Current diagnostic approaches and screening methods for hereditary spherocytosis

3rd Pan-European Conference on Haemoglobinopathies & Rare Anaemias
Limassol, 24 – 26 October 2012

Paola Bianchi
Foundation IRCCS Ca’ Granda Ospedale Maggiore Policlinico
Pathophysiology of Anemia Unit
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
### Results of the survey: “Facilities for patients with rare and very rare anaemias”

Number of registered patients affected by rare anemias (70 centres involved)

<table>
<thead>
<tr>
<th>Disease/Disorders</th>
<th>Number of centres concerned</th>
<th>Total number of patient registered:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>reference centres</td>
</tr>
<tr>
<td>RBC membrane defects</td>
<td>57</td>
<td>1546</td>
</tr>
<tr>
<td>RBC enzymes</td>
<td>56</td>
<td>432</td>
</tr>
<tr>
<td>CDA</td>
<td>36</td>
<td>104</td>
</tr>
<tr>
<td>DBA</td>
<td>38</td>
<td>115</td>
</tr>
<tr>
<td>PNH</td>
<td>38</td>
<td>202</td>
</tr>
<tr>
<td>Hereditary sideroblastic anaemia</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>Very rare anaemia – defective iron utilization</td>
<td>20</td>
<td>93</td>
</tr>
<tr>
<td>Other anemia (ie: FA, AA, Rh null)</td>
<td>11</td>
<td>173</td>
</tr>
</tbody>
</table>

From: “ENERCA WhiteBook for the creation of a European Reference Network of Centres of Expertise on Rare anaemias” (in press).
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
“Vertical interactions”
Adapted from: Liu SC, Derick LH, Semin Hematol 1992
Two factors are implicated in the pathophysiology of HS: an intrinsic red cell membrane defect and an intact spleen that selectively retains, damages, and removes the defective erythrocytes. The diversity of membrane imbalance is likely to result in different red cell clearance mechanisms.
HEREDITARY SPHEROCYTOSIS

- Pathophysiology
- Clinical aspects
- Laboratory investigations
- Results of the ENERCA survey
PATIENTS

- 300 HS pts from 212 families
- 141 M and 159 F
- Age at diagnosis 20 yrs (1-80 yrs): 40% <18 yrs 60%: adults
- Dominant in 70% of case, 55% among families
- 41 pts splenectomised; 21 underwent splenectomy during follow-up and were re-evaluated after surgery.

Haematologica, 2008; 93(9), 1310
SDS-PAGE analysis of red cell membrane proteins

Band 3: 53% cases
Spectrin: 33% cases
Ankyrin: 5% cases
4.2 protein: 2 cases/1fam
No abnormalities: 10%

Combined:
4.2/Band3, 4.2/spectrin
Ankyrin/Spectrin
CLINICAL DATA IN 259 NOT SPLENECTOMIZED HS PATIENTS

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

EXCHANGE TRANSFUSION: 14/82 cases
EFFECTS OF SPLENECTOMY
Haematologic and biochemical data of 21 HS patients before and after splenectomy

<table>
<thead>
<tr>
<th>Haematologic</th>
<th>Pre-splenectomy</th>
<th>Post-splenectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>10.8 (7.6-15.1)</td>
<td>13.9 (12.6-18.8)</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>84 (68-106)</td>
<td>84 (73-95)</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>35.4 (28.3-38.8)</td>
<td>34.8 (33.3-37.1)</td>
</tr>
<tr>
<td>Spherocytes (%)</td>
<td>9 (1-32)</td>
<td>4 (3-16)</td>
</tr>
<tr>
<td>Reticulocytes (x10⁹/L)</td>
<td>337 (96-640)</td>
<td>51 (11-118)</td>
</tr>
<tr>
<td>Unc. bilirubin (mg/dL)</td>
<td>1.9 (0.7-8.9)</td>
<td>0.7 (0.35-1.83)</td>
</tr>
</tbody>
</table>

- Haemoglobin levels: 10.8 g/dL → 13.9 g/dL
- Reticulocytes: 337x10⁹/L → 51x10⁹/L
- Unconjugated bilirubin: 1.9 mg/dL → 0.7 mg/dL

- Effects more evident in Band 3 vs Spectrin
- Effects of splenectomy comparable in young (n=6) and adult patients.
HEREDITARY SPHEROCYTOSIS

- Pathophysiology
- Clinical aspects
- 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 (Ret, Bil, Apto, LDH, Hburia, hemosiderinuria, SF)
- Congenital or acquired
- Extrahematological signs

