An Approach to Hemoglobinopathy Investigations
#everythingyouneedtoknow

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Disclosures

• None. I have no financial or research relationships to disclose, and I will not be talking about or promoting any specific treatment or medications.
Objectives for this one hour presentation:

• **At the end of this session, participants will be able to:**
  
  • Describe a practical approach to the investigation of the hemoglobinopathies.
  
  • List the implications of the common hemoglobinopathies encountered in a clinical setting and how to advise clinicians regarding testing results.
  
  • Demonstrate when to refer samples to a reference lab for further investigations.
  
  • Describe the interpretation of hemoglobinopathy investigations throughout infancy and early childhood.
Realistically....

- I expect you have an approach and good knowledge of the basics, commonly encountered hemoglobinopathies, morphology and various automated platforms in hemoglobinopathy testing. I will *breeze* through theory topics- if you don’t know it well already, that’s great!! Take it as a guide for what to study, using this presentation as a reference.

  *Welcome to your crash course in hemoglobinopathies.*

- My objectives are to mention topics and high-level points that you may not have realized you should know, but treatment and transfusion issues are beyond the scope of this lecture.

- My overall goal is to help you pass the exam AND be a competent independent pathologist, with tips for exam preparedness and real-life practice.
Resources I like

• Color Atlas of Hemoglobin Disorders from the College of American Pathologists Press.

• Variant Haemoglobins: A guide to identification, by Bain.


• Sickle-cell disease. Rees, D., Williams, T., and Gladwin, M. *The Lancet* 376, 2018-2031 (2010). *or some other thorough overview of SC.*
  • In depth review of Sickle cell disease is not included in this lecture.
Basic pathophysiology; Normal Adult Hemoglobin

4 globin chains, each with a heme group consisting of an iron ion (ferrous Fe²⁺) held in a porphyrin ring and carrying one O₂ molecule each.
- Hgb A; 2 alpha globins and 2 beta globins
- Hgb A₂; 2 alpha globins and 2 delta globins
- Hgb F; 2 alpha globins and 2 gamma globins

Normal adult percentages:
- Hgb A 96.8-97.8%
- Hgb A₂ 2.2-3.2%
- Hgb F <0.5%

Chromosome 11:
- 1 beta globin chain gene
- 1 delta globin chain gene
- 1 epsilon globin chain gene
- 2 gamma globin chain genes (γG and γA)

Chromosome 16:
- 2 alpha globin chain genes (α₁ and α₂)
- 2 zeta globin chain genes
Hgb in the embryo (produced in the yolk sac):
- Gower 1 (ζ2ε2)
- Hgb Portland (ζ2γ2)
- Gower 2 (α2ε2)
Embryonic Hgb has higher O2 affinity, permitting O2 transport from amniotic fluid prior to establishment of placental circulation.

In the fetus/newborn:
~ week 14 gestation Hgb F nearly 100%. This starts to decrease at ~30 weeks and then more rapidly after birth.
Hgb F ~60% 10 weeks after birth

By 6 months of age, Hgb F is replaced by Hgb A, and Hgb A2, reaching adult values. Mediated by transcriptional switch in definitive erythroid progenitors from γ to β globin.
At birth; normal newborn
Predominantly Hgb F, small amount of Hgb A

HPLC heel-prick and capillary electrophoresis of umbilical cord blood
**Hgb Function**

Principle function: carry and deliver oxygen from lungs to tissues.

Oxygenation and deoxygenation occur at the heme iron. Binding of oxygen causes a slight conformational shift (R) encouraging oxygen to bind to the remaining heme units, giving the sigmoidal curve (cooperative binding). Relaxed form favors oxygen loading = Hgb with high oxygen affinity. Occurs with high partial pressure of oxygen (lung alveoli).

When deoxygenated, Hgb undergoes confirmation change; Tense (T) form favors oxygen unloading = Hgb with low oxygen affinity, and is stabilized by 2, 3-BPG. Occurs at low partial pressures of oxygen (tissues).

Oxygen affinity of hemoglobin changes based on environment, and shifts the curve; Bohr effect.
Terminology: Hemoglobinopathy umbrella

- Over 950 Hgb variants described, >100 alpha- and >200 beta-thal mutations; thankfully the role of a clinical lab is to identify the relatively small number that are clinically significant and common.

