Recent progress in the diagnosis of RBC membrane and enzyme disorders
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Pathophysiology of Anemias Unit

5th European Symposium on Rare Anemias
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- General overview of RBC membrane and enzyme defects
- Laboratory investigations
- Results of the ENERCA survey
Patient’s and family medical history and clinical examination
- Acute or chronic hemolytic anemia
- Intra or extravascular hemolysis
- Congenital or acquired
- Extrahematological signs

**CONGENITAL CAUSES**

- Blood smear analysis

  RBC morphologic abnormalities
  (spherocytes, elliptocytes, ovalocytes, stomatocytes, marked anisopoikilocytosis)

  **RBC MEMBRANE DEFECTS / CDAs**

  - Osmotic fragility tests
  - Ektacytometry
  - EMA binding
  - SDS-PAGE
  - Molecular analysis

  **Acute hemolysis**
  **Chronic hemolysis**

- Hereditary Spherocytosis
- Hereditary Elliptocytosis
- SAO
- Hereditary Stomatocytosis
- CDAs

- **Hereditary Elliptocytosis**
- **SAO**
- **Hereditary Stomatocytosis**
- **CDAs**

**ACQUIRED CAUSES**

- Direct Antiglobulin Test (DAT)

  **IMMUNE HEMOLYTIC ANAEMIAS**
  - AIHA
  - DHTT (in recently tx pts.)

  **RBC ENZYMOPATHIES**

  - Study of RBC metabolism

  **Acute hemolysis**
  **Chronic hemolysis**

  - **PP-shunt**
  - **Glycolysis**
  - **Nucleotide metab**

  **MECHANICAL HEMOLYSIS**

  - INFECT/TOXIC CAUSES
    - Wilson disease

  - INFECT/TOXIC CAUSES
    - PNH

  - INFECT/TOXIC CAUSES
    - Schistocytes

  - INFECT/TOXIC CAUSES
    - CD55/59

  - INFECT/TOXIC CAUSES
    - positive
    - negative

  - INFECT/TOXIC CAUSES
    - negative
    - positive

  - INFECT/TOXIC CAUSES
    - no

  - INFECT/TOXIC CAUSES
    - Reconsider congenital causes or DAT-negative AIHA
CONGENITAL RED CELL MEMBRANE DISORDERS

Hereditary spherocytosis (HS)  
1:2000 Dom.Tr (75% of cases)

Hereditary elliptocytosis (HE)  
1:4000  Dom. Tr

Hered. Pyropoikilocytosis (HPP)  
Non-Dom. Tr

Hereditary stomatocytosis (HSt)  
1:50000 – 1:100000  Dom. Tr
HEREDITARY SPHEROCYTOSIS

- Dominant transmission in 75% of cases
- Anemia: from very severe to compensated
- Variable splenomegaly and jaundice
- Presence of spherocytes in peripheral blood
- Response to splenectomy
RED CELL MEMBRANE CYTOSKELETON - INTERACTIONS

"Horizontal interactions"
RED CELL MEMBRANE CYTOSKELETON - INTERACTIONS

“Vertical interactions”
SDS- PAGE analysis of red cell membrane proteins

- α-spectrin
- β-spectrin
- Band 3
- Ankyrin

Trypsin digestion

- α 1/80
- β IV/74
- β II/65
- α III/52
- α II/46
- α IV-V/41
- β III/33
- β I/28
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical features</td>
<td>Splenomegaly almost always</td>
</tr>
<tr>
<td>Laboratory red cell indices</td>
<td>(↓Hb, ↓MCV, ↑MCHC, ↑% hyperdense cells, ↑RDW, ↑reticulocyte count)</td>
</tr>
<tr>
<td>Blood film</td>
<td>Abnormal morphology – spherocytes</td>
</tr>
<tr>
<td>Direct antiglobulin test</td>
<td>Negative</td>
</tr>
<tr>
<td>Evidence of haemolysis</td>
<td>Raised bilirubin; reticulocytosis</td>
</tr>
</tbody>
</table>

MCV, mean cell volume; MCHC, mean cell Hb concentration; RDW, red cell distribution width.
CLINICAL DATA IN 259 NOT SPLENECTOMIZED HS PATIENTS
(139 B3; 81 Sp; 9 Ank or Sp/Ank; 2 Band 4.2; 28 undetected)
(< 18 yrs = 121 ; >18 yrs = 138)

