Hemoglobin & Hemoglobinopathies
beyond the peripheral slide
Jene Merschen MT (ASCP), MPH

An Emerging Global Health Burden
Breakdown of the annual number of births with the different hemoglobin disorders

<table>
<thead>
<tr>
<th>Major hemoglobin disorder</th>
<th>No. of annual births</th>
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<tbody>
<tr>
<td>β-thalassemia major</td>
<td>22,989</td>
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<tr>
<td>Hb E thalassemia</td>
<td>19,128</td>
</tr>
<tr>
<td>Hb H disease</td>
<td>9,568</td>
</tr>
<tr>
<td>Hb Bart hydrops</td>
<td>5,183</td>
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<tr>
<td>Hb SS disease</td>
<td>217,331</td>
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<tr>
<td>Hb SS-thalassemia</td>
<td>11,074</td>
</tr>
<tr>
<td>Hb SC disease</td>
<td>54,736</td>
</tr>
</tbody>
</table>

Weatherall DJ, Blood, 2010;115:4331-4336.

What is hemoglobin?
Hemoglobin is a protein in red blood cells that carries oxygen

Hemoglobin structure
Tetrameric structure made by 2 dissimilar pairs of globin chains each linked to a single HEME molecule

- Alpha globin chains: (α)
- Non-alpha globin chains: Beta (β), gamma (γ), delta (δ)

Normal adult Hbs consists of 2 (α) chains + another pair
Hemoglobin structure

Highly conserved protein
Proteins are made of amino acids
Gas transporting molecule that makes up 95% of the RBC
Carries Oxygen

Adult Hemoglobins

HbA (α2 β2)
- Major adult hemoglobin
- 95% of total

HbA2 (α2 δ2)
- ~ 2.5% of adult Hb
- δ chain differs from β at ~ 10% of residues
- function unclear

HbF (α2 γ2)
- 'about' 1%

Fetal Hemoglobins

Embryonic: Gower I (ζ2 ε2), Gower II (α2 ε2), Portland (ζ2 γ2)

Fetal: HbF (α2 γ2)

> When synthesis is un-balanced:
- β, γ, δ, ε chains can all form tetramers: Hb Bart's (γ4)
- HbH (β4)
- α does not form tetramers

Globin Synthesis and Hemoglobin Assembling

Transition of the hemoglobin chains

Newborn chromatogram on HPLC
HEMOGLOBIN SWITCHING

Embryonic  Fetal  Adult

Hb Gower1 (ζ2ε2)  Hb F (α2γ2)  Hb A (α2β2)
Hb Gower2 (α2ε2)  Hb A (α2β2)
Hb Portland (ζ2γ2)

HEMOGLOBIN TESTING

HEALTHY ADULT CHROMATOGRAM ON HPLC

Genetics

Chromosome 11

Chromosome 16

Genetic Gene Clusters

Genes encoding for globin chains

From Gene to Hemoglobin

We have 4 α genes and only 2 β genes

Presumptive or Definitive Identification?

ANALYTICAL METHODS OF ANALYSIS RESULTS

ELECTROPHORESIS TO SCREEN HEMOGLOBINS

IEF
CE
MICROCOLUMNS
CE-HPLC
HPLC
TO DIFFERENTIATE GLOBINS
MS
DNA ANALYSIS TO CONFIRM AA SEQUENCE
TO IDENTIFY GENE MUTATIONS
Hemoglobinopathies

Structural variants:
- Mutation changes an amino acid in the globin chain sequence
- Change may alter the normal hemoglobin function
- Change may also cause thalassaemia

The thalassemias:
- Mutation results in either the absence or a reduction in the synthesis of one or more globin chains
- Causes an imbalance in the ratio of the amounts of the two globin chains that make up a Hb (eg Hb A: a/b chains)