**Thalassemia (quantitative)**
- Alpha thalassemia
- Beta thalassemia

**Hgb variant (qualitative)**
- Alpha variant
- Beta variant
Approach to Hemoglobinopathies

Abnormal α or β globin or regulatory elements

No abnormality in production or function
- No clinical significance
  - e.g. Hb G-Coushatta

Abnormal function
- Altered Function
  - O₂ affinity
    - e.g. Hb Iwate, Hb M-Saskatoon
- Altered Solubility
  - Hemolytic Anemia
    - e.g. Hb SS, SC, CC
- Altered Stability
  - Unstable Hb
    - e.g. Hb Koin

Decreased production
- Thalassemia syndrome
  - e.g. Hb E, Hb Lepore, Hb Constant Spring, α-thal, β-thal

Adapted from McFarlane A. (2005) “Current Good Practice Guidelines for the Laboratory Investigation of Hemoglobinopathies”
Racial origin

- African; Alpha thal, Hgb S, C, G and delta variant
- South Asia; Hgb variants D-Punjab and D-Iran
- South-East Asia; Alpha and beta thal, and Hgb E (~50-60% of population)
- Mediterranean, Middle East, India; Beta thal common
- Aboriginal; Hgb G-Coushatta

- Hgb S; Caribbean, South America, Southern Europe, North Africa, Middle East, Indian subcontinent. (malaria resistance!)
- Thalassemia; Caribbean, South American, Southern Europe, North Africa, Africa, Middle East, Indian subcontinent, Afghanistan, Pakistan, Bangladesh, South East Asia.
Newborn screening program

- Screening based on ethnicity would miss cases, thus newborn screening is offered on all babies. Program intended to allow confirmatory testing at an earlier age, and to allow for early critical intervention/treatment in potentially devastating disorders.

- Includes:
  - 16 inborn errors of metabolism
  - Hemoglobin testing (IFE or HPLC)- as a screen for sickle disease/related conditions
  - Congenital hypothyroidism
  - Cystic Fibrosis
  - SCID (TEC DNA circles within T-cells)
Indications for work-up

• Preconception screening/Prenatal (family history or high-prevalence ethnicity)
  • To permit genetic counseling of prospective parents
  • To identify fetuses at risk and offer parents informed choice

• Newborn screening
  • To identify an abnormality prior to the onset of sequelae

• CBC with thalassemic indices, suspicious PB morphology or abnormal peak on Hgb A1C testing found incidentally
  • To explain a hematological abnormality... so what if clinically insignificant to the patient? May have importance in the context of family planning, including patient’s family members.

• Clinical evidence of thalassemia or SCD
  • For appropriate treatment, precautions and family planning

• Unexplained hemolytic anemia, erythrocytosis, or cyanosis
  • For appropriate treatment, precautions and family planning
Laboratory testing includes:

- CBC: RBC, Hgb, MCV, MCHC, and RDW
- Peripheral smear morphology
- Ferritin and iron studies
- Low tech: sickle solubility test, H-body prep, and Hgb stability test.
- High tech: IFE, acid and alkaline gel electrophoresis, HPLC, capillary electrophoresis, and molecular gene analysis.
Why do we use two testing modalities?

• Tests used to identify variant hemoglobins typically do not give specific results but rather seek to recognize different hemoglobins by means of their physico-chemical characteristics, in the context of clinical, family history, ethnic origin, blood count, and blood film.

• Hemoglobins have different properties and performance characteristics based on different methods of analysis. Multiple methods allow for more reliable categorization. A method which would allow separation of all variants does not exist!

• Know how tests are performed, causes of false positive/negative results, the benefits and limitations, and how results are interpreted in conjunction with other test modalities.
Sickle solubility test

• A lysate of RBC are placed in a high phosphate buffer solution. Sodium hydrosulfite is added, which lowers the oxygen tension of the solutions. Hgb S, if present, will form a cloudy solution as it precipitates.

• Benefits: it’s fast, cheap, easy/low tech, widespread use, minimal skill needed to perform and read results, and can be an adjunct for other test modalities to differentiate Hgb variants or confirm presence of Hgb S.
Sickle solubility test

• Limitations: can’t differentiate heterozygous trait vs homozygous disease, can’t quantify Hgb fractions, can’t tell difference between compound heterozygous sickling diseases (Hgb S/C, S/D, S/O-Arab, S/Beta thal°, and Hgb C-Harlem).