ANEMIA: severe 6%, moderate 16%, mild 40%, compensated 38%

EXCHANGE TRANSFUSION: 14/82 cases

Mariani et al, Haematologica, 2008
Haematologic parameters of 259 not splenectomized HS patients

Not always standard hematologic parameters give specific diagnostic indications!
## Screening tests for the diagnosis of Hereditary Spherocytosis

<table>
<thead>
<tr>
<th>Test</th>
<th>Description</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Osmotic fragility (OF) test</strong></td>
<td>Measure absorbance at 540 nm for fresh blood and after 24 h incubation. Plot a graph of % haemolysis versus NaCl concentration</td>
<td>Affected by elevated reticulocyte counts Also increased in AIHA</td>
</tr>
<tr>
<td><strong>Acidified glycerol lysis test (AGLT)</strong></td>
<td>Measure the time taken for absorbance of red cell suspension at 625 nm in glycerol to fall to half of its original value before glycerol addition (AGLT50)</td>
<td>Also positive in AIHA, enzyme deficiency, pregnant women, chronic renal failure and myelodysplastic syndrome.</td>
</tr>
<tr>
<td><strong>The Pink test</strong> (Vettore &amp; Zanella, 1984)</td>
<td>Measure the time taken for absorbance of red cell suspension at 625 nm in glycerol to fall to half of its original value before glycerol addition (AGLT50)</td>
<td>Also positive in AIHA, enzyme deficiency, pregnant women, chronic renal failure and myelodysplastic syndrome.</td>
</tr>
<tr>
<td><strong>Hypertonic cryohaemolysis test</strong></td>
<td>% cryohaemolysis at 540 nm after transfer of red cells from 37°C to 0°C for 10 min</td>
<td>Positive results for HS, some CDAII and Melanesian elliptocytosis</td>
</tr>
<tr>
<td><strong>Eosin-5-maleimide (EMA) binding</strong></td>
<td>Reduced fluorescence (green) intensity of EMA-labelled red cells by flow cytometry</td>
<td>Distinct histograms for red cells of HS. Reduced in CDAII, cryohydrocytosis, SAO.</td>
</tr>
</tbody>
</table>

Note: Bolton-Maggs et al, Br J Haematol 126:455-474, 2004
EMA-binding test

Rapid flow cytometric test for the diagnosis of membrane cytoskeleton-associated haemolytic anaemia

MAY-JEAN KING,^1^ JUDITH BEHRENS,^2^ CHRIS ROGERS,^3^ CLAIRE FLYNN,^4^ DAVID GREENWOOD^5^ AND KEITH CHAMBERS^6^

- Direct test

- Measures the fluorescence intensity of intact red cells labelled with the dye eosin-5-maleimide, interacting with the protein band 3 complex Lys 430

- A decrease of fluorescence intensity is also detected with spectrin- and protein 4.2-deficient HS red cells.

Sensitivity = 92.7%
Specificity = 99.1%
Sensitivity of diagnostic tests according to biochemical defect
150 HS patients

<table>
<thead>
<tr>
<th></th>
<th>EMA-binding</th>
<th>GLT</th>
<th>AGLT</th>
<th>Pink</th>
<th>OF NaCl fresh</th>
<th>OF NaCl inc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total HS patients</td>
<td>140/150 (93%)</td>
<td>92/150 (61%)</td>
<td>143/150 (95%)</td>
<td>136/150 (91%)</td>
<td>102/150 (68%)</td>
<td>122/150 (81%)</td>
</tr>
<tr>
<td>HS with biochemical defect</td>
<td>132/141 (94%)</td>
<td>90/141 (64%)</td>
<td>135/141 (96%)</td>
<td>131/141 (93%)</td>
<td>100/141 (71%)</td>
<td>119/141 (84%)</td>
</tr>
<tr>
<td>Spectrin</td>
<td>68/73 (93%)</td>
<td>45/73 (61%)</td>
<td>70/73 (96%)</td>
<td>67/73 (92%)</td>
<td>51/73 (70%)</td>
<td>62/73 (85%)</td>
</tr>
<tr>
<td>Band 3</td>
<td>55/59 (93%)</td>
<td>38/59 (64%)</td>
<td>56/59 (93%)</td>
<td>55/59 (93%)</td>
<td>43/59 (73%)</td>
<td>49/59 (83%)</td>
</tr>
<tr>
<td>Combined spectrin/ankyrin</td>
<td>9/9 (100%)</td>
<td>7/9 (78%)</td>
<td>9/9 (100%)</td>
<td>9/9 (100%)</td>
<td>6/9 (67%)</td>
<td>8/9 (89%)</td>
</tr>
<tr>
<td>HS with undetectable defect</td>
<td>8/9 (88%)</td>
<td>2/9 (22%)</td>
<td>8/9 (88%)</td>
<td>2/9 (22%)</td>
<td>3/9 (33%)</td>
<td>4/9 (44%)</td>
</tr>
</tbody>
</table>

