HEMOLYTIC ANEMIAS: Laboratory Diagnosis

Behzad Poopak, DCLS PhD
bpoopak@yahoo.com

CME, 21.11.1389
Hemolytic anemias

The hemolytic anemia is the disease condition of short erythrocyte survival partially offset by increased activity of the bone marrow erythropoiesis.
The hemolytic disease and hemolytic anemia

With optimal marrow compensation, the survival of red cells in the circulation can decrease from the normal 120 days to as few as 15 to 20 days without anemia developing.

Such an increase in both destruction and production of erythrocytes can result in a compensated hemolytic state without anemia being present, so-called compensated hemolytic disease.

When red cell survival is so short that anemia develops despite a vigorous erythropoietic response, however, the term hemolytic anemia is appropriate.
### PATHOGENESIS AND CLASSIFICATION OF HEMOLYTIC ANEMIAS

<table>
<thead>
<tr>
<th>The course of the disease</th>
<th>acute</th>
<th>chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td>The place of RBC distraction</td>
<td>intravascular</td>
<td>extravascular</td>
</tr>
<tr>
<td>The whence</td>
<td>acquired</td>
<td>inherited</td>
</tr>
</tbody>
</table>
Lab findings differ, depending on:

- The site of blood destruction
- The amount of destroyed blood
- The rate of destruction
LABORATORY MANIFESTATIONS

- **Test findings**
  1. Related to the *increase in erythrocyte destruction*,
  2. Related to the *compensatory increase* in the rate of erythropoiesis,
  3. Found only in *particular varieties* of hemolytic anemia, which therefore are useful in differential diagnosis.
I. Signs of Excessive Red Cell Destruction

1. Erythrocyte Survival
2. Catabolism of Heme
3. Lactate Dehydrogenase increase
4. Disappearance / Decrease of Haptoglobin
5. Glycosylated Hemoglobin decrease
6. Signs of Intravascular Hemolysis
7. Signs of intracellular Hemolysis
I. Signs of Excessive Red Cell Destruction

1. *Erythrocyte Survival*

   - The life span of the red cell can be measured by random labeling with **51 chromium**.
   - Erythrocyte life span determinations are *time-consuming* and *expensive*.
   - This test is rarely necessary because the approximate amount of red cell destruction usually can be determined by the *serial observations of the degree of anemia, reticulocytosis, and jaundice*.
   - For these reasons, determination of red cell survival should be reserved for use in evaluating patients with especially *difficult diagnostic problems*. 
I. Signs of Excessive Red Cell Destruction

2. Catabolism of Heme

Serum Bilirubin unconjugated (indirect-reacting),

The increased serum bilirubin level in hemolysis almost always consists of the unconjugated (indirect-reacting) pigment. The conjugated fraction remains within normal limits, and no bilirubin is evident in the urine.

Rate of Heme Catabolism. endogenous carbon monoxide 

fecal urobilinogen excretion
Bilirubin

- Absence of hyperbilirubinemia **does not exclude** HA
- Unconjugated bilirubin also corrected for anemia

**Upper limit of normal** = \(1.0 \text{ mg/dL} \times \text{patient's Hct/45}\)

- Serum bilirubin **1 - 4 mg/dL** especially if unconjugated implies hemolysis or Gilbert's syndrome or ineffective erythropoiesis such as megaloblastic anemia; serum bilirubin > **4 mg/dL** hemolysis plus liver dysfunction
I. Signs of Excessive Red Cell Destruction

3. Lactate Dehydrogenase increase.

- Serum LD activity often is increased in patients with hemolytic anemia, as in megaloblastic anemia.
- Of the LDH, LDH-2 isozymes predominates in hemolytic anemia, LDH-1 predominates in megaloblastic conditions.
- The increase in LDH probably results from liberation of the erythrocyte enzyme into the plasma during hemolysis.
- The usefulness of LDH values in the detection of hemolysis is limited by this lack of specificity.

