Laboratory Diagnosis of Hemoglobinopathies and Thalassemia

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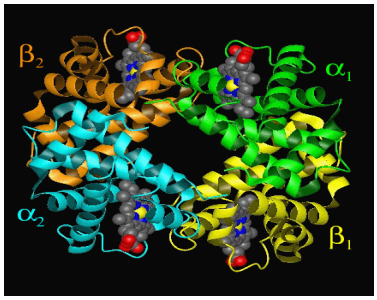
Learning Objectives

- Understand the pathophysiology of hemoglobinopathies
- Recognize the most important expected test results in hemoglobinopathies and thalassemias
- Understand different testing methodologies
- To be able to direct ordering physician to appropriate tests for these disorders

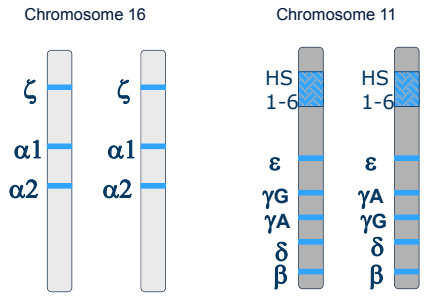
Hemoglobin (Heme+Globin)

- Hemoglobin is a tetramer composed of 4 globin molecules; 2 alpha globins and 2 beta globins or beta like globins
- The alpha globin chain is composed of 141 amino acids and the beta globin chain is composed of 146 amino acids
- Each globin chain also contains one heme molecule

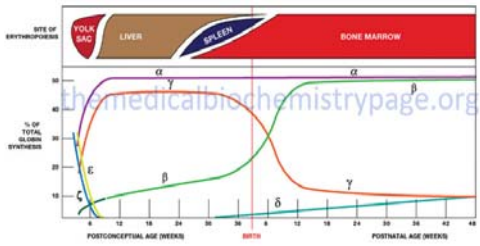
Ribbon Diagram of Hemoglobin



Genetics of Globin Genes



Hemoglobin-Development Switching



Normal Adult Human Hemoglobin Composition

Hemoglobin	Structure	% of Normal Adult Hb
Hb A	$\alpha_2\beta_2$	>96%
Hb A2	$\alpha_2\delta_2$	~2.5%
Hb F	$\alpha_2\gamma_2$	<1%

Hemoglobinopathy (structural)

- Due to mutations in either alpha or beta globin
- **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
  - Large deletions, point mutations, small insertion/deletion that leads to decreased transcription or an unstable transcript
- Beta thalassemia results from mutations in beta gene(s)
  - Pathogenesis a result of the ***free alpha subunits***
  - Two classes**: β0 and β+**
- Alpha thalassemia results from large deletions 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	αα/αα
• Silent carrier	- α/αα
• Minor /trait	-α/-α
	--/αα
• Hb H disease	--/-α
• Barts hydrops fetalis	--/--

Clinical Presentations of Alpha Thalassemia

- A **single** deletion (α-thalassemia minor)
  - silent carrier state
  - 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 β/β
- Minor / trait β/β^0
  β/β^+
- Intermedia β^0/β^+
- Major β^0/β^0
  β^+/β^+

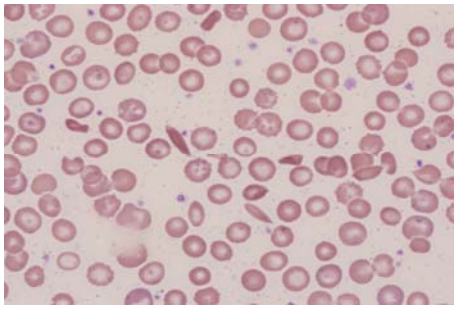
Clinical Significance of β Thalassemia

- Heterozygous asymptomatic
- Homozygous β^0 is a severe disorder associated with transfusion dependent hemolytic anemia
- Homozygous β^+ is a heterogenous disorder
  - severity depending on mutation and % of HbA
  - Increased HbA = decreased severity

Sickle Cell Anemia

- Single nucleotide base change codes for valine instead of glutamic acid at the 6th position from the N-terminus of the ß-globin chain
- Affects the shape and deformability of the red blood cell
- Leads to veno-occlusive disease and hemolysis