• **False-negative:** % of Hgb S is below 15-20% (post-exchange transfusion, severe anemia or infant less than 6 months). Old or outdated reagents

• **False-positive:** other causes of turbidity (nRBCs, extreme leukocytosis, Heinz body hemolytic anemia/unstable hemoglobins, hyperlipidemia or marked hypergammaglobulininemia.
H-body prep (supravital stain)

• New methylene blue, brilliant cresyl blue, crystal violet or methyl violet.
• Must be done within 1hr of venipuncture (live, non-fixed cells), is technically challenging to perform and read (difficult to keep up stain quality and proficiency skills with low test volume), may be confused with Heinz bodies.
• Hgb H (β tetramer) can’t transport O2. It’s unstable and precipitate in RBC giving the appearance of many small dots inside cells. Causes variable degree of hemolytic anemia.
Hgb instability/precipitation test

• Heat or isopropanol test; premise is to weaken van der Waals bonds in Hgb, and unstable Hgb variants will precipitate but stable Hgb will not. Not widely used.

• False positives occur with aging samples due to formation of methemoglobin over time. Also, Hgb H, S, E or increased Hgb F will precipitate as they are less stable than Hgb A.

• Hgb Köln is the most common; β chain variant causing hereditary Heinz body hemolytic anemia. Autosomal dominant, Dutch or German heritage. Hgb Olmstead (β chain variant) is more rare.

• Hgb Terre Haute is an extremely unstable Hgb leading to a thalassemic phenotype.
Gel electrophoresis

• Hgbs separate based on charge and ability to move through agar gel. Amino acid substitutions alter charge and mobility.

• Combo of acid (citrate agar with pH 6.2) and alkaline (cellulose acetate at pH 8.6; at alkaline pH, all Hgb have a negative charge and migrate towards the anode proportional to their charge) read together.

• It’s super cheap and separates many common variants, but labor intensive, not quantifiable and poor separation of many variants.

• On alkaline; many variants move with S (D, G, Lepore and Korle Bu), and A2 (C, C-Harlem, E and O-Arab).

• When read with acid gel, Hgb S can be confirmed, and C is separated! Unfortunately, many variants show no altered mobility from Hgb A (including A2).
Hgb F will run between S and A.  
Hgb C-Harlem runs with Hgb C/A2.  

**Lots** of things run with Hgb A; A2, D, E, G, Lepore, H, I, N, and J.  
Hgb C-Harlem runs with Hgb S.  
Hgb O-Arab migrates between A and S, and can be misdiagnosed.
Gel electrophoresis- exam studying tip

Example of how you could quiz yourself for practice:
Alkaline gel shows a single band in A2, then:
  Acid; single band in C = Hgb CC or C/Beta thal°
  Acid; single band in A = Hgb EE or E/Beta thal°
  Acid; band in C and band in A = Hgb C/E
  Acid; band in S = Hgb C-Harlem, or between S and A = Hgb O-Arab

• Do the same for single band in S on alkaline, a band in C and in A on alkaline....
• Situations when you are left with a differential, think about how additional testing would resolve; would results of a sickledex or knowing if CBC indices were thalassemic help? For each Ddx what would you expect on HPLC?
HPLC (high performance liquid chromatography)

• Automated cation exchange; diluted RBC lysate is placed in an exchange column. HgbS are adsorbed onto the negatively charged column (stationary phase), and then eluted off by a positively charged solution added in increasing concentration (competing with Hgb for binding). HgbS are differentially eluted at a rate related to their affinity for the column, and detected by a photometer. The resulting peaks are graphically represented in defined windows based on their retention time. The size of the Hgb fraction can be quantified as area under the curve.

• Benefits: It’s automated, precise, and fast, with high throughput. It requires very small sample and can be used for newborn screening. It can identify and quantify normal HgbS and many common Hgb variants.

• Limitations: Capital cost, interpretation requires technical skill*, can’t distinguish some common variants including Hgb E, Korle Bu, and Lepore from Hgb A2. Fast eluting Hgb variants can spuriously raise Hgb A1c, interfering with DM management.
Normal pattern
Fast eluting Hgb variants with minimal hematologic consequence, like Hgb F, I, J-Baltimore, N-Baltimore and Hgb Hope, can interfere with Hgb A1C monitoring.