93%  61%  95%  91%  68%  81%

Bianchi et al, Haematologica 2012
Sensitivity of diagnostic tests according to clinical phenotype

Bianchi et al, Haematologica 2012
Combined tests’ sensitivity in total HS cases

- All HS patients were positive to at least two different tests with the exception of two who were EMA-binding positive only.

- The combination of EMA & AGLT enabled to identify the totality of HS patients

<table>
<thead>
<tr>
<th>Test Combination</th>
<th>Number Positive/Total</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMA + AGLT</td>
<td>150/150 (100%)</td>
<td></td>
</tr>
<tr>
<td>EMA + OF NaCl fresh</td>
<td>143/150 (95%)</td>
<td></td>
</tr>
<tr>
<td>EMA + OF NaCl inc.</td>
<td>143/150 (95%)</td>
<td></td>
</tr>
<tr>
<td>EMA + Pink</td>
<td>149/150 (99%)</td>
<td></td>
</tr>
<tr>
<td>OF NaCl inc. + AGLT</td>
<td>146/150 (97%)</td>
<td></td>
</tr>
</tbody>
</table>

Bianchi et al, Haematologica 2012
### Definition of cut-off limits for EMA binding tests

<table>
<thead>
<tr>
<th>% 30</th>
<th>% 30</th>
<th>% 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
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</tr>
<tr>
<td>28</td>
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<td>10</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

- **HS**: Hereditary Spherocytosis
- **Norm**: Normal
- **“Grey zone”**: Intermediate values between HS and Norm
- **Cut-off Normal/Hs**: Threshold for distinguishing between normal and HS
- **Cut-off Hs/Other hemolytic dis**: Threshold for distinguishing between HS and other hemolytic disorders

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**References**

- Bianchi et al, *Diagnostic power of laboratory tests for hereditary spherocytosis… and Replays*. Haematologica 2012 97: 516-23
Disease specificity of diagnostic tests (87 not HS-hemolytic pts)
DIFFERENTIAL DIAGNOSIS OF HS AND CDAII

SDS-PAGE analysis of RBC membrane proteins

13% of patients referred with a suspect of HS were CDAII
Ectacytometry
Laser-assisted Optical Rotational Cell Analyzer

Clark et al, Blood 1984
Osmoscan Curves in patients with red cell membrane disorders
Molecular characterization of RBC membrane disorders

<table>
<thead>
<tr>
<th>Band</th>
<th>Protein</th>
<th>Associated haemolytic anaemias</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-Spectrin</td>
<td>HE, HS</td>
</tr>
<tr>
<td>2</td>
<td>β-Spectrin</td>
<td>HPP</td>
</tr>
<tr>
<td>2.1</td>
<td>Ankyrin</td>
<td>HE, HS, HS in mice</td>
</tr>
<tr>
<td>2.9</td>
<td>Adducin</td>
<td>HS</td>
</tr>
<tr>
<td>3</td>
<td>Band 1</td>
<td>HE, SAO, HAC</td>
</tr>
<tr>
<td>4</td>
<td>Band 4</td>
<td>HE</td>
</tr>
<tr>
<td>4.1</td>
<td>Protein</td>
<td>HE</td>
</tr>
<tr>
<td>4.2</td>
<td>Protein</td>
<td>HE</td>
</tr>
<tr>
<td>5</td>
<td>β-Actin</td>
<td>?</td>
</tr>
<tr>
<td>6</td>
<td>Ga3PD</td>
<td>?</td>
</tr>
<tr>
<td>PAS-1</td>
<td>Glycos</td>
<td>?</td>
</tr>
<tr>
<td>PAS-2</td>
<td>Glycos</td>
<td>?</td>
</tr>
<tr>
<td>PAS-3</td>
<td>Glycos</td>
<td>?</td>
</tr>
</tbody>
</table>

*Band numbers refer to the position on SDS-PAGE electrophoresis.

