Molecular Diagnosis in Neonatal Hereditary Hemolytic Anemia

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Learning Objectives

List the causes of neonatal hereditary hemolytic anemia (HHA)

Identify the limitations of routine diagnostic tests in these disorders

Discuss the association of HHA with neonatal hyperbilirubinemia/acute bilirubin encephalopathy

Recognize the advantages of molecular diagnostic tests in these disorders
What is Hemolytic Anemia?

• Characterized by premature destruction of red blood cells (RBC’s) within the circulatory system

  » RBC’S normally survive 60-120 days
  » Bone marrow has the capacity to increase the production of RBCs 6-8 times than normal

• Anemia develops when the bone marrow cannot adequately compensate for the shortened life span of the red blood cells in the circulation
Causes of Hereditary Hemolytic Anemia (HHA)

» RBC membrane defects
  ▪ Hereditary spherocytosis
  ▪ Hereditary elliptocytosis/Hereditary pyropoikilocytosis
  ▪ Hereditary stomatocytosis

» Red cell enzyme deficiencies
  ▪ G6PD deficiency
  ▪ Pyruvate kinase deficiency

» Hemoglobin synthesis abnormality
  ▪ Thalassemias
  ▪ Hemoglobin S, C and E disorders
Red Cell Membrane Cytoskeleton

PMID: 28698843 Slide courtesy Dr Coumarane, ARUP
**Hereditary Spherocytosis (HS)**

- Common inherited hemolytic anemia (1/1000 - 1/3000)

- All ethnic groups; common in Northern Europeans

- AD (75%) and AR (25%)

- The disease is diagnosed in only one third of affected infants during the first year of life
HS: Clinical Presentation

• Highly variable depending on the underlying defect

• 60% - intermediate-severity phenotype
  » Moderate hemolysis (Hb 8-11 g/dL)
  » Reticulocyte count > 8%
  » Normal hemoglobin at birth that may sharply and transiently decline in the first 3 weeks of life

• 20% - mildly affected/asymptomatic
  » May not be identified until there is a hemolytic crisis in childhood
  » Often triggered by viral infection
  » May be diagnosed due to aplastic crisis with parvovirus B19

• 20% - have severe disease
  » Usually, AR inheritance due to abnormality of α-spectrin
• Hereditary elliptocytosis (HE)/ Hereditary pyropoikilocytosis (HPP)
HE/HPP

• HE /HPP- heterogenous clinical presentations
• HE- mostly asymptomatic, but HPP is a severe form of HE that presents with hemolytic anemia and jaundice during the infantile period
• Erythrocyte morphology in HPP resembles that of blood smears in thermal burns with poikilocytes, red blood cell fragments, microspherocytes, and elliptocyte
• HE - AD while HPP is an AR disorder
Hereditary Elliptocytosis

https://imagebank.hematology.org/image/61156/hereditary-elliptocytosis
Hereditary Pyropoikilocytosis
HE/HPP Pathophysiology

• Principal defect is mechanical weakness or fragility of the erythrocyte membrane skeleton

• Due to defects in various membrane proteins, including $\alpha$- and $\beta$-spectrin, protein 4.1, and glycophorin C

• Spectrin integrity is dependent on the self-association of heterodimers of $\alpha$- and $\beta$-spectrin into mature spectrin molecules that are critical for membrane stability and erythrocyte shape and function
Hereditary Stomatocytosis

• Stomatocytosis is associated with abnormalities in red cell cation permeability

• A mouthlike or slitlike pattern replaces the normal central zone of pallor
  » Only seen in a subset

• Changes in red cell volume, which may be either increase (overhydrated) or decreased (xerocytosis), or, in some cases, near normal

• The pathobiology of the stomatocytic shape is poorly understood
Dehydrated Hereditary Stomatocytosis (DHST)

- Also known as hereditary xerocytosis - most common inherited red cell cation permeability disorders
- Characterized by a mild increase in potassium permeability that is sufficient to lead to the gradual loss of red cell $K^+$ and water, and to red cell dehydration, stiffness, and hemolysis
- High MCHC, and resistant osmotic fragility are characteristics
- Hemoglobin and hematocrit values are often normal (compensated hemolysis) and patient blood smears are surprisingly normal, featuring mostly a few target
- Unexplained iron overload

Hereditary Stomatocytosis: Genetic Etiology

- Most reported DHS cases are caused by gain-of-function mutations in the gene **PIEZ01** (16q24.3) which encodes part of a mechanosensitive ion channel

- Rarely **KCNN4** gene that encodes a Gardos channel
Diagnosis of HS, HE/HPP and Stomatocytosis

• Peripheral blood smear- elliptocytes, spherocytes, stomatocytes and fragmented cells (in HPP) are seen

• Osmotic fragility
  » A laboratory test used in the diagnosis of HS, is sensitive but not specific. The test measures the *in vitro* lysis of RBCs suspended in solutions of decreasing osmolarity
  » Spherocytes are characterized by membrane loss and less redundancy
  » Not very specific for any of these disorders
Osmotic Fragility Test

Osmotic Fragility Graph

% Hemolysis

% NaCl

- PATIENT
- Low Range
- High Range
Diagnosis Continued!