Peripheral Smear: Sickle Cell Anemia

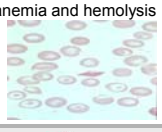


Hb E

- 2nd most prevalent hemoglobin variant
  - 30,000,000 world wide
  - 80% in Southeast Asia
- Hb E trait: microcytosis (mean MCV=65fl). No anemia
- Hb E disease: MCV =55-65fl with minimal anemia
- *On HPLC has similar migration pattern as Hb A2

Hb C

- Mutation in β-globin gene β(6glu->lys)
- Seen predominantly in blacks: Gene prevalence in US black population is 2 to 3%
- May confer malaria resistance
- Often asymptomatic, mild anemia, splenomegaly
- Blood smear shows many target cells, rare intracellular crystals
- Hb S/C disease causes moderate to severe anemia and hemolysis



Diagnosis

- **Indications for Testing**
  - Hemolytic anemia; family history of hemoglobinopathy
- **Laboratory Testing**
  - Initial testing – CBC with peripheral smear
  - Polychromasia, spherocytes, schistocytes, sickle cells, Heinz bodies, basophilic stippling; however, the lack of any of these cells does not rule out hemolytic anemia
  - Many hemoglobinopathies can be diagnosed using electrophoretic or high performance liquid chromatography (HPLC) techniques, but some may be missed
  - Genetic testing

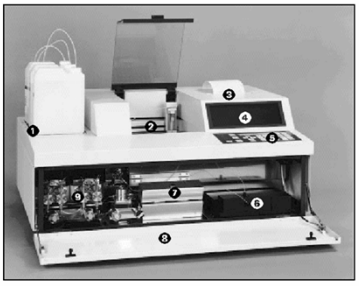
Importance of CBC

- Thalassemias
  - Red cell indices are critical to diagnosis
  - Hypochromic microcytic anemia
    - MCV (mean corpuscular volume or size of the cell) is key
    - RDW (red cell distribution width) changes are variable
    - Increased RBC count → one distinguishing factor between thalassemias and other microcytic anemias

Distinguishing Features Between Iron Deficiency and Thalassemia

- The RBC count in thalassemia is either normal or on higher side of normal
- MCV usually less than 70 in
- The RDW is usually in the normal range
- Low RBC count
- MCV usually more than 70
- RDW is usually more than 17

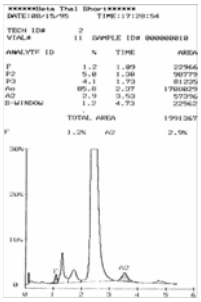
Diagnosis of Thalassemias

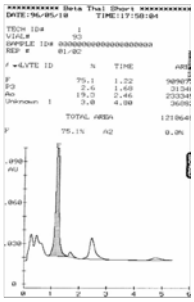


High-Pressure Liquid Chromatography

- Cation Exchange
- Analytical cartridge contains negatively charged silica
- Buffers contain Na+ and K+ ions
- Hemolysates contain positively charged hemoglobin
- Hemoglobin binds to negatively charged silica at injection
- Na+ and K+ concentration increased and separates hemoglobin fragments from silica

Normal Patient Chromatograms





Summary of HPLC

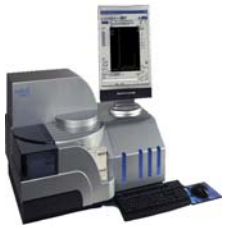
Advantages

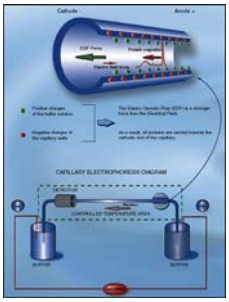
- Fast
- Small amounts of sample
- Accurate quantitation of A2

Disadvantages

- Hemoglobin E cannot be separated from A2
- Hemoglobin H and Barts elute too quickly from column