Hemoglobinopathies

More than 80% result from a substitution mutation
- Many populations affected
- Multiple abnormalities can be inherited
- Less than 30% of variants result in clinical outcomes
- Some hemoglobinopathies are 'screen negative'

www.globin.cse.psu.edu

Hb Variants - Types

- β-chain variants 362
- α-chain variants 217
- γ-chain variants 70
- δ-chain variants 32

They can be:
- Variants with one amino acid replacement
- Variants with two amino acid replacements
- Variants with hybrid chains
- Variants with elongated chains
- Variants with deletions (17), insertions (6), and deletions + insertion (4)

Common Hb Variants

HbS is the commonest variant
Exceeds 20% in parts of Africa. Also found in Turkey, Iran, Saudi Arabia, & India

HbE is the second commonest variant
Exceeds 50% in the "Hb triangle" – ie parts of Thailand, Laos and Cambodia

HbC is found mainly in West Africa
Found at a frequency of up to 50% on the Ivory Coast

High frequencies are due to heterozygotes having increased resistance to infection by malaria (P. falciparum)

Inheritance of Hemoglobin Disorders
What is autosomal recessive inheritance?

Limitations of Hemoglobin Electrophoresis

Identification dependent on technical performance
- Hemoglobin concentration
- Amperage
- Running temperature
- Length of electrophoresis run

Identical or similar migrations
- Discrete differences
- Lack of control specimens

METHODOLOGIES

QUALITATIVE
- Cellulose Acetate Electrophoresis (pH 8.6)
- Citrate Agar Electrophoresis (pH 6.2)
- Isoelectric Focusing (pH gradient)
- Globin Chain Electrophoresis (Alkaline and Acid)

QUANTITATIVE
- Column Chromatography (Hb A)
- Alkal Denaturation (Hb F)
- Radial Immunodiffusion (Hb F)
- Densitometry (Hb A, Hb F, and hemoglobin fractions)
- Kleihauer-Betke Staining (Hb F)

AUTOMATED (Qualitative and Quantitative)
- Capillary Zone Electrophoresis (CZE)
- High Performance Liquid Chromatography (HPLC)
The thalassemias

Did you Know...
- Thalassemia is sometimes called Mediterranean anemia or Cooley’s anemia.
- The name “thalassemia” comes from the Greek thalassa which means “sea” and haema, which means “blood”.
- Thalassemia is prevalent in people from the Mediterranean region and middle east.
- India, Central Asia, and Southeast Asia also have a high prevalence of thalassemia.
- The highest prevalence of thalassemia is geographically located in the world where there is or was malaria.

What are α- and β- thalassemias?

Thalassemia: Quantitative defects:
- Reduced synthesis of a normal globin chain
  - α⁺ or β⁺ thalassemia
- Absent synthesis of a normal globin chain
  - α₀ thalassaemia or β₀ thalassaemia

Clinical Information: thalassemias

Range of Clinical Symptoms
- β-thalassemia minor
  - Asymptomatic to mild microcytic anemia
- β-thalassemia intermedia
  - Range of symptoms
- β-thalassemia major
  - Fatal if untreated
    - Transfusion Dependency
    - Hepatosplenomegaly
    - Severe Anemia
    - Skeletal Deformities
- α-thalassemia trait (-α/-α) (-a/-a)
  - Asymptomatic
- Hemoglobin H Disease (–/–)
  - Chronic hemolytic anemia
- Hydroa Fetalis
- Stillbirth

Transfusion therapy
**Thalassemia**

- A group of inherited disorders in which globin chain synthesis is impaired.