- LDH: specificity 61%; predictive value 40%
- LDH + haptoglobin: specificity 92%
I. Signs of Excessive Red Cell Destruction

4. Disappearance of Haptoglobin

- A low haptoglobin level indicated an 87% probability of hemolytic disease. When hemoglobin enters the plasma, it binds to haptoglobin, and the hepatocyte removes the complex.

- Haptoglobin tends to disappear from the plasma in individuals with hemolytic disease with the intravascular site and with predominantly extravascular hemolysis, such as sickle cell anemia, hereditary spherocytosis, hereditary elliptocytosis, and pyruvate kinase deficiency.

- In hereditary spherocytosis, the characteristically reduced haptoglobin levels are restored after splenectomy.
Serum Haptoglobin

- Reduced both with intra and extravascular hemolysis

  - Sensitive (reduced when hemoglobin destruction exceeds 2-3 times normal) but may require greater degrees of hemolysis in states of increased synthesis (acute phase reactant/inflammation, neoplasia and steroid therapy). Low levels congenitally (rare) and severe hepatocellular disease

- Not usually decreased following transfusion
I. Signs of Excessive Red Cell Destruction

5. Glycosylated Hemoglobin decrease
   The averaged 6.7% (range, 6.0 to 8.0%) normal
   the average value fell to 3.9% (range, 2 to 5.5%) - hemolytic anemias

6. Signs of Intravascular Hemolysis
   Hemoglobinemia
   Hemoglobinuria
   Urine Iron Excretion,
   Hemosiderinuria

7. Signs of intracellular Hemolysis
   Splenomegaly
Hemoglobinemia
Hemoglobinuria
Hemosiderinuria-urine sediment
Hemosiderinuria-urine sediment
Iron stain
II. Signs of Accelerated Erythropoiesis

Blood

- Reticulocytosis (Polychromasia, Basophilic stippling)
- Macrocytosis
- Erythroblastosis (Nrbc)
- Leukocytosis and thrombocytosis

Bone marrow

- Erythroid hyperplasia (more then 20-25% normoblasts in BM aspirate)
Polychromasiasia
Polychromasia
Reticulocyte Counts

- Rule of thumb: *uncorrected reticulocyte count* > 5% suspect hemolysis; > 10% *hemolysis very likely*

- Differential diagnosis: blood loss and recent treatment of a megaloblastic anemia
Erythroid Hyperplasia
III. Laboratory Test Findings Useful in Differential Diagnosis

1. Specific Morphologic Abnormalities of RBC
2. Hb Electrophoresis
3. The Antiglobulin (Coombs) Test
4. The Osmotic Fragility Test
4. Tests for Hemolytic Disorders Associated with Heinz-body Formation
5. Other Tests
Hereditary spherocytosis is characterized by numerous spherocytes
The presence of spherocytes is not diagnostically specific, since this may result from:

1. **Hereditary spherocytosis,**
2. **Autoimmune hemolytic anemia,** or
3. **Alloimmune hemolytic anemia (e.g., hemolytic disease of the newborn or a delayed transfusion reaction).**

Nevertheless, consideration of the **clinical features,** together with the results of a **direct antiglobulin test,** in patients with spherocytes will generally indicate the correct diagnosis.
Spherocytosis

- Warm autoimmune hemolytic anemia
- Acute and delayed hemolytic transfusion reactions
- ABO hemolytic disease of newborn/Rh hemolytic disease of newborn
- Hereditary spherocytosis
- Clostridium sepsis
- Intravenous water infusion or drowning (fresh water)
- Hypophosphatemia
- Bartonellosis
- Snake bite
- Cold autoimmune hemolytic anemia/paroxysmal cold hemoglobinuria
- Hyposplenism
- Rh-null phenotype
Acute hemolysis in glucose-6-phosphate dehydrogenase (G6PD) deficiency, with the presence of a “bite” cell, or keratocyte (arrow).
"blister" cells
Oxidized Hb accumulates

blister cell
Hemolytic anemia

- **Microspherocytes** (i.e., cells that are both hyperchromic and significantly reduced in size and therefore in diameter) may be present in low numbers in patients with a spherocytic hemolytic anemia but are also characteristic of burns and of microangiopathic hemolytic anemia.