Low expression α spectrin alleles (Sp α<sub>Lely</sub>)

SNP: IVS45 -12c/t

Alpha LELY in trans + HE Sp mutations

Hereditary Pyropoikilocytosis

Genetic counselling

HAC, hereditary acanthocytosis; HE, hereditary elliptocytosis; HS, hereditary spheroctytosis; HPP, hereditary pyropoikilocytosis; SAO, Southeast Asian ovalocytosis; Ga3PD, gyceraldehyde-3-phosphate dehydrogenase.
Survey on red cell membrane disorders and enzyme defects
Centres involved: 26
Use of diagnostic tests performed for diagnosis of red cell membrane defects

% of centers

ENERCA from: “White Book for the creation of a European Reference Network of Expert Centers in Rare Anaemias.”
Method with best specificity and sensitivity

Combination of tests:
- RBC Morphology + EMA
- EMA + AGLT
- AGLT + Cryo
- RIA + EMA + AGLT
- OF + EMA + Cryo
- Cryo + EMA + SDS
- EMA + pink + OF
- RBC Morphology + Pink
- RIA + AGLT + OF
- EMA + AGLT + SDS

Not known: 8
EMA-binding test: 3
Pink test: 1
OF: 1
Ectacytometer: 1

ENERCA from: “White Book for the creation of a European Reference Network of Expert Centers in Rare Anaemias.”
RBC metabolism

Glycolysis

Glucose

Hexose monophosphate shunt

2 NADPH

Glutathione

reducing power

Nucleotide metabolism

2 NADH

methemoglobin reduction

Rapoport-Luebering shunt

2,3-DPG

Hb-O₂ affinity

2 ATP

metabolic energy

Lactate
The type and degree of haemolysis in CNSHA depends on:
- the metabolic cycle involved
- the relative importance of the affected enzyme
- the functional properties of the mutant enzyme with regard to kinetic abnormalities and/or instability
- the ability to compensate for the enzyme deficiency by over-expressing isoenzymes or using alternative pathways
I. ENZYME DEFICIENCIES OF THE HEXOSE MONOPHOSPHATE SHUNT AND GLUTATHIONE METABOLISM

→ Inadequate levels of reduced glutathione (GSH): inability to withstand oxidative stress

→ **Acute hemolysis** induced by oxidant drugs, food, infection, stress

  Associated deficiencies:
  - Glucose-6-phosphate dehydrogenase (G6PD) (except for class I variants)
  - \( \gamma \)-Glutamylcysteine synthetase (GCS)
  - Glutathione synthetase (GSH-S)
  - Glutathione reductase (GR) (?)
II. ENZYME DEFICIENCIES OF GLYCOLYSIS AND NUCLEOTIDE METABOLISM

→ Continuously impaired ATP generation: lack of sufficient energy
→ Chronic hemolysis exacerbated by infection, pregnancy

Glycolisis
- pyruvate kinase (PK)
- hexokinase (HK)
- glucosephosphate isomerase (GPI)
- phosphofructokinase (PFK)
- aldolase
- triosephosphate isomerase (TPI)
- phosphoglycerate kinase (PGK)

Nucleotide Metabolism
- pyrimidine 5′-nucleotidase (P5N)
- adenylate kinase (AK)
Clinical heterogeneity

- Splenomegaly
- Janudice
- Iron overload (even in absence of transfusion)
- Not hematological symptoms
### Chronic hemolysis and non-hematologic signs

<table>
<thead>
<tr>
<th>Ubiquitous enzyme defect</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycolysis</strong></td>
<td>Hemolysis +</td>
</tr>
<tr>
<td>Phosphofructokinase (PFK)</td>
<td>+ Myopathy</td>
</tr>
<tr>
<td>Triose Phosphate Isomerase (TPI)</td>
<td>+ Neurom. dysfunctions</td>
</tr>
<tr>
<td></td>
<td>Susceptib. to infections</td>
</tr>
<tr>
<td>Phosphoglycerate Kinase (PGK)</td>
<td>+ Mental retardation</td>
</tr>
<tr>
<td></td>
<td>Neuromuscular abnorm</td>
</tr>
<tr>
<td>Aldolase</td>
<td>+ Mental retardation</td>
</tr>
<tr>
<td><strong>Glutathione metabolism</strong></td>
<td></td>
</tr>
<tr>
<td>(\gamma)-glutamylcysteine synthetase</td>
<td>+ Mental retardation</td>
</tr>
<tr>
<td>Glutathione synthetase</td>
<td>+ Mental retardation</td>
</tr>
</tbody>
</table>
## Frequency of RBC enzyme defects