Common variants
Remember, Hgb E, Lepore and Korle Bu usually elute in the Hgb A2 window. **TIP:** use expected Hgb fraction to differentiate Hgb E vs Lepore vs elevated A2 in beta thal.
Hgb O-Arab runs between S and C.
Hgb G runs in D window.
Study practice: What runs with Hgb C on alkaline gel and how you can figure this out with acid gel or HPLC results

<table>
<thead>
<tr>
<th>Haemoglobin</th>
<th>Relevant globin chain</th>
<th>Usual percentage</th>
<th>Mobility on agarose gel at acid pH</th>
<th>HPLC</th>
<th>Usual ethnic origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₂</td>
<td>δ</td>
<td>2–3.5*</td>
<td>With A</td>
<td>A₂ window</td>
<td>Normal minor haemoglobin</td>
</tr>
<tr>
<td>C</td>
<td>β</td>
<td>40–45†</td>
<td>C</td>
<td>C window</td>
<td>West African ancestry</td>
</tr>
<tr>
<td>E</td>
<td>β</td>
<td>30–35</td>
<td>With A</td>
<td>A₂ window</td>
<td>South-east Asian</td>
</tr>
<tr>
<td>O-Arab</td>
<td>β</td>
<td>40–45</td>
<td>Slightly on C side of S‡</td>
<td>Between S and C windows but closer to C window</td>
<td>Eastern European, Afro-American, Afro-Caribbean</td>
</tr>
<tr>
<td>C-Harlem</td>
<td>β</td>
<td>40–45†</td>
<td>With S‡</td>
<td>Between S and C windows but closer to C window</td>
<td>West African ancestry</td>
</tr>
<tr>
<td>E-Saskatoon</td>
<td>β</td>
<td>35–40</td>
<td>With A</td>
<td>S window</td>
<td>Scottish, Turkish</td>
</tr>
</tbody>
</table>

HPLC, high performance liquid chromatography.
*3.5–8% in most β thalassaemia trait.
† Lower if coexisting 8 thalassaemia trait.
‡ O-Arab and C-Harlem are more readily distinguished from each other on citrate agar than on agarose; on citrate agar at acid pH, C-Harlem moves with S and O-Arab is between S and A but closer to A.
What runs with Hgb S on alkaline gel?

<table>
<thead>
<tr>
<th>Haemoglobin</th>
<th>Abnormal globin chain</th>
<th>Usual percentage</th>
<th>Mobility on agarose gel at acid pH</th>
<th>HPLC</th>
<th>Usual ethnic origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>β</td>
<td>40–45*</td>
<td>S</td>
<td>S window</td>
<td>African ancestry, Arab, Indian</td>
</tr>
<tr>
<td>D-Punjab</td>
<td>β</td>
<td>40–45*</td>
<td>With A</td>
<td>D window</td>
<td>Punjabi, Northern European, Greek, Turkish, Yugoslav, Afro-American, Afro-Caribbean, Chinese</td>
</tr>
<tr>
<td>G-Philadelphia</td>
<td>α</td>
<td>20–25‡, 25–35‡, 35–45‡</td>
<td>With A</td>
<td>D window†</td>
<td>African ancestry, Chinese, Italian</td>
</tr>
<tr>
<td>Lepore</td>
<td>δβ fusion</td>
<td>7–15</td>
<td>With A</td>
<td>A₂ window</td>
<td>Greek, Italian, Turkish, Cypriot, Eastern European, English, Spanish, Afro-Caribbean</td>
</tr>
<tr>
<td>Korle Bu</td>
<td>β</td>
<td>40–45</td>
<td>With A</td>
<td>A₂ window</td>
<td>West African ancestry</td>
</tr>
<tr>
<td>G-Coushatta</td>
<td>β</td>
<td>40–45</td>
<td>With A</td>
<td>A₂ window</td>
<td>Native American, Chinese, Korean, Japanese, Thai, Italian, Turkish, Algerian</td>
</tr>
<tr>
<td>D-Iran</td>
<td>β</td>
<td>36–45</td>
<td>With A</td>
<td>A₂ window</td>
<td>Iranian, Pakistani, Italian, Jamaican</td>
</tr>
<tr>
<td>Zurich</td>
<td>β</td>
<td>22–35</td>
<td>With A</td>
<td>A₂ window</td>
<td>Swiss, Japanese</td>
</tr>
<tr>
<td>Hasharon</td>
<td>α</td>
<td>15–20 (if Jewish) or 30–35 (if Italian)</td>
<td>With S and C†</td>
<td>Between S and C†</td>
<td>Ashkenazi Jewish, Italians from Ferrara district</td>
</tr>
</tbody>
</table>
Capillary electrophoresis

- In a narrow bore capillary tube, higher voltages can be used to separate charged molecules based on their electrophoretic mobility in alkaline buffer (pH 9.4; electroosmotic flow at it’s greatest). Electrophoresis principle; differential movement or migration of ions by attraction or repulsion in an electric field. Higher voltages allows for shorter running times. This is done at a constant temperatures (maintenance of buffer viscosity is crucial), and bands are then visualized by UV optics.