**CONGENITAL CAUSES**

Blood smear analysis

**RBC morphologic abnormalities**
(spherocytes, elliptocytes, ovalocytes, stomatocytes, marked anyso-poikilocytosis)

**RBC MEMBRANE DEFECTS / CDAs**

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

**Hereditary Spherocytosis**
**Hereditary Elliptocytosis**
**SAO**
**Hereditary Stomatocytosis**
**CDAs**

**ACQUIRED CAUSES**

Direct Antiglobulin Test (DAT)

**unremarkable**

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

**RBC ENZYMOPATHIES**

Study of RBC metabolism

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

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

**Molecular analysis**

**RBC MEMBRANE DEFECTS / CDAs**

**Hereditary Spherocytosis**
**Hereditary Elliptocytosis**
**SAO**
**Hereditary Stomatocytosis**
**CDAs**

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

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

**CD55/59**

**positive**

**negative**

**Schistocytes**

**PNH**

**null**

**INFECT/TOXIC CAUSES**
Wilson disease

**MECHANICAL HEMOLYSIS**

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

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

Reconsider congenital causes or DAT-negative AIHA
Table IV. Diagnostic parameters for hereditary spherocytosis.

<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.
RBC morphology

residual Sp=81%

residual Sp=53%
RBC morphology

HS (B3 deficiency)  HS (4.2 deficiency)
Haematological parameters of 259 not splenectomized HS patients

Not always standard hematologic parameters give specific diagnostic indications!
## Guidelines for the diagnosis and management of hereditary spherocytosis

Br J Haematol 126:455-474, 2004

P. H. B. Bolton-Maggs¹, R. E. Stevens², N. J. Dodd³, G. Lamont⁴, F. Tittensor⁵ and M. J. King⁶ on behalf of the General Haematology Task Force of the British Committee for Standards in Haematology

---

### Table VI. 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> (Parpart et al, 1947)</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> (Zanella et al, 1980)</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>A modified AGLT.</td>
<td></td>
</tr>
<tr>
<td><strong>Osmotic gradient ektacytometry</strong> (Clark et al, 1983)</td>
<td>A laser diffraction viscometer that measures red cell deformability at constant shear stress as a continuous function of suspending osmolality (hypotonic to hypertonic).</td>
<td>Distinct deformability curves for red cells from patients with HS, hereditary elliptocytosis, hereditary pyropoikilocytosis, stomatocytosis and sickle disease. (Mohandas et al, 1980)</td>
</tr>
<tr>
<td><strong>Hypertonic cryohaemolysis test</strong> (Streichman &amp; Gescheidt, 1998)</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> (King et al, 2000)</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>
EMA-binding test

- 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

<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><strong>Total HS patients</strong></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><strong>HS with biochemical defect</strong></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><strong>HS with undetectable defect</strong></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>

**Sensitivity:**
- **Total HS patients:** 93%
- **GLT:** 61%
- **AGLT:** 95%
- **Pink:** 91%
- **OF NaCl fresh:** 68%
- **OF NaCl inc.:** 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

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

- 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
  
  - 133/150 (88%) EMA+AGLT+
  - 7/150 (5%) EMA+AGLT-
  - 10/150 (7%) EMA-AGLT+

Bianchi et al, Haematologica 2012
Disease specificity of diagnostic tests

**EMA binding**

- HS
- CDAII
- CDAI
- AIHA
- Enzyme def
- HE (4.1 def)
- PNH
- Mechanical
- Stomatocytosis
- Unknown

% decrease in fluorescence

**Acidified Glycerol Lysis Test (AGLT)**

- HS
- CDAII
- CDAI
- AIHA
- Enzyme def
- HE (4.1 def)
- PNH
- Mechanical
- Stomatocytosis
- Unknown

seconds

**Pink Test**

- HS
- CDAII
- CDAI
- AIHA
- Enzyme def
- HE (4.1 def)
- PNH
- Mechanical
- Stomatocytosis
- Unknown

**Osmotic Fragility curve after incubation (OF NaCl inc)**

- Decreased OF
- Slightly decreased OF
- Normal OF
DIFFERENTIAL DIAGNOSIS OF HS AND CDAII

SDS-PAGE analysis of RBC membrane proteins

13% of patients referred with a suspect of HS were CDAII
Hereditary Spherocytosis

- Pathophysiology
- Clinical aspects
- Laboratory investigations
- Results of the ENERCA survey
**SURVEY TO ESTABLISH AN EUROPEAN NETWORK FOR RED BLOOD CELL ENZYME AND MEMBRANE DISORDERS**

**Structure of the questionnaire**

**ORGANIZATION:**

**DEPARTMENT:**

**CONTACT PERSON NAME:** FirstName Surname

**CITY/TOWN:**

**ZIP CODE:**

**PHONE (including country and area code):**

**FAX:**

**PROFESSION (e.g. physician, researcher):**

<table>
<thead>
<tr>
<th>Red cell disorders</th>
<th>Mean number of new laboratory diagnosis in one year</th>
<th>Number of patients in regular follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hereditary spherocytosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hereditary elliptocytosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pyropoikilocytosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stomatocytosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Congenital dyserythropoietic anemias type II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glucose-6-phosphate dehydrogenase deficiency</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pyrurate kinase deficiency</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Other red blood cell enzyme deficiencies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Other red blood cell membrane disorders</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**RED CELL MEMBRANE DISORDERS**