- Flow cytometry
  - Measures the fluorescent intensity of intact red blood cells labelled with EMA (eosin-5-maleimide)
  - EMA binds specifically with band 3 protein
  - EMA binding is affected by all sorts of membrane protein abnormalities, not just band 3 deficiency
  - Greater than 95% sensitive and specific for HS
Ektacytometry

- Briefly, RBC are subjected to a defined value of shear stress and an osmotic gradient, and the laser diffraction pattern generated by the RBC suspension is recorded.
Bryce Canyon
Enzyme deficiency
# Enzymopathies

<table>
<thead>
<tr>
<th>Clinical phenotypes</th>
<th>Genes</th>
<th>Location</th>
<th>Inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC enzymopathies</td>
<td></td>
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</tr>
<tr>
<td>G6PD deficiency</td>
<td>G6PD</td>
<td>Xq28</td>
<td>XR</td>
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<tr>
<td>Pyruvate kinase deficiency</td>
<td>PKLR</td>
<td>1p22</td>
<td>AR</td>
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<tr>
<td>Enolase deficiency</td>
<td>ENO1</td>
<td>1p36.23</td>
<td>AD</td>
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<td>Adenylate kinase deficiency</td>
<td>AK1</td>
<td>9q34.11</td>
<td>AR</td>
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<td>Glucose phosphate isomerase deficiency</td>
<td>GPI</td>
<td>19q13.11</td>
<td>AR</td>
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<tr>
<td>Pyrimidine 5’ nucleotidase (UMPH1) deficiency</td>
<td>NT5C3A</td>
<td>7p14.3</td>
<td>AR</td>
</tr>
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<td>Gamma-glutamylcysteine synthetase deficiency</td>
<td>GCLC</td>
<td>6p12.1</td>
<td>AR</td>
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<tr>
<td>Glutathione peroxidase deficiency</td>
<td>GPX1</td>
<td>3p21.31</td>
<td>AR</td>
</tr>
<tr>
<td>Glutathione reductase deficiency</td>
<td>GSR</td>
<td>8p12</td>
<td>AR</td>
</tr>
<tr>
<td>Glutathione synthetase deficiency</td>
<td>GSS</td>
<td>20q11.22</td>
<td>AR</td>
</tr>
<tr>
<td>Hexokinase deficiency</td>
<td>HK1</td>
<td>10q22.1</td>
<td>AR</td>
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<tr>
<td>Bisphosphoglycerate mutase deficiency</td>
<td>BPGM</td>
<td>7q33</td>
<td>AR</td>
</tr>
<tr>
<td>Phosphoglycerate kinase 1 deficiency</td>
<td>PGK1</td>
<td>Xq21.1</td>
<td>XR</td>
</tr>
<tr>
<td>Triosephosphate isomerase deficiency</td>
<td>TPI1</td>
<td>12p13.31</td>
<td>AR</td>
</tr>
</tbody>
</table>

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; XR, X-linked recessive.
Glucose 6 phosphate Dehydrogenase Deficiency

• The most common human enzyme defect! >400-600 million people
• X linked
• Most prevalent in populations with endemic malaria
• Enzyme deficiency leading to ↓ NADPH-glutathione (GSH) depletion
  » ↑ Oxidative stress, ↑ Hemolysis

• **Diagnosis**
  » ↓ G6PD (false negative with ↑ reticulocytes)
  » Heinz bodies (denatured Hb) and Bite cells in PB smear
Heinz (denatured Hb) and bite cells
Pyruvate Kinase Deficiency

• Common cause of congenital non-spherocytic chronic hemolytic anemia
• Autosomal recessive disorder
• Found in north Europeans; high frequency in Pennsylvania Amish population
• Diagnosis
  » Direct measurement of enzyme activity in RBCs - ↓ PK activity
• Severe disease may require frequent red cell transfusion throughout infancy and into adulthood.
• Splenectomy ameliorates the severity of hemolysis
PK Deficiency