- The detection of a microangiopathic hemolytic anemia is of considerable clinical significance, since this type of anemia may indicate pregnancy-associated hypertension, disseminated cancer, chronic disseminated intravascular coagulation, the hemolytic–uremic syndrome, or thrombotic thrombocytopenic purpura.

- The latter two conditions both require **urgent diagnosis** so that appropriate management can be initiated.
shows microangiopathic hemolytic anemia resulting from cyclosporine therapy, with numerous red-cell fragments.
Mechanical Trauma - Schistocytes
Schistocytes
<table>
<thead>
<tr>
<th>Table 1.14</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fragmentation</strong></td>
</tr>
<tr>
<td><strong>With thrombocytopenia</strong></td>
</tr>
<tr>
<td>- Disseminated intravascular coagulopathy (DIC)</td>
</tr>
<tr>
<td>- Thrombotic thrombocytopenic purpura (TTP)</td>
</tr>
<tr>
<td>- Hemolytic uremic syndrome (HUS)</td>
</tr>
<tr>
<td>- HELLP syndrome</td>
</tr>
<tr>
<td>- Preeclampsia/eclampsia</td>
</tr>
<tr>
<td>- Malignant hypertension</td>
</tr>
<tr>
<td>- Systemic lupus erythematosus (SLE)</td>
</tr>
<tr>
<td>- Vasculitis</td>
</tr>
<tr>
<td>- Scleroderma crisis</td>
</tr>
<tr>
<td>- Antiphospholipid antibody crisis</td>
</tr>
<tr>
<td>- Drugs (cyclosporine, tacrolimus, mitomycin C, gemcitabine)</td>
</tr>
<tr>
<td>- Sepsis</td>
</tr>
<tr>
<td>- Disseminated carcinoma (mucin secreting)</td>
</tr>
<tr>
<td>- Extracorporeal circulation devices</td>
</tr>
<tr>
<td>- Vascular malformations</td>
</tr>
</tbody>
</table>

| **Without thrombocytopenia** |
| - Damaged native and prosthetic heart valves |
| - Malignant hypertension |
| - Acute glomerulonephritis |
| - Rejection of transplanted kidney |
| - Renal cortical necrosis |
| - Drugs (cyclosporine, tacrolimus) |
| - Vasculitis |
| - Systemic lupus erythematosus (SLE) |
Hereditary Elliptocytosis
Stomatocytes
Sickle cell anemia, PBS
Sickle cell anemia, PBS
Sickle cells
Diagnostic recommendations regarding the laboratory investigation of abnormal Hbs and thalassemias

1. **Initial tests:**
   - Complete blood count (CBC),
   - Electrophoresis at pH 9.2,
   - Tests for solubility and sickling,
   - & quantification of Hb A2 and Hb F.

2. **If an abnormal Hb is identified on the preliminary tests:**
   - Electrophoresis at pH 6.0–6.2,
   - Globin chain separation,
   - and isoelectric focusing (IEF).
   - Additional testing, including heat and isopropanol stability tests, was recommended for detection of unstable Hbs or Hbs with altered oxygen affinity.
The elements of one approach include

- CBC
- Hb H test
- Ferritin
- HPLC or capillary Elect for Hb A2 and F quantification

The use of HPLC or Capillary Elect streamlines the recommended preliminary and follow-up tests for the identification of hemoglobinopathies and thalassemias and provides for rapid and complete diagnostic work up in a majority of cases.
**Patient Information**

- **Test:** Hemoglobin Electrophoresis
- **Date:** 1384/07/24
- **Lane:** 2
- **ID No.:** 2445
- **Age/Sex:**
- **Patient:** [Name]
- **Physician:** [Name]

