Laboratory Diagnosis of Hemolytic Anemia

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Program Objectives

• Discuss causes of hemolytic anemia
• Understand the pathophysiology of hemolytic anemia
• Discuss appropriate tests for the underlying disorder causing hemolytic anemia
• Discuss the clinical utility of the G6PD enzyme activity test, HPLC evaluation for hemoglobinopathies, and molecular diagnostics
What is Hemolytic Anemia?

• Characterized by premature destruction of circulating red blood cells within the circulatory system
• Anemia develops when the bone marrow cannot adequately compensate for the shortened life span of the red blood cells in the circulation
Classification of Hemolytic Anemia

• Hereditary
  – Membrane defects
  – Red cell enzymes
  – Hemoglobin synthesis abnormality (thalassemia/hemoglobinopathies)

• Acquired
  – Immune
    • Infections
    • Alloantibodies
    • Autoantibodies
  – Non-immune
    • Mechanical damage
    • Physiochemical damage
    • Membrane abnormalities
Classification of Hemolytic Anemia-Continued

• Intrinsic
  – Red blood cell membrane defects
  – Red blood cell enzyme defects
  – Hemoglobinopathies

• Extrinsic
  – Immune mediated hemolysis
  – Physical damage to red blood cells like toxins, thermal injury and mechanical disruption

Practical Diagnosis of Hematologic Disorders (Kjeldsberg, Practical Diagnosis of Hematologic Disorders)
Clinical Presentations of Hemolytic Anemia

- New onset of pallor and anemia
- Jaundice
- Gallstones
- Splenomegaly and later hepatomegaly
Lets Elaborate on the Hereditary Causes of Hemolytic Anemia

– Membrane defects
  • Hereditary spherocytosis
  • Hereditary elliptocytosis/pyropoikilocytosis

– Red cell enzymes
  • G6PD deficiency
  • Glycolytic pathway enzyme deficiencies
  • Glutathione pathway deficiency

– Hemoglobin synthesis abnormality
  • Thalassemias
  • Hemoglobin S, C and E disorders
Hereditary Spherocytosis (HS)

- Most common hemolytic anemia due to red cell membrane defect
- Alteration of one of the five genes which encode for proteins involved in the vertical association
- Occurs in all racial groups and is particularly common in individuals of northern European ancestry, affecting approximately one person in 3000
Red Cell Membrane Structure

- Five interconnected proteins are involved in the coupling of the cytoskeleton to the lipid bilayer
  - Spectrin (composed of alpha, beta heterodimers)
  - Ankyrin
  - Pallidin (band 4.2)
  - Band 4.1 (protein 4.1)
  - Band 3 protein (the anion exchanger, AE1)
  - RhAG (the Rh-associated glycoprotein)
Peripheral Smear of Hereditary Spherocytosis

Jaffe et al., Hematopathology. Elsevier, 2011
Hereditary Spherocytosis, Continued

• It is a common inherited hemolytic anemia (1 in 1000 to 1 in 3000)
• Dominant inheritance (75%)
• Although non-dominant and recessive inheritance (25%) have been described
• The disease is diagnosed in only one third of affected infants during the first year of life
• The clinical manifestations of HS vary widely
• Mild, moderate, moderately severe
Hereditary Spherocytosis, Continued

• Ankyrin mutation is the most common cause of HS in Northern European populations accounting for approximately 50–60% of cases but it is found in only 5–10% of HS cases in Japan

• Spectrin deficiency is often present in HS

• Even in those conditions where primary mutation is in non-spectrin protein as alteration in these proteins adversely affect the assembly of spectrin onto the membrane protein

• The clinical severity is correlated well with the spectrin deficiency
Hereditary Elliptocytosis

- It is a relatively common, clinically and genetically heterogeneous disorder
  - elliptically-shaped red cells on peripheral blood smear
- HE has a worldwide distribution but is more common in malaria endemic regions with prevalence approaching 2% in West Africa
- Inheritance of HE is autosomal dominant
- The clinical presentation of HE is heterogeneous
Hereditary Elliptocytosis (HE)
Hereditary Pyropoikilocytosis (HPP)

- Increased thermal sensitivity of the red cells and the unusual morphological features
- Recent molecular studies have clearly established it as a subset of HE due to either homozygous or compound heterozygous mutations in spectrin leading to severe disruption of spectrin self association
Hereditary Pyropoikilocytosis (HPP)
Diagnosis of HS, HE and HPP

- Peripheral blood smear - Spherocytes, elliptocytes and fragmented cells (in HPP) are seen

- An abnormally high (MCHC) is almost always found. In fact, among non-neonates, an MCHC >35.5 g/dL is said to be pathognomonic for spherocytes

- 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 to withstand
Example of Osmotic Fragility
Diagnosis Continued!