**α-Thalassemia**

- Normal
- Increased HbA (4-6%)
- Increased HbF (10-70%)

**β-Thalassemia**

- Hb A\(^{0}\) or Hb A\(^{+}\)
- Hb A\(^{0}\) or Hb A\(^{+}\)

**PATHOPHYSIOLOGY OF THALASSEMIA**

- Thalassemia genes
- Defective α- or β-chain synthesis, Excess α- or β-chains
- Membrane damage
- Iron excess
- Increased bilirubin production
- Jaundice
- Gallstones
- Blood transfusion
- Increased RBC destruction
- Hyperuricemia
- Anemia
- Massive erythropoiesis
- Hepato-splenomegaly
- Increased RBC production
- Extramedullary hematopoiesis
- Inadequate nutrition
- Bone changes
- Iron overload
- Increased infection
- Anemia
- Massive erythropoiesis
- Hepato-splenomegaly
- Increased infection
- Anemia
- Massive erythropoiesis
- Hepato-splenomegaly
- Bone changes
- Iron overload
- Increased infection
- Anemia
- Bone changes

**Beta thalassemia**

- Inherited mutation defect on beta gene
- Mutation results in an either the absence or a reduction in the synthesis of b-globin chains:
  - β\(^{+}\) type: reduced expression of b-globin
  - β\(^{0}\) type: no expression of b-globin

Causes an imbalance in the ratio of the amounts of the two globin chains that make up a Hb

**β-thalassemia Genotypes**

- β-thalassemia TRAIT (β\(^{0}\) or β\(^{+}\) type)
  - both types can be mild or severe

- β-thalassemia Intermedia (β\(^{0}\)β\(^{0}\) or β\(^{0}\)β\(^{+}\))
  - β\(^{+}\) is a mild type (β\(^{+}\) type)

- β-thalassemia MAJOR (β\(^{0}\)β\(^{0}\) or β\(^{0}\)β\(^{+}\))
  - β\(^{+}\) is a severe type

**Phenotypes of β-thalassemia**

- β\(^{+}\)-thalassemia (trait)
  - Silent
  - normal MCH & normal HbA2
- normal HbA2 (reduced MCH & normal HbA2)
- Mild
  - reduced MCH & raised HbA2
- Severe
  - reduced MCH & raised HbA2

- β\(^{0}\)-thalassemia (trait)
  - Mild
  - reduced MCH & raised HbA2
  - Severe
  - reduced MCH & raised HbA2
  - Dominant
  - reduced MCH & raised HbA2, plus anemia and splenomegaly
There is great variety in β Thal alleles—and approx 20 diff alleles comprise 80% of the mutations worldwide.

### Frequencies of common β-thalassemia mutations

<table>
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<tr>
<th>Country</th>
<th>IVS110 (G→A)</th>
<th>CD39 (C→T)</th>
<th>IVS1-6 (T→C)</th>
<th>IVSII-745 (C→G)</th>
<th>IVS1-1 (G→A)</th>
<th>CD8/9 (+G)</th>
<th>IVSI-5 (G→C)</th>
<th>619 bp deletion</th>
<th>IVSI-1 (G→T)</th>
<th>CD41/42 (-TCTT)</th>
<th>-28 (A→G)</th>
<th>CD17 (A→T)</th>
<th>CD71/72 (+T)</th>
<th>IVSII-654 (C→T)</th>
<th>-29 (A→G)</th>
<th>-88 (C→T)</th>
<th>CD24 (T→G)</th>
<th>CD41/42 (-TCTT)</th>
<th>-28 (A→G)</th>
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</tbody>
</table>

### Alpha thalassemia

- Inherited deletion or mutation defect on alpha genes
- Deletion or mutation results in an either the absence or a reduction in the synthesis of α-globin chains:
  - α⁺ type: reduced expression of α-globin genes
  - α⁻ type: no expression of α-globin genes
- Causes an imbalance in the ratio of the amounts of the two globin chains that make up a Hb (eg Hb A: αβ chains):
  - Excess of γ chains in the fetal stage can generate γ₄ tetramers: Hb Bart’s
  - Excess of β chains in the adult stage can generate β₄ tetramers: HbH

### Genetics of α-thalassemia

- α⁺-thalassemia
  - Caused by the loss of both the α1 and the α2 genes on the same chromosome
  - 5 common mutations: --SEA, --FIL, --THAI, --MED, --20.5
- α₀-thalassemia
  - Single gene deletion:
    - 3.7kb (-α²) Mediterranean, Middle east, Africa, India
    - 4.2kb (-α¹) India, Southeast Asia, Pacific populations
  - Non-deletion mutation on either α1 or α2 gene
    - 60 mutations – some result in hyper-unstable globins
Hb Constant Spring