• Treatment - supportive

• Data from a phase II study in PK deficiency patients treated with AG-348 showed that approximately half of treated subjects experienced a rise in hemoglobin (Hb)

Arches National Park, Utah
Clinical Features of HHA

• Onset may be acute or chronic
• Symptoms and signs of anemia-pallor, fatigue
• RBC break down-increased bilirubin (hyperbilirubinemia)
  » Jaundice and gall stones
  » Neonatal toxicity
• Crises (chronic hemolytic disease)
  » Hemolytic (increased splenic activity)
  » Aplastic (B19 Parvo-virus)
  » Megaloblastic
Hyperbilirubinemia and Its Consequences

• Bilirubin needs to be conjugated and excreted in bile

• Conjugation in the liver happens by the enzyme uridine diphosphoglucuronic acid (UDP) glucuronyltransferase

• Gene involved in **UGT1A1**
  » Gilbert (usually due to additional TA repeats) and Crigler-Najjar syndrome (due to AR mutations)

• Solute carrier organic anion transporter family members 1B1 and 1B3 (**SLCO1B1** and **SLCO1B3**)
  » Rotor syndrome
Hyperbilirubinemia: Neonates

• Increased unconjugated bilirubin can pass blood brain barrier - neurotoxicity in neonates

• Pathophysiology
  » Increased bilirubin production (twice/kg body weight as adults)
  » Delayed UGT1A1 induction

• Risk factors:

<table>
<thead>
<tr>
<th>Genetic factors</th>
<th>Maternal/Perinatal/Other neonatal factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6PD deficiency</td>
<td>Diabetes, Rh incompatibility, Age, Race</td>
</tr>
<tr>
<td>RBC membrane defects (HS, HE)</td>
<td>Mode of delivery, birth trauma, delayed cord clamping</td>
</tr>
<tr>
<td>Hemoglobinopathies</td>
<td>Congenital infections</td>
</tr>
<tr>
<td>Gilbert's syndrome (UGT1A1 deficiency)</td>
<td>Nutritional deficiencies</td>
</tr>
<tr>
<td>Crigler-Najjar syndrome (UGT1A1 deficiency)</td>
<td></td>
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</table>
Park City, Utah
Why Do We Need Molecular Testing?
Utility of this testing during neonatal period

- HS of AR variant without family history
- Screening tests not that useful during neonatal period
- Spherocytes on the blood smear are not specific for HS and peripheral smear findings are not informative in all cases
- Some patients may require transfusion, making the biochemical testing unreliable and uninformative
- G6PD/PK deficiency of mild-to-moderate severity can be missed in neonates when reticulocyte counts are high
- To diagnose complex interactions (HHA +Bilirubin metabolism disorders)
Molecular Approach

• Focused targeted Next-generation sequencing (NGS) provides a cost-effective and relatively rapid approach

• Evaluated since 2012 with gene counts on these panels varying from 28-70

• Diagnostic yield - 30-75%

Table 1 Recent studies performed by targeted NGS panels in patients with hemolytic anemia. Number of the genes included in the panel, patient studied, overall sensitivity and, when available, sensitivity of panel in patient with undefined hemolytic anemia are reported.

<table>
<thead>
<tr>
<th>Studies</th>
<th>No. of genes analysed</th>
<th>No. of cases</th>
<th>Overall sensitivity</th>
<th>Sensitivity in hemolytic patients with no previous diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chonat et al., 2019 (21)</td>
<td>32 (membrane defects)</td>
<td>11 (HS)</td>
<td>100%</td>
<td>Not studied</td>
</tr>
<tr>
<td>van Vuren et al., 2019 (16)</td>
<td>7 (membrane defects)</td>
<td>95 (HS)</td>
<td>89%</td>
<td>Not studied</td>
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<tr>
<td>Xue et al., 2019 (1)</td>
<td>10 (membrane defects)</td>
<td>10 (HS)</td>
<td>90%</td>
<td>Not studied</td>
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<tr>
<td>Pang et al., 2018 (20)</td>
<td>n.a.</td>
<td>51 (HS)</td>
<td>72%</td>
<td>Not studied</td>
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<tr>
<td>Li et al., 2018 (17)</td>
<td>217</td>
<td>46 (CHA)</td>
<td>60.9%</td>
<td>n.a.</td>
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<tr>
<td>Russo et al., 2018 (14)</td>
<td>34 and 71</td>
<td>74 (CHA)</td>
<td>64.9%</td>
<td>45.8%</td>
</tr>
<tr>
<td>Agarwal et al., 2010 (18)</td>
<td>20</td>
<td>17 (CHA)</td>
<td>70%</td>
<td>70%</td>
</tr>
<tr>
<td>Roy et al., 2016 (13)</td>
<td>33</td>
<td>57 (CHA)</td>
<td>38.6%</td>
<td>11%</td>
</tr>
</tbody>
</table>