- Flow cytometry
  - Greater than 95% sensitive and specific for HS
  - Labels patient’s intact RBCs with EMA (eosin-5-maleimide)
  - EMA reacts covalently with Lys-430 on the first extracellular loop of band 3 protein
  - EMA binding is affected by all sorts of membrane protein abnormalities, not just band 3 deficiency
Example of EMA

A. Normal Neonate

B. Neonate With HS
Glucose 6-phosphate Dehydrogenase
Glucose 6 phosphate Dehydrogenase Deficiency

• The most common human enzyme defect!
• Affects 400-600 million people worldwide (about 10% of the world’s population)
• Most prevalent in populations with endemic malaria
  – African, Asian, Mediterranean, Middle Eastern
  – G6PD deficient patients have lower morbidity and mortality from malaria, probably because parasites reproduce less efficiently in G6PD deficient RBCs
Glucose 6 phosphate Dehydrogenase Deficiency

• More than 400 variants with at least 140 point mutations and small deletions
  – Most of these mutations cause decreased substrate affinity or decreased protein stability
  – Large deletions or expression defects are not detected, suggesting that complete absence of G6PD enzyme is lethal
• X-linked inheritance
• Fully expressed only in males and homozygous females; heterozygous females may have milder G6PD deficiency (worse if imbalanced X-inactivation)
• Most cases are familial but some patients have spontaneous mutations
WHO Classification of G6PD Disease

• Five classes - the first three are deficiency states
  – Class I: severe deficiency (<10% activity) with chronic (nonspherocytic) hemolytic anemia independent of oxidative stressors
    • Rare, most mutations involve NADP binding domain or G6P binding domain
    • Patients have chronic hemolytic anemia and can be transfusion dependent
  – Class II: severe deficiency (<10% activity), with intermittent hemolysis that responds to removal of stressors
    • Some patients are asymptomatic
  – Class III: mild deficiency (10-60% activity), self-limited hemolysis with stressors only
    • Most common, many patients are asymptomatic
  – Class IV: Non-deficient variant, no clinical sequelae
  – Class V: Increased enzyme activity, no clinical sequelae
Common Variants of G6PD Deficiency

- **G6PD A**
  - 11% of blacks
  - Unstable enzyme with shortened half life
  - Moderate hemolysis (class III)

- **G6PD\(^{\text{Med}}\)**
  - Greeks, Arabs, Sicilians, Sephardic (Spanish/middle eastern) Jews
  - Gene frequencies from 2-20% in Italy, Turkey, Greece
  - 70% gene frequency in Kurdish (mesopotamian) Jews
  - Severe hemolysis (class II)

- **G6PD\(^{\text{Canton}}\)**
  - Asians, moderate hemolysis (class III)
Why Does G6PD Deficiency Selectively Affect RBCs?

- Erythrocytes have no other way of generating NADPH
- Other tissues in G6PD deficient patients compensate for the deficiency by increasing G6PD gene transcription, and/or generating NADPH through other metabolic pathways
- Erythrocytes can’t compensate because they have no nucleus, ribosomes, or mitochondria
- Active G6PD decreases rapidly in aging RBCs, making them increasingly susceptible to oxidative stress
Symptoms of G6PD deficiency

• Neonatal jaundice, kernicterus
• Chronic hemolytic anemia (less common, seen in class I mutations)
• Episodic hemolytic anemia (more common, class II-III)
  – Hemolytic episodes are triggered by oxidative stress
  – Hemolysis usually occurs 24-72 hours after ingestion of offending agent, and resolves in 4-7 days
• Symptoms of hemolysis include abdominal pain, back pain, jaundice, hemoglobinuria, transient splenomegaly
  – Jaundice usually doesn’t occur until >50% of RBCs have been hemolyzed (assuming normal liver function)
• Patients may be asymptomatic
Common triggers of hemolysis in G6PD deficiency

- **Drugs and chemicals**
  - Primaquine
  - Dapsone
  - Aspirin
  - Tylenol
  - Ibuprofen, other NSAIDS
  - Ciprofloxacin, other quinolone antibiotics
  - Sulfa drugs
  - Ethanol
  - Furosemide
  - Many others

- **Foods**
  - Fava beans (contain vicine, convicine and isouramil)
  - Other legumes
  - Sulfites
  - Menthol
  - Blue artificial food coloring (methylen and toluidine blue)
  - Vitamin C and K
  - Certain Chinese herbs
  - Many others

- **Stress (i.e. infections)**
  - Salmonella, Escherichia coli, beta-hemolytic streptococci, rickettsial infections, viral hepatitis, influenza A.