- **Benefits**: automated with high throughput, can identify and quantify normal HgbS and many common Hgb variants. Separates Hgb A2 from E (unlike HPLC). Small sample required but can’t use dried blood spot.

- **Limitations**: capital cost, technical skill and expertise to interpret, poor separation of Hgb S from D, electrophoresis zones *shifted in absence of Hgb A, and decreased resolution with sample age.
Capillary electrophoresis

* Do remember every analyzer is different and in practice, may have slight variations in zones.

Other rare Hgb may fall in these zones too...

Common variants

<table>
<thead>
<tr>
<th>H</th>
<th>Bart's</th>
<th>A</th>
<th>F</th>
<th>D/S</th>
<th>E</th>
<th>A2</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lep</td>
<td>CSpring</td>
<td>G</td>
<td>O</td>
<td>CSpring</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
IFE (isoelectric focusing)

- Polyacrylamide or agarose gel containing low molecular weight molecules that allow a pH gradient.
- Hgbs in the gel migrate in an electric field to their isoelectric point (the pH at which their net charge is 0).
- This differs from most other techniques which rely on speed at which protein moves.
- Benefits: suitable for small samples including dried blood spot (newborn screening), higher resolution (more separation of Hgb variants) thus allows distinction of Hgb D from G for example.
- Disadvantages: expensive, can’t precisely quantify variants, not widely used.

**Don’t memorize this!**
Thalassemia; variable clinical consequence

• Imbalanced globin gene synthesis causes
  • RBC hemolysis: precipitated globin chains like Hgb H and Bart’s, or alpha chain inclusion bodies damage the cell membrane (removal by spleen).
  • Ineffective hematopoiesis

• Leading to “Thalassemic indices”; microcytic, hypochromic red cells +/- anemia and normal or elevated RBC count.

• Alpha globin is required to make Hgb F, thus why alpha thal can present in utero, while beta thal does not have clinical manifestations until a ~6 months after birth (when Hgb F drops and production of Hgb A increases).

• Mentzer Index: RBC/MCV (estimation to help distinguish IDA from thal)
  • <13.9: more suspicious for thalassemia
  • >14.0: normal/iron deficiency
Alpha thal

- Type 1= A° (--/aa) SEA, Mediterranean vs Type 2= A+ (a-/aa) usually Africans
- 1 gene affected: asymptomatic carrier, CBC may be normal
- 2: asymptomatic trait usually with thalassemic indicies
- 3: Hgb H disease with chronic hemolytic anemia and reticulocytosis, may be transfusion dependent
- 4 (--/-- or --/a*CS): Hgb Bart’s (Hydrops Fetalis) incompatible with life

- Alpha thal minor/trait has no electrophoretic abnormalities in adults. At birth, you would see a small amount of fast-moving Hgb Bart’s (γ tetramer). Hgb H disease at birth would show 20-40% Hgb Bart’s, and then in adulthood 5-30% Hgb H.

- Acquired Hgb H disease (MDS, AML, MPN); somatic mutation of ATRX gene on X chromosome. ATRX mutations are also cause of congenital X-linked syndrome affecting males, causing alpha thalassemia and intellectual disability.
Adult with **Hgb H disease** caused by deletional alpha thal --/aa* and an unstable α chain variant. The percentages for Hb H and Hb Bart's were 14.7% and 19.0%, respectively.

**TIP: Artifacts**
Bilirubin can run in the Bart’s window! Wash the cells. Also injection artifact (where the sample was loaded) may be seen here.

*Figure 1 - Cation-exchange HPLC chromatogram of the patient’s blood sample showing hemoglobins Bart’s and H beside the normal hemoglobins (Hb A₂ and Hb A).*
Constant-Spring

• Most common non-deletional alpha thal (1-8% in SEA). Caused by mutation in stop codon of α2 gene such that 31 additional amino acids are added. The variant Hgb itself is functional, but the mRNA is very unstable and the rate of alpha chain synthesis is less than 1%.

• It elutes with Hgb C on HPLC and runs with C on Capyllaris.

• Heterozygotes have marked anemia (MCV may be normal) and basophilic stippling. HPLC shows 2-11% Hgb Constant-Spring and usually 1-3% Hgb Bart’s.

• Co-inherited with Hgb H disease is severe, similar to alpha thal (--/--).
Molecular analysis (PCR, NGS, Sanger, MLPA...)