<table>
<thead>
<tr>
<th>Red cell enzyme defects associated chronic with hemolytic anemia</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate kinase deficiency</td>
<td>&gt;500 families</td>
</tr>
<tr>
<td>Pyrimidine-5’-nucleotidase deficiency</td>
<td>&gt;60 families</td>
</tr>
<tr>
<td>Triosephosphate isomerase deficiency</td>
<td>50 – 100 cases</td>
</tr>
<tr>
<td>Phosphofructokinase deficiency</td>
<td>50 – 100 cases</td>
</tr>
<tr>
<td>Phosphoglycerate kinase deficiency</td>
<td>40 cases</td>
</tr>
<tr>
<td>Class I glucose-6-phosphate dehydrogenase deficiency</td>
<td>&gt;50 families</td>
</tr>
<tr>
<td>Glucose-6-phosphate isomerase deficiency</td>
<td>&gt;50 families</td>
</tr>
<tr>
<td>Glutathione synthetase deficiency</td>
<td>&gt;50 families</td>
</tr>
<tr>
<td>Hexokinase deficiency</td>
<td>20 cases</td>
</tr>
<tr>
<td>Adenylate kinase deficiency</td>
<td>12 families</td>
</tr>
<tr>
<td>Glutamate cysteine ligase deficiency</td>
<td>12 families</td>
</tr>
<tr>
<td>Aldolase deficiency</td>
<td>6 cases</td>
</tr>
<tr>
<td>Adenosine hyperactivity</td>
<td>3 families</td>
</tr>
<tr>
<td>Glutathione reductase deficiency</td>
<td>2 families</td>
</tr>
</tbody>
</table>

ENERCA from: “White Book for the creation of a European Reference Network of Expert Centers in Rare Anaemias.”
Laboratory diagnosis of HNSHA

- P5’N-I deficiency (Wright stained): marked basophilic stippling (2-12% of RBCs)
- PK deficiency, presence of echinocytes
Laboratory diagnosis of HNSHA

Exclusion of other causes of hemolytic anemia

Demonstration of a specific enzyme defect
(spectrophotometric assay, Beutler, 1984)

Other clinical symptoms may be helpful (e.g. neuromuscular symptoms, myopathy)

Molecular characterization of the defect
Factors that may influence enzyme activity

- Reticulocytosis (HK, PK)
- Contamination with donor RBCs in transfused patients
- Incomplete leukocyte removal
- Storage and shipment of samples (e.g. instability of PFK, TPI)
- Expression of isoenzyme in mature RBCs
- Mutant PKs with normal catalytic activity “in vitro”

RBC enzyme defects: molecular heterogeneity

<table>
<thead>
<tr>
<th>Red cell enzyme defects</th>
<th>No of cases</th>
<th>Gene</th>
<th>Chromosomal Localization</th>
<th>No. of mutations Described</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK</td>
<td>&gt;500</td>
<td>PKLR</td>
<td>1q22</td>
<td>&gt;200</td>
</tr>
<tr>
<td>GPI</td>
<td>&gt;50 fam</td>
<td>GPI</td>
<td>19q13</td>
<td>31</td>
</tr>
<tr>
<td>TPI</td>
<td>31 fam</td>
<td>TPI1</td>
<td>12p13</td>
<td>18</td>
</tr>
<tr>
<td>PFK</td>
<td>&gt;40 cases</td>
<td>PFKM</td>
<td>12p13/21q22</td>
<td>17</td>
</tr>
<tr>
<td>PGK</td>
<td>&gt; 40 cases</td>
<td>PGK-1</td>
<td>Xq13</td>
<td>20</td>
</tr>
<tr>
<td>HK</td>
<td>20 cases</td>
<td>HK-1</td>
<td>10q22</td>
<td>5</td>
</tr>
<tr>
<td>Aldolase</td>
<td>6 cases</td>
<td>AldoA</td>
<td>16q22</td>
<td>4</td>
</tr>
</tbody>
</table>

Usefulness if genotyping:
- Prenatal testing
- Diagnosis confirmation
...If genotype is not complete → exclusion of other causes of hemolysis!
ENERCA SURVEY – 26 Centers involved
Availability of PK activity assay
ENERCA SURVEY – 26 Centers involved
Availability of rare glycolytic enzyme assay