- Non-deletional form of α-thalassemia
- α2 globin allele contains a point mutation
- The globin chains α^CS is elongated by 31 amino acids
- Hb Constant Spring is highly unstable
- HbCS in heterozygotes constitutes only 1-2% of the total hemoglobin
- HbCS interacting with deletional α°-thalassemia can cause a severe HbH disease (−/− α^CS α°)

Alpha Thalassemia genetics

- 4 alleles = Normal
- 1 gene deletion = silent carrier
  - Asymptomatic
  - May see small amount of Hb Bart's (γ4) at birth
  - Normal red cell indices – normal looking morphology
  - Detected only by DNA methods

Alpha Thalassemia genetics

- 2 gene deletion = α-thalassemia trait
  - Mild anemia
  - Small red blood cells (low MCV)
  - Hb Bart's at birth
  - Difference between cis and trans becomes important when considering inheritance
- 3 gene deletion = hemoglobin H disease
  - Moderate to marked anemia
  - Very small red blood cells
  - Hb Bart's (γ4) present at birth, replaced by HbH (β4)
- 4 gene deletion = hydrops fetalis
  - Extreme anemia produces congestive heart failure, edema in utero
  - Stillbirth or early neonatal death

Risk for HbH

A pregnancy is at risk for HbH disease (3 gene deletion) if:

- a) One parent has 1 α gene deletion and the other parent has 2 α gene deletion (CIS or α°-thalassemia heterozygote)
  \[
  \begin{align*}
    \text{cis} &+ \text{cis} \\
    &+ \text{cis}
  \end{align*}
  \]

- b) One parent has 1 α gene deletion and the other parent has HbH disease
  \[
  \begin{align*}
    \text{cis} &+ \text{cis} \\
    &+ \text{cis}
  \end{align*}
  \]

Risk for Hydrops Fetalis

A pregnancy is at risk for Hydrops Fetalis if:

- a) Both parents have a 2 α gene deletion (CIS or α°-thalassemia heterozygote)
  \[
  \begin{align*}
    \text{cis} &+ \text{cis} \\
    &+ \text{cis}
  \end{align*}
  \]

- b) One parent has 2 α gene deletion (CIS) and the other parent has HbH disease
  \[
  \begin{align*}
    \text{cis} &+ \text{cis} \\
    &+ \text{cis}
  \end{align*}
  \]
Alpha thalassemia hematology

### Red Blood Cell Indices in Adults with Alpha-Thalassemia

<table>
<thead>
<tr>
<th>Red Blood Cell Index</th>
<th>Normal</th>
<th>Affected</th>
<th>Carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Corpuscular Volume (MCV, fL)</td>
<td>89.1 ± 5.0</td>
<td>87.6 ± 5.0</td>
<td>136.1 ± 5.0</td>
</tr>
<tr>
<td>Mean Corpuscular Hemoglobin (MCH, pg)</td>
<td>30.9 ± 1.9</td>
<td>30.2 ± 2.1</td>
<td>31.5 ± 1.9</td>
</tr>
<tr>
<td>Hemoglobin (Hb, g/L)</td>
<td>15.8 ± 1.0</td>
<td>14.0 ± 1.0</td>
<td>13.8 ± 1.0</td>
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</tbody>
</table>

Note: α-thalassemia carriers with the [-/-] genotype have slightly lower RBC indices.