**Results**

<table>
<thead>
<tr>
<th>No.</th>
<th>Fraction</th>
<th>(%)</th>
<th>Flag</th>
<th>Normal (%)</th>
<th>g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A1</td>
<td>94.2</td>
<td>L</td>
<td>95 - 98.5</td>
<td>11.6</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>1</td>
<td></td>
<td>0 - 2</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>A2</td>
<td>4.8</td>
<td>H</td>
<td>1.5 - 3.5</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**Chemical Methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A2 (Ion-Exchange chromatography)</td>
<td>5 %</td>
</tr>
<tr>
<td>Hb F (Alkali Denaturation)</td>
<td>0.5 %</td>
</tr>
</tbody>
</table>

**Comments**

HB A2 checked by column chromatography method.

**CBC**

- **WBC:** 4.9 x1000/mm3
- **RBC:** 5.64 mil/mm3
- **HGB:** 12.3 g/dl
- **HCT:** 37.3 %
- **MCV:** 66.1 Fl
- **MCH:** 21.8 Pg
- **MCHC:** 33 %
- **RDW:** 14.7

**Sickling Test:**

**Solubility Test:**

**Heinz body:**

**Golf ball RBC:**

**%F-cells Acid Elution:**

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Control

- A **control** sample containing Hb A, F, S, and C should be applied to each strip containing the unknown samples.
- With each analysis, slight variations in the migration rate may be caused by slight current fluctuations, different buffer lots, or variations in application.
- Participation in an inter laboratory trial or proficiency check system is recommended.
- Results should occasionally be rechecked using duplicate samples.
Separation

- With every new lot of cellulose acetate, samples containing a combination of Hbs A & F and of Hbs S & F should be applied and tested for separation properties.

- Distinct separation with a small, clear area between these hemoglobins must be obtained.

- It may be necessary to reduce the concentration of hemoglobin in the sample and also to adjust time & voltage to obtain these distinct separations.
Principle of capillary electrophoresis

Thermal bridge
Temperature controlled by Peltier elements

Detector
Deuterium Lampe
High voltage
Migration
Cathode -
Anode +
Capillaries: advantages

- Fully automated and very fast by application of very high voltage
- No support, free migration in liquid medium
- **Very good resolution** and reproducibility
- Very low analyzed sample volume (very good sensitivity)
- No dye, direct measurement at 200 nm / 415 nm
- **Curves similar to those obtained by densitometry**
- Advanced software capabilities
SPECIALISTS

Technology
Sample No. 25

Fraction values:
- Hb A: 97.2%
- Hb A2: 2.8%

Potential variants:
- Hb Bart's
- Hb J-Providence
- Hb J-Broussais
- Hb J-Mexico
- Hb J-Baltimore
- Hb J
- Hb D-Punjab
- Hb U-Ulved Kaban
- Hb Lesore
- Hb G-Philadelphia
- Hb Korte-Bu
- Hb Hedin
- Hb Winni
- Hb Sjorissen
- Hb E denatured
- "Hasharon" Hb A2 variant
- "Winnipeg" Hb A2 variant
- Other Hb A2 variants
- Hb Constant Spring

O.D. Mode: Final

Patient data:
- Rack: HBA
- Position: 1

Reagents:
- LOT: 29044/01
- Exp.
- LOT: 04092/01
- Exp.

Ratio 1: 1.00
Ratio 2: 1.00

Conc.