• **Most common alpha thal deletions;** 3.7kB deletion and 4.2kB.

• Protein-based methods are widely available with extensive clinical experience, but it’s not possible to make a definitive diagnosis of thalassemia.

• DNA-based methods determine genotype and are *diagnostic of alpha thal*, but are expensive and only done in reference labs.

• Main utility is in the **context of family planning;** person with thalassemic indices but normal HPLC or electrophoresis (suspected alpha thal) and screening their partners even if normal CBC.
Beta thal

• **Minor/trait**: 1 abnormal β allele (usually asymptomatic with microcytosis)

• **Intermedia**: compound heterozygotes for 2 different abnormal β allele (variable clinical significance, generally symptomatic but do not require transfusion during childhood). Severity ameliorated by presence of alpha globin mutations or presence of increased Hgb F.

• **Major**: homozygous for mutations associated with absent or severely reduced production of β chains (becomes clinically apparent after **6 months** of age with effects of severe hemolytic anemia, extramedullary hematopoiesis with skeletal abnormalities and ineffective erythropoiesis). Dependent of chronic transfusions and suffer effects of end-organ damage from iron overload. Mortality is high due to the various long-term consequences.

• Electrophoresis findings in trait/intermedia; Hgb A2 increased (**3.5-7%**), and ~50% of patient’s have slightly increased Hgb F. In beta thal major; Hgb A is absent, thus only have Hgb A2 and 10-100% Hgb F.
Beta thal major

The toxicity of the excess beta globin chains (which can form soluble tetramers) on the RBC membrane skeleton appears to be less than that of the excess, partially oxidized alpha globin chains in beta thalassemia.

Unstable alpha chain precipitates are more deleterious to the RBC membrane because they can’t form soluble tetramers. This provides some explanation of why Beta thal seems to be clinically more significant than alpha thal.
Beta thal major

Skeletal abnormalities are dramatic, producing the characteristic "chipmunk facies" and delayed skeletal maturation. This is due to expansion and invasion of erythroid bone marrow, which widen the marrow spaces, attenuate the cortex, and produce osteoporosis. Extramedullary hematopoiesis can break through cortex and behave like benign tumour in severe cases.

Common X-ray finding of skull shows “hair-on-end” appearance.
Good lists

- **Alternative causes of increased Hgb A2 besides beta thal?**
  - Hyperthyroidism
  - Megaloblastic anemia
  - Treatment/drugs for HIV (zidovudine)
  - Hgb S

- **Causes of decreased Hgb A2?**
  - **Iron deficiency**
  - Alpha thal
  - Delta globin variants
  - HPFH
  - Sideroblastic anemia (including lead poisoning)

- **Alternative causes of thalassemic indices with normal Hgb A2 besides alpha thal?**
  - PV with iron deficiency
  - Beta thal with iron deficiency
  - Beta thal with co-inherited alpha thal/Hgb H disease
  - Beta thal post-transfusion
  - Delta/Beta thal
  - Gamma/Delta/Beta thal

- **Name 3 methods to accurately quantify Hgb A2**
  - HPLC
  - Capillary zone electrophoresis
  - Immunoaffinity chromatography
HPFH/Delta-Beta thal

- Generally classified by the cellular distribution of Hgb F (heterocellular vs homocellular).
- Most HPFH are caused by deletional mutations spanning both the beta and delta globin genes. This causes pan/homocellular distribution of Hgb F in all red cells, with *balanced* alpha and non-alpha chain synthesis. Hgb F increased to ~15-35%.
- Non-deletional forms involve activating point mutations in promoter region of gamma globin gene, thus can show either a pan- or heterocellular distribution of Hgb F.

- Delta-Beta thal occurs with large deletions in the *HBB* gene cluster that remove the beta- and delta-globin genes, sparing both gamma-globin genes or *HBG2* alone. Think about this if you see thalassemic indices with elevated Hgb F and normal Hgb A2. There is *unbalanced* alpha and non-alpha chain synthesis. Hgb F increased ~5-15%
- Homozygosity will yield 100% Hgb F with a mild thalassemia intermedia phenotype
Other causes of increased Hgb F

- Developmental: Trisomy 13, infants of diabetic moms
- Bone marrow regeneration: TEC, BMT, chemo, iron repletion
- BM failure syndromes: FA, DKC, DBA
- Leukemia: JMML, Erythroleukemia
- Other: Choriocarcinoma
Hgb Variants in general

• Be able to describe the PB findings, CBC indices and clinical implications of commonly encountered hemoglobinopathies. Know the patterns of these on gels, HPLC, and capillary zone electrophoresis. Clinical effect and interpretation of patterns during neonatal/childhood. As well, know the Hgb fraction and clinical effect of the variant if co-inherited with a thalassemia or another variant Hgb.

