- Serum TxB₂
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Light transmission platelet aggregation in citrated platelet rich plasma
## Platelet aggregation in citrated PRP

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<tr>
<th></th>
<th>ADP</th>
<th>Collagen</th>
<th>AA</th>
<th>Ristocetin</th>
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<tbody>
<tr>
<td>Normal</td>
<td><img src="image1" alt="Graph" /></td>
<td><img src="image2" alt="Graph" /></td>
<td><img src="image3" alt="Graph" /></td>
<td><img src="image4" alt="Graph" /></td>
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<tr>
<td>Glanzmann</td>
<td><img src="image5" alt="Graph" /></td>
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<td><img src="image15" alt="Graph" /></td>
<td><img src="image16" alt="Graph" /></td>
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<td>Aspirin</td>
<td><img src="image17" alt="Graph" /></td>
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<td><img src="image20" alt="Graph" /></td>
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<td><img src="image21" alt="Graph" /></td>
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Boneu & Cazenave
Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH

M. CATTANE0, * C. CERLETTI, † P. HARRISON, ‡ C. P. M. HAYWARD, § D. KENNY, ¶ D. NUGENT, ** P. NURDEN, †† A. K. RAO, †‡ A. H. SCHMAIER, §§ S. P. WATSON, ¶¶ F. LUSSANA, * M. T. PUGLIANO* and A. D. MICHELSON***
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Flow cytometry
- Measurement of activation markers
- Measure Responsiveness to agonists
- VASP phosphorylation

Thromboxane A₂ metabolites
- Serum TxB₂
- Urinary Tx metabolites
Platelet aggregation (upper tracings) and secretion (lower tracings) induced by ADP at the indicated concentrations (μM), obtained with the lumiaggregometer.
Platelet function tests - 2

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**Thromboxane A\(_2\) metabolites**
- Serum TxB\(_2\)
- Urinary Tx metabolites
firm adhesion and aggregation of platelets on the sensor surface enhances the electrical resistance between the 2 sensor wires
MULTIPLATE

LIGHT TRANSMISSION AGGREGOMETRY

Normal

P2Y$_{12}$ defect
MULTIPLATE

**Aggregation [AU]**

- **Normal**
- **P2Y\_12 defect**

**Light Transmission (%)**

- **Normal**
- **P2Y\_12 defect**
MULTIPLATE

Light transmission (%)

0 1 2 3 4 5

0 1 2 3 4 5

time [min]

time [min]

Normal

P2Y_{12} defect

Normal

P2Y_{12} defect
Effects of the platelet count on the extent of aggregation of washed human platelets, measured by Light Transmission Aggregometry and by Impedance Aggregometry (Multiplate®)

*Light Transmission Aggregometry*

- **TRAP-6 (32μM)**
  - n=5
  - p=NS

- **COLLAGEN (3.2 μg/mL)**
  - n=8
  - p=NS

- **ADP (20 μM)**
  - n=6
  - p=NS

*Multiplate®*

- **TRAP-6 (32μM)**
  - n=5
  - p<0.001

- **COLLAGEN (3.2 μg/mL)**
  - n=8
  - p<0.001

- **ADP (20 μM)**
  - n=6
  - p<0.001

Femia et al, JTH 2013
Platelet function tests - 2

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Thromboxane A$_2$ metabolites
- Serum TxB$_2$
- Urinary Tx metabolites
Flow Cytometry for Platelet Function Studies

- Active conformation of GPIIb/IIIa
- Secretion of granules/lysosomes
- Procoagulant activity
- Platelet-derived microparticles
- Platelet aggregation (dual-color FC)
- Platelet-leukocytes heteroaggregates
- VASP phosphorylation
- …
Vasodilator Stimulated Phosphoprotein (VASP) Assay for the Measurement of $P2Y_{12}$ Antagonism

Modified from Cattaneo in PLATELETS (Michelson, 2nd ed, 2007)
Vasodilator Stimulated Phosphoprotein (VASP) Assay for the Measurement of P2Y_{12} Antagonism

Modified from Cattaneo in PLATELETS (Michelson, 2nd ed, 2007)
Platelet Reactivity Index, measured by VASP phosphorylation assay in healthy controls and patients with inherited P2Y\textsubscript{12} defects.
Vasodilator Stimulated Phosphoprotein (VASP) Assay for the Measurement of P2Y$_{12}$ Antagonism

In the presence of both PGE$_1$ and ADP, VASP-P is directly proportional to the degree of P2Y$_{12}$ antagonism

Modified from Cattaneo in PLATELETS (Michelson, 2nd ed, 2007)
Correlations between plasma concentration of Clopidogrel Active Metabolite (CAM) with PRI (VASP phosphorylation assay): *in vivo vs in vitro* experiments

Danese et al, JTH 2016
Correlations between plasma concentration of Clopidogrel Active Metabolite (CAM) with platelet aggregation in whole blood (MEA): *in vivo vs in vitro* experiments

Danese et al, JTH 2016
Correlations between plasma concentration of Clopidogrel Active Metabolite (CAM) with platelet aggregation in whole blood (MEA): 
*in vivo* vs *in vitro* experiments

\[ R^2 = 0.087 \] 

\[ R^2 = 0.580 \] 

\[ R^2 = 0.175 \] 

\[ R^2 = 0.548 \]
Diagnosis of Congenital Disorders of Platelet Function

Need for standardized, official Guidelines
Guidelines for the Diagnosis of Inherited Platelet Function Disorders – ISTH-SSC Platelet Physiology

• Working Group members
  – Paolo Gresele (ITALY) - Chair
  – Marco Cattaneo (ITALY) - CoChair
  – Christian Gachet (FRANCE)
  – Paul Harrison (UK)
  – Catherine Hayward (CANADA)
  – Dermot Kenny (IRELAND)
  – Diego Mezzano (CHILE)
  – Andrew David Mumford (UK)
  – Diane Nugent (USA)

Sara Orsini, Emanuela Falcinelli, Loredana Bury, Manuela Sebastiano
Diagnosis of suspected inherited platelet function disorders: results of a worldwide survey

P. GRESELE,* P. HARRISON,† L. BURY,* E. FALCINELLI,* C. GACHET,† C. P. HAYWARD,§
D. KENNY,¶ D. MEZZANO,** A. D. MUMFORD,†† D. NUGENT,‡‡ A. T. NURDEN, §§ S. ORSINI* and
M. CATTANEOP¶
2 - Approach to the Diagnosis

2.1-Do you perform clinical interview?