- **Random things (mothballs, henna)**

http://g6pddeficiency.org/index.php?cmd=contraindicated
Morphologic Features of Hemolysis in G6PD Deficiency

- Bone marrow: erythroid hyperplasia
- Peripheral blood: anisopoikilocytosis, polychromasia, bite cells, Heinz bodies

Heinz bodies = denatured hemoglobin precipitate

Jaffe et al., Hematopathology. Elsevier, 2011
Tests for G6PD deficiency

- Enzyme activity assay (spectrophotometric assay)
- Fluorescent spot test
  - Quick and cheap
  - Detects generation of NADPH from NADP+
  - G6P and NADP added to a drop of patient blood
  - Blood spot fluoresces at 340 nm if NADPH is generated
  - Only detects severe G6PD deficiency (enzyme levels below 30%)
- Mutation testing by PCR
  - Useful in families with known mutation
  - Prenatal diagnosis
  - Also useful in targeted screening of populations with high frequency of common mutations, such as G6PD A- in Africans & African-Americans
- *Caveats for non-PCR-based tests:
  - don’t perform right after a hemolytic episode or blood transfusion!
  - May not detect heterozygous females or mild deficiencies
G6PD Enzyme Level

• The diagnosis of G6PD deficiency is made by adding a measured amount of red cell hemolysate to an assay mixture that contains substrate (glucose-6-phosphate) and cofactor (NADP)
• The rate of NADPH generation is measured spectrophotometrically
• Normal values differ according to the methodology employed and are directly related to the temperature at which the assays are performed.
• Usual values are in the following range
  - Lower limit of normal: 5.5 to 8.8 Units/gram of hemoglobin
  - Upper limit of normal: 8.8 to 20.5 Units/gram of hemoglobin
When to Test?

- Children and adults with acute hemolysis related to infection, drug exposure, or “trigger food” ingestion
- Especially males of African, middle Eastern, or Asian descent
- Consider G6PD deficiency in cases of chronic hemolytic anemia in all ethnic groups
- Test in neonates who develop hyperbilirubinemia within the first 24 hours of life, a history of jaundice in a sibling, bilirubin levels greater than the 95th percentile, and in Asian males
  - 2x increased risk of neonatal hyperbilirubinemia in male infants (and homozygous females) with G6PD deficiency
- WHO recommends newborn screening for G6PD deficiency in populations where the prevalence is >3-5% in males
Pyruvate Kinase

• It is the most common cause of congenital non-spherocytic chronic hemolytic anemia caused by an erythrocyte enzyme defect
• Inherited as an autosomal recessive disorder
• Diagnosis of PK deficiency, or any glycolytic enzymopathy, is made by direct measurement of enzyme activity in RBCs.
• Severe disease may require frequent red cell transfusion throughout infancy and into adulthood.
• Splenectomy ameliorates the severity of hemolysis
Pyrimidine 5'-nucleotidase Deficiency

• P5N participates in RNA degradation in reticulocytes.
• P5N deficiency results in the accumulation of pyrimidines in the red cells, which is presumed to be toxic and a cause of hemolysis.
• Autosomal recessive inheritance and is the only congenital hemolytic anemia due to a red cell enzyme deficiency that results in a specific, consistent morphological abnormality—basophilic stippling
• Lead is a powerful inhibitor of P5N, and determination of lead levels should be included whenever a constellation of hemolytic anemia, P5N deficiency, and basophilic stippling is found
Basophilic Stippling
Molecular Approach

• Currently, tests for the diagnosis of these disorders are restricted to the straightforward screening, based on PCR on targeted sequencing

• Why???
  – Many cases of hemolytic anemia are not diagnosed
  – All these disorders can be combined together
  – For genetic counseling purposes

• Our panel is designed to diagnose the common causes of hemolytic anemia
Next Generation Sequencing

• Takes advantage of miniaturization to engage in massively parallel analysis
  – Essentially carrying out millions of sequencing reactions simultaneously in each of 10 million tiny wells

• Sophisticated computer analysis of huge amounts of information allows “assembly" of a given sequence
### Genes involved in hereditary membrane disorders

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<th>Genes</th>
<th>Inheritance</th>
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<td>Hereditary spherocytosis</td>
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<td>Hereditary elliptocytosis/pyropoikilocytosis</td>
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<td>Hereditary stomatocytosis</td>
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## Genes involved in RBC enzyme deficiency

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• We used Illumina HiSeq with 100bp paired-end read sequencing with a capacity for 19.8Gb/lane (divide by half since we will be performing paired-end reads = 9.9Gb).