• Heterozygous and homozygous Hgb S, C, E, D, O-Arab, Lepore, G (most common alpha variant), SC disease and HPFH.

• Know a little about the uncommon ones: Hgb C-Harlem, Constant-Spring, J, I, A2’ (delta variant), Köln, Hgb M-Saskatoon and M-Hyde Park, Chesapeake and Kempsey, and co-inherited variants S/G, S/E, S/HPFH...
Hgb Lepore

- Hgb Lepore results from non-homologous meitic crossover and recombination between delta and beta globin genes. The product is a fused (delta-beta) globin polypeptide. 3 types depending on breakpoint, but Lepore Boston is most common.

- Lepores are stable but under-produced (under control of delta globin promoter), thus function as a thalassemic allele. Should represent ~5-15% of total Hgb.

- Homozygous state is a severe thalassemic disorder similar to beta thal intermedia or major.

- Runs with S on alkaline gel (but negative sickledex), and with A on acid gel. It elutes with Hgb A2 on HPLC and migrates to the D window on Capyllaris.
Considering variant Hgb fraction

• As there are 4 alpha genes, an alpha chain variant is expected to total less than 25% of total Hgb. Remember, α2 is produced more than α1 (2.5:1 ratio), so it depends which gene is mutated (an α1 variant should represent 12.5% and α2 variants 37.5%).

• With only 2 beta genes, beta chain variant fraction usually totals more than 25%. Why not 50%? Usually the alpha chains preferentially bind the normal beta chain.

• Hgb fractions may differ from expectations for various reasons; the variant chain may be produced at a reduced rate or may be unstable, the variant may have greater or lesser affinity for normal Hgb chains (preferential binding), or there may be coexisting thalassemia or variant.
Tips for HPLC/capillary electrophoresis interpretation

• Think about what’s going on and what is supposed to be there.

• A heterozygous beta variant in an adult will split the Hgb A peak by nearly half ($\alpha2\beta2$ and $\alpha2$ with the variant). In an infant, they have mostly Hgb F and will thus only show a tiny blip of split Hgb A (or blip of variant only in homozygous state).

• A delta variant (A2’) in an adult will split the Hgb A2 peak by nearly half ($\alpha2\Delta2$ and $\alpha2$ with the variant). The only significance is diagnostic confusion; you have to add the A2 and A2’ fractions together to get the appropriate percentage of “Hgb A2” when considering a beta thal.

• An alpha variant (G) will split all peaks… can be hidden if falls in the zone of another Hgb with larger fraction, and variant fraction increases with concurrent alpha thal.

• Most of the common variants are of the Beta chain. Know the expected Hgb fraction of the main ones; if your case has less, that is suggestive of co-inherited alpha thal. If more, that’s suggestive of beta thal+. If too many peaks, suggestive of an co-inherited alpha variant.
## Hgb S, C and E

- **Hgb S**: point mutation substituting glutamic acid by **valine** at the 6th AA position of the β chain.
- **Hgb C**: same as S, but replaced by **lysine**.
- **Hgb E**: glutamic acid replaced by **lysine** at the 26th AA position.

<table>
<thead>
<tr>
<th>Variant %</th>
<th>Hgb AE</th>
<th>Hgb AC</th>
<th>Hgb AS</th>
<th>Hgb SS</th>
<th>Hgb SC</th>
<th>Hgb S/β⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30-35%</td>
<td>35-40%</td>
<td>35-40</td>
<td>80-95+ 10-15%Hgb F (S/HPFH 20-30% F)</td>
<td>~90S +Hgb F (S/β⁺; S% &gt;50)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Hgb</th>
<th>N</th>
<th>N</th>
<th>N</th>
<th>65-85</th>
<th>~100</th>
<th>Variable</th>
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</table>

<table>
<thead>
<tr>
<th>MCV</th>
<th>↓</th>
<th>N/↓</th>
<th>N</th>
<th>N</th>
<th>N/↓</th>
<th>↓↓↓↓↓</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Significant</th>
<th>Nothing</th>
<th>Nothing</th>
<th>N</th>
<th>Severe sickling disease</th>
<th>More mild sickling disease</th>
<th>More mild sickling disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE; mild thal</td>
<td></td>
<td>CC; mild hemolytic anemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Normal</th>
<th>Normal</th>
<th>N</th>
<th>Sickle cells, hyposplenic changes ++nRBC</th>
<th>Target cells, SC poik (boat/pita)</th>
<th>++poik, sickles, ++nRBC, hyposplenic changes</th>
</tr>
</thead>
</table>
Know tracings associated with Hgb S well; adults and neonate!