Diagnostic tests performed (please indicate if performed in your lab (A) or elsewhere (B)*

<table>
<thead>
<tr>
<th><strong>WHEN THEY ARE USED</strong></th>
<th><strong>ON ALL CASES</strong></th>
<th><strong>ONLY WHEN...</strong></th>
<th><strong>NEVER USED</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A/B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**RED CELL ENZYME DISORDERS**

<table>
<thead>
<tr>
<th><strong>Total no. of deficiencies detected</strong></th>
<th><strong>Enzymatic assay (method used</strong>)**</th>
<th><strong>Molecular characterization</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzymes of glycolysis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Enzymes of hexose-monophosphate shunt and glutathione metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Enzymes of nucleotide metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Other red blood cell enzyme activities</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glycolysis intermediates</strong></td>
<td>Method used**</td>
<td></td>
</tr>
</tbody>
</table>

15 different tests considered

26 different enzymes considered
Survey on red cell membrane disorders and enzyme defects
Centres involved: 26
Centers involved the diagnosis of red cell membrane defects

- Centers performing laboratory diagnosis (median n. of diagnosis in 1 year)
- Centers performing clinical follow-up (n. of patients in regular follow up)

- About 100 cases
- About 50 cases
- About 30 cases
- About 1-3 cases
Use of diagnostic tests performed for diagnosis of red cell membrane defects

% of centers
Number of diagnostic tests / Center

Most centres use a “battery of tests” (3-6 different tests)

*Tests considered: NaCl on fresh and incubated blood (2 different concentrations or curve); glycerol lysis tests; flow cytometric Ema-binding test; SDS-PAGE analysis; Ecktacytometer/Lorca
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

EMA-binding test

- Pink test
- OF
- Ectacytometer

8
3
1
1
1
11
The diagnosis of HS can be easy in typical cases but it could be difficult in atypical/mildest cases.

Family history/clinical examination/red cell morphology evaluation are very important for the diagnosis of HS.

As observed from ENERCA surveys no systematic register is available for this disorder and its frequency may be underestimated.

As confirmed by ENERCA survey a battery of tests (or at least one direct and one indirect) is strongly suggested to reach a correct diagnosis.
THANKS!

- ERASME, Clinical Chemistry
- CHU Liege, Human Genetics
- Specialized Hospital for Active Treatment of Haematologic Diseases
  Laboratory of Cytogenetics and Molecular Biology
- CHU of Montpellier, Laboratory of Haematology
- AP-HP, Hôpital Bicêtre, Hematology Lab
- Centre Hospitalier Universitaire (CHU)
  Laboratoire de génétique moléculaire et biochimie
- Robert Debré Hospital, AP-HP, Hematology laboratory
- University of Würzburg (Universitätsklinikum Würzburg)
  Department of Paediatrics and Internal Medicine II (Red Cell Laboratory)
- Zentrum für Kinder- und Jugendmedizin, Universitätsklinikum Heidelberg
  Kinderheilkunde III
- Universitätsmedizin Göttingen (UMG), Pädiatrie I
- Istituto Superiore di Sanita’,
  Dep. Haematology, Oncology and Molecular Medicine
- E.O. Ospedali Galliera, Ematologia,
  Centro della Microcitemia e Anemie Congenite
- 2a Clinica Pediatrica Università degli Studi Cagliari-Ospedale Micrctemico
- Università Cattolica del Sacro Cuore, Dipartimento di Ematologia
- University Federico II Naples - Pediatrics
  CEINGE Biochemistry and Medical Biotechnologies
- Ospedali Riuniti Villa Sofia Cervello - UOC Ematologia per le Malattie
  Rare del Sangue e degli Organi Ematopoietici
- Foundation IRCCS Ca’ Granda Ospedale Maggiore - Hematology 2 Unit
- University Medical Center Utrecht, Clinical Chemistry and Haematology
- Sanquin Diagnostic Services, Red Cell Diagnostics
- Centro Hospitalar de Coimbra - Hematology
- Medical University of Warsaw, Pediatric Hematology/Oncology
- Hospital Clinic . University of Barcelona, Red Cell Pathology Unit
- Hospital Clinico San Carlos – Servicio de Hematologia y Hemoterapia
  King College Hospital, Red Cell Laboratory
- International Blood Group Reference laboratories, NHS Blood and Transplant
  Membrane Biochemistry