• This provided us greater than 1200 fold coverage of the target regions for analysis
Case example

• 2yr, 3 month old with congenital hemolytic anemia

• At baseline, HB <6gm/dL. Retic anywhere from 10-20. She was transfusion dependent with significant hepatosplenomegaly and also had iron overload. She also had sickle cell trait.
Further testing's

- R/O RBC membrane defect: osmotic fragility normal. MCHC's are normal. Peripheral smear shows normal RBC morphology

- R/O RBC enzyme defect: a full RBC enzyme panel was normal

- R/O unstable hemoglobinopathy: No Heinz bodies. Hb electrophoresis did not demonstrate evidence of unstable Hb's.
- R/O thalassemia: Alpha thal mutation analysis negative. Hb electrophoresis consistent only with sickle cell trait. Beta globin gene sequencing identified only sickle cell trait, otherwise negative

- Extensive evaluation had also ruled out occult bleeding (GI as well as pulmonary)
We reported these results

• Abnormal = EMA

• NGS Panel picked up mutations:
Follow up...

• She had a splenectomy performed a few months ago and since then her baseline hemoglobin is 10-11. She has been doing ok for last 6 months.
Hemoglobin (the basics)

• Hemoglobin is a tetramer composed of 2 alpha globin's and 2 non alpha globin's
• Each globin chain also contain one heme molecule
• Alpha chains
  – All forms of adult hemoglobin will have two alpha chains
  – Coded by 4 genes (2/chromosome 16)
• Non-alpha chains
  – Beta (1 gene/chromosome)
  – Delta
  – Gamma (2 genes/chromosome)
Ribbon Diagram of Hemoglobin

http://www.psc.edu/science/Ho/Ho.html#hemoglobin
Building the hemoglobin tetramers

- Hemoglobin gene expression changes throughout development and continues to evolve for the first few years of life
- Gene expression defines the types of circulating hemoglobins
- Understanding the developmental stages of hemoglobin helps with understanding of the heterogeneity of hemoglobin tetramers
Hemoglobin-Development Switching
Chromosome 16

Chromosome 11

\[\begin{align*}
\alpha_1 & \quad \alpha_2 \\
\zeta & \quad \zeta
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Hemoglobinopathy (Structural)

• Due to mutations in either alpha or beta globulin
• **Structural** – substitution, addition or deletion of one or more AAs in the globin chain
  • i.e HbS, HbC, HbE, HbD, HbO, etc...

• Over 1000 identified
  • Majority are benign & discovered incidentally
  • Pathogenic mutations can cause
    • Change in physical properties (sickling, crystalizes)
    • Globin instability (Heinz body formation, lower expression)
    • Altered oxygen affinity
Thalassemia (quantitative)

• A quantitative decrease in the production of alpha or beta globin chain
  – Generally deletions
  – Point mutation that leads to decreased transcription or an unstable transcript

• Beta thalassemia results from deletions/mutations in beta gene(s)
  – Pathogenesis a result of the free alpha subunits

• Alpha thalassemia results from deletions/mutations in the alpha gene(s)
  – Pathogenesis a result of the free beta subunits
Demographics: Thalassemias

• Found most frequently in the Mediterranean, Africa, Western and Southeast Asia, India and Burma

• Distribution parallels that of *Plasmodium falciparum*
Classification & Terminology: Alpha Thalassemia

- Normal: $\alpha\alpha/\alpha\alpha$
- Silent carrier: $-\alpha/\alpha\alpha$
- Minor /trait: $-\alpha/-\alpha$
- Hb H disease: $--/\alpha\alpha$
- Barts hydrops fetalis: $--/--$
Clinical presentations of alpha thalassemia

- **A single deletion** (α-thalassemia minor)
  - silent carrier state, mild
  - RBC morphology and hemoglobin concentrations are usually normal
- **Two gene deletion** (α-thalassemia minor)
  - Mild microcytic anemia
- **Three gene deletion** (hemoglobin H disease)
  - Precipitated β chains—Hb H
  - Patients have moderate anemia, marked microcytosis, splenomegaly, and bone marrow erythroid hyperplasia
- **Four gene deletion** (Hydrops fetalis)
  - Not compatible with life (barring very early intervention)
  - Hemoglobin is primarily comprised of γ4 (Bart’s), which has a very high affinity for O2 and is a poor oxygen transporter
Classification & Terminology: Beta Thalassemia