Capillary Electrophoresis





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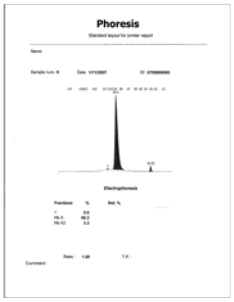
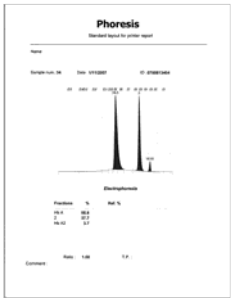
Phoresis Reports





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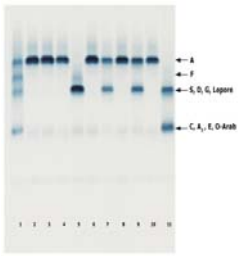
Alkaline and Acid Gel Electrophoresis

- Electrophoresis (pH 8.4 (alkaline) and pH 6.2 (acid) on agarose gels)
- Slow, labor-intensive, and inaccurate in the quantification of low-concentration Hb variants (e.g., Hb A_2) or in the detection of fast Hb variants (Hb H, Hb Barts)
- The precision and accuracy of Hb A_2 measurements using densitometric scanning of electrophoretic gels is poor, especially when compared with HPLC techniques



Isoelectric Focusing

- IEF is an electrophoretic technique with excellent resolution
- IEF is an equilibrium process in which Hb migrates in a pH gradient to a position of 0 net charge
- The Hb migration order of IEF is the same as that of **alkaline electrophoresis** with better resolution

Molecular Analysis

- Alpha thalassemia
  - Multiplex ligation dependent probe amplification (MLPA) and multiplex PCR
  - Alpha globin sequencing
- Beta thalassemia
  - Beta globin sequencing
    - 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
  - Beta globin del/dup testing by MLPA

α–Thalassemia Diagnosis

- Hb gel/HPLC migration patterns
  - Not helpful for α–Thalassemia, unless β4 (Hb H) and γ4 (Hb Barts) are present
- Genetic analysis
  - MLPA: will identify all deletions and duplications
  - Multiplex PCR for 7 common deletions-only 7 common deletion
  - 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
    - Only useful in 5-10% of cases where alpha thal is due to point mutation

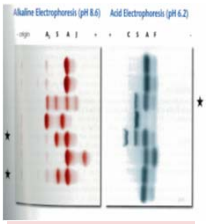
β–Thalassemia Diagnosis

- **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
- Beta globin del/dup in some cases (about 5%) where beta thalassemia is due to large deletions

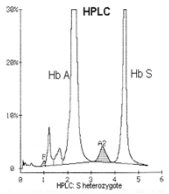
Sickle Cell Disease Diagnosis

- Sickledex test (Screening test)
  - Deoxygenated Hb-S is insoluble in a concentrated phosphate buffer solution and forms a turbid suspension
  - Normal Hemoglobin A and other hemoglobins remain in solution
  - It does not differentiate between Sickle Cell Disease (S/S) and Sickle Cell Trait (A/S)

Sickle Cell Disease Diagnosis



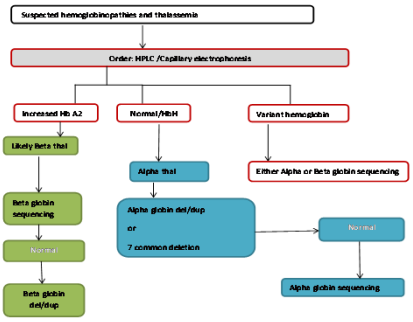
Electrophoresis



HPLC

Color Altas of Hemoglobin Disorders: A compendium Based on Proficiency Testing (2003), updated in 2010

Simplified Algorithm



References and Acknowledgement

- Kaushansky K, Lichtman MA, Beutler E, Kipps TJ, Prchal J, Seligsohn U. Willam's Hematology. Ninth Edition. McGraw Hill Professional. 2015.
- Steinberg MH, Forget BG, Higgs DR, Nagel RL. Disorders of Hemoglobin. Genetics, Pathophysiology, and Clinical Management, 2nd ed. Cambridge University Press, New York, 2009
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- **Acknowledgement:**
  - Josef T. Prchal, M.D, Professor of Medicine, Genetics and Pathology. University of Utah and ARUP Laboratories
  - Dottie Hussie, M.T, ARUP Laboratories

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