CHA, chronic hemolytic anemias; HS, hereditary spherocytosis; n.a., not available.
Targeted NGS Panels: Importance

• Russo et al. - 74 patients with HHA with an emphasis on congenital dyserythropoietic anemia (CDA)

• One of their interesting findings was CDA misdiagnosed as enzyme deficiency particularly PK deficiency

  » Out of 22 patients initially diagnosed as CDA, 10/22 were later diagnosed with enzyme deficiencies
# Genes Involved in Hereditary Membrane Disorders

<table>
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<tr>
<th>Condition</th>
<th>Genes</th>
<th>Inheritance</th>
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</thead>
<tbody>
<tr>
<td>Hereditary spherocytosis</td>
<td>ANK1, SLC4A1, SPTB, EPB42, SPTA1</td>
<td>AD/AR</td>
</tr>
<tr>
<td>Hereditary elliptocytosis/pyropoikilocytosis</td>
<td>SPTA1, SPTB, EPB41</td>
<td>AD/AR</td>
</tr>
<tr>
<td>Hereditary stomatocytosis</td>
<td>PIEZO1, KCNN4</td>
<td>AD</td>
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## Liver Related

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<tr>
<th>SLC01B1</th>
<th>Hyperbilirubinemia, rotor type</th>
<th>AR (digenic)</th>
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<tbody>
<tr>
<td>SLC01B3</td>
<td>Hyperbilirubinemia, rotor type</td>
<td>AR (digenic)</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>Crigler-Najjar syndrome, types I and II</td>
<td>AR</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>Hyperbilirubinemia, transient familial neonatal</td>
<td>AR</td>
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</table>

## Additional Conditions

<table>
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<tr>
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<th>Genes</th>
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<td>XR</td>
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<tr>
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<td>PKLR,</td>
<td>AR</td>
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<tr>
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<td>AR</td>
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<td>PGK1</td>
<td>XL</td>
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Our Recent Findings:

https://doi.org/10.1182/blood-2018-99-112589

• 268 patients evaluated using targeted NGS panel with 28 genes

• Age - newborn to 62 years

• Mild lifelong anemia to severe hemolytic anemia with extreme hyperbilirubinemia
Continued!

- We identified pathogenic and likely pathogenic variants in 64/268 (24%) patients that were clearly responsible for the disease phenotype (e.g., moderate to severe HA)
- Complex interactions (12/64)
- 11% (29/268) - UGT1A1 gene
Case Study

• Caucasian ethnicity, lacking regular well child visits
• Presented at 3.5 yrs -with mild anemia and splenomegaly
• The peripheral blood smear—anemia with many spherocytes
• Past history-significant for hyperbilirubinemia in the newborn period
• Parents declined childhood immunizations and transfusions; the patient’s anemia and splenomegaly worsened
• Genetic testing was permitted
  » after dilated cardiomyopathy developed
Case Study: Continued

• Two variants - \textit{SPTA1} gene identified
  » A novel mutation (c.7134+2T>G, p.?), predicted to cause abnormal splicing of \textit{SPTA1} gene, was identified in addition to heterozygous low expression variants \textit{αLEPRA}

• \textit{αLEPRA} in \textit{trans} to a pathogenic \textit{SPTA1} variant- associated with autosomal recessive HS

• A third heterozygous variant in the \textit{SPTB} gene (c.4564-4G>A)
  » \textit{SPTB} variant - one child with HS and reduced \textit{SPTB} mRNA level.
  » Previous computational study predicted this \textit{SPTB} variant would result in abnormal splicing of \textit{SPTB} gene; however its effect on splicing remains to be determined experimental
Case Study: Continued

• Given the genetic testing results, the parents consented to immunizations, and splenectomy

• Four months after splenectomy, the patient’s hemoglobin improved to 15.2g/dL and echocardiogram changes were resolving
Summary

• Our results demonstrate that many patients with hemolytic anemia harbor complex combinations of known and novel mutations in RBC cytoskeleton/enzyme genes.

• Their clinical significance can be further augmented by polymorphisms of UGT1A1 gene contributing to severe neonatal hyperbilirubinemia and its consequences in pediatric population.

• Molecular diagnosis can be helpful to understand the pathophysiology.
Lake Powell, Utah
ARUP is a nonprofit enterprise of the University of Utah and its Department of Pathology.