- Normal: $\beta/\beta$
- Minor /trait: $\beta/\beta^0$
- Intermedia: $\beta^0/\beta^+$
- Major: $\beta^0/\beta^0$
  $\beta^+/\beta^+$
Diagnosis of hemoglobinopathies & thalassemias

• Largely dependent on the clinical laboratory

• Thalassemias
  – For alpha, depends on the number of genes that are deleted
  – For beta, look for an increase in % Hb A2 on HPLC
Diagnosis of Hemoglobinopathies/thalassemia

• Historically: slab-gel electrophoresis based techniques used for it all
  • Labor intensive
  • Densitometry is semiquantitative at best (and quantifying is key to accurate diagnosis!)

• Currently: Screen with a quantitative biochemical technique like **HPLC, Capillary Zone, Electrophoresis, Acid/Alkaline/IEF**
  – Definitive conformation using molecular assays (when necessary)

• CBC has always and will always play a critical role
Value of CBC

• Hemoglobinopathies
  – Anemia common in deleterious mutations
  – Important, but initial diagnosis is often made with NBS

• Thalassemias
  – Red cell indices are critical to diagnosis
  – Hypochromic microcytic anemia
    • MCV is key
    • RDW changes are variable
    • Increased RBC count → one distinguishing factor between thals and other microcytic anemias
Distinguishing features between iron def and thalassemia

**Thalassemia**
- The RBC count in thalassemia is more than 5.0 x 10^6/μL
- MCV usually less than 70 in
- The RDW is usually in the normal range

**Iron deficiency anemia**
- Low RBC count
- MCV usually more than 70
- RDW is usually more than 17
Diagnosis of Thalassemias
High throughput screening

- HPLC
- Capillary Zone Electrophoresis
- Increased Hb A2 is diagnostic of beta thalassemia trait
- However, can be falsely elevated in certain conditions...
High Performance Liquid Chromatography (HPLC)

- Sample is injected into a buffer flow, which carries the Hb through the column
- Separation via cation exchange chromatography
- Spectrophotometric detection
Normal Patient Chromatograms

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**Note:** The chromatograms show the analysis of Beta Thal Short samples, indicating the percentage and time of each analyte with associated area values.
Molecular Analysis

• Alpha thalassemia
  • Multiplex PCR/MLPA for common deletions
  • Alpha globin sequencing

• Beta thalassemia
  • The test examines the complete beta globin coding sequence, the splice sites and other intronic regions known to harbor mutations, the proximal promoter region, and the 5’ and 3’UTR regions.
  • Clinical sensitivity is up to 97% based on the ethnicity
α–Thalassemia Detection

- Hb gel/HPLC migration patterns
  - Not helpful for α–Thalassemia, unless β^4 (Hb H) and γ^4 (Hb Barts) are present

- Genetic analysis
  - Multiplex PCR for seven common deletions/Multiplex ligation dependent probe assay
  - Alpha globin sequencing
    - PCR amplification followed by bidirectional sequencing of the complete protein coding sequence with exon/intron boundaries, proximal promoter region, 5’ and 3’ untranslated regions, and polyadenylation signal
    - Clinical sensitivity up to 10 percent, depending on ethnicity
    - Analytical sensitivity and specificity are 99 percent
β–Thalassemia Detection

- **CBC**: Always microcytosis (mild anemia and microcytosis, usually β-thalassemia)
- **HPLC**: Elevated Hb A2 diagnostic
- **Molecular analysis**: Complete beta globin coding sequence, the splice sites and other intronic regions known to harbor mutations, the proximal promoter region, and the 5’ and 3’UTR regions
- Clinical sensitivity is up to 97% based on the ethnicity
Quick Algorithm

• Suspicion for hemoglobinopathy
  
  CBC (decreased MCV)
  
  HPLC
  
  Increased Hb A2 → beta globin sequencing
  
  Normal HPLC → alpha thalassemia 7 common deletion testing/MLPA testing
    - alpha globin sequencing
References and Acknowledgement

- Color Altas of Hemoglobin Disorders: A compendium Based on Proficiency Testing (2003), updated in 2010
- **Acknowledgement:**
  - Josef T. Prchal, M.D, Professor of Medicine, Genetics and Pathology. University of Utah and ARUP Laboratories
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Questions!!!