- YES: 64.5%
- NO: 35.5%

N. of respondents: 186/202 (92.1%)

2.2-Do you perform standardized bleeding questionnaire?

- YES: 30.1%
- NO: 69.9%

ISTH Questionnaire: 42.8%

N. of respondents: 200/202 (99.0%)
Inherited Platelet Function Disorders

Laboratory Diagnosis

A

Simple laboratory tests

Diagnostic hypothesis

B

Confirmatory/specific tests: molecular biology, SDS-PAGE, flow cytometry, EM, PF3…
4.1 - First step laboratory tests

What kind of first step (screening) tests do you perform in patients with a suspected inherited platelet function disorder?

- Blood platelet count: 97.0%
- Peripheral blood smear: 80.2%
- Light transmission aggregometry: 59.4%
- PFA-100: 53.3%
- Bleeding time: 28.4%
- Flow cytometry: 23.3%
- Lumiaggregometry: 21.3%
- Impedance aggregometry: 11.7%
- Others: 11.7%
- Clot retraction: 10.7%

Other: Electron microscopy, Thromboelastogram, Coagulation and other routine tests

N. of respondents: 197/202 (97.5%)
1. Light microscopy of whole-blood smear: platelet size and morphology

2. Lumiaggregometry on citrated PRP: explores platelet aggregation and secretion simultaneously
   - ADP 2 μM (if abnormal, ADP 10 μM)
   - Collagen 2 μg/mL (if abnormal, 10 μg/mL)
   - U46619 1μM
   - Adrenaline 5 μM
   - Ristocetin 1.2 mg/mL (if normal, 0.6 mg/mL)
   - Artachidonic acid 1 mM
   - No agonists (SPA)

3. Clot retraction (save serum for TxB₂ assay, to confirm abnormalities of AA pathway or rule out NSAID)
Diagnosis of inherited platelet function disorders: guidance from the SSC of the ISTH.

Abstract
Although rare, the prevalence of inherited platelet function disorders (IPFD) is probably underestimated due to underdiagnosis [1]. IPFD are heterogeneous in severity, mechanisms, and frequency and few are characterized at the molecular level. While severe IPFD, like Glanzmann Thrombasthenia (GT) or Bernard-Soulier Syndrome (BSS), are now rather straightforward to identify, the diagnosis of most other forms is cumbersome and requires complex assays. …
1. Light microscopy of whole-blood smear
2. Light transmission aggregometry on citrated PRP:
   - ADP
   - Collagen
   - Adrenaline
   - Arachidonic acid 1 mM
   - Ristocetin
3. Granule secretion (luminometry, ELISA)
4. Flow cytometry
   - GPIIb/IIIa expression
   - GPIIIa expression
   - GPIb/IX
   - GPIb
   - GPIIb/IIIa activation (PAC-1 binding)
DIAGNOSTIC ALGORITHM

Flowchart

PROBAND

Clinical evaluation:

Personal and family history and bleeding score: bleeding manifestations typical of IPFD

Physical examination: bleeding manifestations typical of IPFD

Syndromic forms: hearing loss; immunodeficiency; renal function; cardiac function; mental retardation; facial dysmorphism; eyes; bone; skin

ABNORMAL

Preliminary laboratory investigation

Potential platelet function disorder

NORMAL/LOW

Platelet count

LOW

Thromobocytopenia

Abnormal

VWF screening

ABNORMAL

VWD

Blood clotting defect

Afibrinogenemia

DIAGNOSIS

NORMAL

Routine coagulation tests

PLATELET FUNCTION STUDIES

NEXT GENERATION SEQUENCING

Gresele et al, JTH 2015
DIAGNOSTIC ALGORITHM

Flowchart

PROBAND

Clinical evaluation:

- Personal and family history and bleeding score: bleeding manifestations typical of IPFD
- Physical examination: bleeding manifestations typical of IPFD
- Syndromic forms: hearing loss; immunodeficiency; renal function; cardiac function; mental retardation; facial dysmorphism; eyes; bone; skin

No further studies

NORMAL

Preliminary laboratory investigation

- Potential platelet function disorder
  - NORMAL/LOW
  - NORMAL

PLATELET FUNCTION STUDIES

- NEXT GENERATION SEQUENCING

Routine coagulation tests
- VWF screening

DIAGNOSIS

- Platelet count
  - LOW
  - Thrombocytopenia
  - ABNORMAL
  - VWD
    - Blood clotting defect
    - Afibrinogenemia
Title: A comprehensive high-throughput sequencing test for the diagnosis of inherited bleeding, thrombotic and platelet disorders

Running title: The ThromboGenomics platform

Author List

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Key points

• We have developed a targeted sequencing platform covering 63 genes linked to heritable bleeding, thrombotic and platelet disorders.

• The ThromboGenomics platform provides a sensitive genetic test to obtain molecular diagnoses in patients with a suspected etiology.
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The future?