### Hemoglobin Patterns in Alpha-Thalassemia (After 12 Months of Age)

<table>
<thead>
<tr>
<th>Hemoglobin Type</th>
<th>Normal</th>
<th>Affected</th>
<th>Carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA</td>
<td>96-98%</td>
<td>0</td>
<td>96-98%</td>
</tr>
<tr>
<td>HbF</td>
<td>&lt;1%</td>
<td>0</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Hb Bart's</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HbH</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HbA2</td>
<td>2.3%</td>
<td>&lt;2.0%</td>
<td>1.5-3%</td>
</tr>
</tbody>
</table>

Newborns chromatograms on HPLC

- **Healthy**
- **Affected**

Hb Bart’s

- **δ-thalassemia & HPFH phenotypes**
  - **δ-thalassemia**
    - Reduced MCH: Hb F 4-20% Heterocellular
  - **Deletion HPFH**
    - Normal MCH: Hb F 20-35% Pancellular
  - **Non-deletion HPFH**
    - Normal MCH: Hb F 3-35% Heterocellular
**HPFH**

**Hereditary Persistence of Fetal hemoglobin**

- Deletional and non-deletional (deletional can have a Hb F of 25-35%)
- Cellular distribution (pancellular or homocellular)
- D-10 (A1c) can report A1c in the presence of Fetal hemoglobin up to 10%. Using the Dual Mode, the c/o for Fetal is 17%.

**Prenatal Diagnosis**

Significant hemoglobinopathies that require prenatal diagnosis by DNA analysis:
- Sickle cell diseases: Hb SS, SD-Punjab, SC, SO-Arab
- β-thalassemia major
- β-thalassemia intermedia (severe genotypes)
- HbE/β-thalassemia
- HbS/β-thalassemia
- α-thalassemia
  - Hb Bart’s hydrops fetalis syndrome
  - Hb H hydrops fetalis syndrome

**Alpha Thalassemia genetics**

- 4α genes
  - Normal
- 3α genes deleted
  - α-thalassemia major
- 2α genes deleted
  - α-thalassemia minor
- 1α gene deleted
  - α-thalassemia intermedia
- 0α genes deleted
  - Hb H disease

Alpha thalassemia main deletions:
- -3.7 Kb
- -4.2 Kb

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. Genes Functional</th>
<th>Phenotype</th>
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<tbody>
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<td>Normal</td>
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<td>α/αα</td>
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<td>MCV, MCH</td>
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<tr>
<td>α - α - -</td>
<td>0</td>
<td>Hb Barts hydrops fetalis</td>
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</table>
Normal RBCs

Beta-thal minor RBCs

Beta-thal major RBCs

**High Performance Liquid Chromatography (HPLC)**

• Separates Hbs based on charge differences

• Positively charged Hbs are separated by their absorption on a negatively charged stationary phase in a column

• The cations (positive charge) in the mobile phase (buffers with increasing ionic strength) compete with the absorbed Hbs eluting them off

• The fractions are detected optically by a spectrophotometer that measures the concentration of Hgb in each fraction which is quantified by calculating the area under the peaks

Polyaspartic acid column bound to a silica gel support, producing a weak cation exchange resin

Two mobile phases (buffers) produce gradient of increasing ionic strength

Blending of the phases, the flow rate and the physical characteristics of the hemoglobin determine the retention time on the column

Elution from column measured at 415 nm. The resulting chromatograph is integrated and the % hemoglobins calculated
26 M  RBC 3.77  MCV 97.4  HB 12.8  MCH 29.4  HCT 36.8  Dx: Hb E trait

Infant  Father  Mother

3.77  97.4

MCV

98

12

1.2

12.8

29.4

40

32.0

13.0

Infant  Father  Mother

11.2  29.3

Hb

12

2.3

3

Dx:

Hb E trait

Hb D-­‐Punjab

Dx:

Hb D-­‐Punjab trait

Dx:

Hb D-­‐Punjab trait + α-thal

1 y.o. M  RBC 4.95  MCV 60.6  HB 10.5  MCH 19.7  HCT 32.0  RDW 12.8  Dx: Hb D-Punjab trait + α-thal

Bunn and Forget, Hemoglobin: Genetics and Clinical Aspects, 1986

THANK YOU

Jane_merschen@bio-rad.com