Capillaris Sickle trait **Hgb AS**

HPLC Homozygous **Hgb SS** (or Hgb S/B°)

Remember: presence of Hgb S spuriously raises Hgb A2 levels (usually <4%), this alone is not indicative of Hgb S/beta+ thal.
Easy cases...

HPLC Hgb S/C

HPLC S/Beta⁺ thal

RBC 5.28
Hgb 121 g/L
MCV 66 fl
MCHC 346 f/L
Asymptomatic black woman with normal CBC and PB morphology. Sickledex positive.

18 yr old black male with thalassemic indices. PB morphology shows target cells and basophilic stippling. Alkaline gel shows a band in the S window and a band in A. Sickledex negative. Acid gel shows a single band in A position. HPLC shows a 35% peak eluting in the D window.

Asymptomatic black woman with normal CBC and PB morphology. Sickledex positive.
General approach

• What is age of patient (what types of Hgb should be present?)
  • In a neonate, even homozygous beta variant will show mostly Hgb F, with only a small blip of the variant Hgb (and no Hgb A obviously).

• Look at CBC (anemia? Thalassemic indices?) correlate with iron studies (rule out IDA), sickledex results, etc.

• Look at results of your institutions testing method;
  • No abnormal peaks but microcytic; A2= 3.5-8% (beta thal), A2= normal (presumed alpha thal)
  • Abnormal peak? Use multiple modalities to determine the variant, as discussed in this lecture.
  • If heterozygous, then look at the CBC indices and the variant fraction of total Hgb:
    • Is it lower than expected? Co-inherited thalassemia of the opposite chain (or is it Hgb SS post-transfusion?)
    • Higher than expected? Co-inherited thalassemia of the same chain
  • If you have no Hgb A; is it homozygous or compound heterozygous variants? Are CBC values appropriate? Hgb SS vs Hgb S/B° look the same on HPLC; difference will be thalassemic indices.
  • Look at peripheral blood morphology; does it make sense with your diagnosis?

• Send for further confirmation testing as required.
Hb A + PRESENCE OF A COMMON VARIANT (Hb S, Hb C, Hb E)

Hb A + Hb S
- Hb S = 35 - 40%
  - Heterozygote A/S
- Hb S < 35%
  - Heterozygote A/S + α-thalassemia or Iron deficiency
  - Hb S > Hb A
    - Compound Heterozygote S/β⁺-thalassemia?

Hb A + Hb C
- Hb C = 35 - 40%
  - Heterozygote A/C
- Hb C < 35%
  - Heterozygote A/C + α-thalassemia or Iron deficiency
  - Hb C > Hb A
    - Compound Heterozygote C/β⁺-thalassemia?

Hb A + Hb E
- Hb E = 25 - 30%
  - Heterozygote A/E
- Hb E < 25%
  - Heterozygote A/E + α-thalassemia or Iron deficiency
  - Hb E > Hb A
    - Compound Heterozygote E/β⁺-thalassemia?

Vinatier I. CERBA recommendations (2010)
Practical approach for further testing

• Most variant Hgb when heterozygous, are clinically insignificant to the patient, and usually hematologically silent. Even in homozygous state some have minimal consequences (Hgb DD, EE, and OO). Similarly to alpha thal trait, the main concern is co-inheritance of other variants/thalassemia in family planning can lead to offspring with significant disease.

• Not all variants are identifiable; if no clinical significance than does it matter?

• My practice in regards to sending samples to a reference lab for molecular confirmation; advise clinicians to send a new sample for DNA analysis if clinically significant to the patient or when indicated for *family planning/prenatal testing.

• Many ethical and healthcare utilization issues with reflex testing.
Questions?

• This power point literally contains **everything** I know about hemoglobinopathy testing. Hopefully you’ve picked up some useful tips, now go look at some gels/HPLC/ Capyllaris cases for practice.

• What’s not included, and I recommend you study, is a complete review of complications and treatment in sickling disease and severe thalassemia. Management of these patient’s is major topic in transfusion, which you should know well.

• I know this was an overwhelming amount of information. I’d hoped this powerpoint would be a very comprehensive study reference for you.