Comment:
- Sang normal
300 points with 16 zones Curve

<table>
<thead>
<tr>
<th>Zone</th>
<th>Hemoglobins (Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z1</td>
<td>Hb δA', Hb αA', other Hb A2 variants, Hb A2 Hasharon and Hb A2 Winnipeg variants</td>
</tr>
<tr>
<td>Z2</td>
<td>Hb C, Hb A2 Setif variant, Hb Constant Spring</td>
</tr>
<tr>
<td>Z3</td>
<td>Hb A2, Hb O-Arab</td>
</tr>
<tr>
<td>Z4</td>
<td>Hb E, Hb Köln, Hb A2 variants (and degraded Hb C)</td>
</tr>
<tr>
<td>Z5</td>
<td>Hb S (and degraded Hb O-Arab), Hb Hasharon</td>
</tr>
<tr>
<td>Z6</td>
<td>Hb D-Punjab, Hb D-Ouleed Rabah, Hb Lepore, Hb G-Philadelphia, Hb Korle-Bu, Hb Köln, Hb Setif, Hb G-Taipei Hb Winnipeg (and degraded Hb E).</td>
</tr>
<tr>
<td>Z7</td>
<td>Hb F, Hb Porto-Alegre, Hb Richmond, Hb G-San Jose (and degraded Hb S)</td>
</tr>
<tr>
<td>Z8</td>
<td>Hb Atlanta, acetylated Hb F</td>
</tr>
<tr>
<td>Z9</td>
<td>Hb A, Hb Okayama, Hb Camperdown, Hb Fontainebleau, Hb Toulon</td>
</tr>
<tr>
<td>Z10</td>
<td>Hb Hope</td>
</tr>
<tr>
<td>Z11</td>
<td>Hb J-Kaohsiung (and degraded Hb A)</td>
</tr>
<tr>
<td>Z12</td>
<td>Hb Bart's, Hb J-Providence, Hb J-Mexico, Hb J-Baltimore, Hb J, Hb J-Broussais</td>
</tr>
<tr>
<td>Z13</td>
<td>Hb N-Baltimore</td>
</tr>
<tr>
<td>Z15</td>
<td>Hb H</td>
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Normal Pattern

<table>
<thead>
<tr>
<th>Fractions</th>
<th>%</th>
<th>Ref. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A</td>
<td>97.2</td>
<td>96.5 - 98.5</td>
</tr>
<tr>
<td>Hb A2</td>
<td>2.8</td>
<td>1.5 - 3.5</td>
</tr>
</tbody>
</table>

CBC

<table>
<thead>
<tr>
<th>Name</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>11.1</td>
<td>x 1000/mm³</td>
</tr>
<tr>
<td>RBC</td>
<td>4.87</td>
<td>mil/mm³</td>
</tr>
<tr>
<td>Hb</td>
<td>14.2</td>
<td>g/dl</td>
</tr>
<tr>
<td>Hct</td>
<td>42.3</td>
<td>%</td>
</tr>
<tr>
<td>MCV</td>
<td>86.9</td>
<td>FL</td>
</tr>
<tr>
<td>MCH</td>
<td>29.2</td>
<td>Pg</td>
</tr>
<tr>
<td>MCHC</td>
<td>33.6</td>
<td>g/dl</td>
</tr>
<tr>
<td>RDW</td>
<td>14.3</td>
<td>%</td>
</tr>
</tbody>
</table>

Comment: Hb F <= 0.1 (Ref: <= 0.5)
Homozygous or double Heterzygous HbD- Iran or HbD-Punjab

Capillary Zone Electrophoresis
Name carousel: 1 Pos.: 4
Date: 2010/11/23
ID:
Physician:
Age:
Sex:

<table>
<thead>
<tr>
<th>Fractions</th>
<th>%</th>
<th>Ref. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb F</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Hb D</td>
<td>91.5</td>
<td></td>
</tr>
<tr>
<td>Hb A2</td>
<td>5.8</td>
<td></td>
</tr>
</tbody>
</table>

Comment: Homozygous HbD-Punjab or D Iran or double Heterozygous

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Web site: www.payvandlab.com   Email: info@payvandlab.com
Hb H disease
Case

A 28 year old pregnant female.

<table>
<thead>
<tr>
<th>RBC $x10^{12}$/L</th>
<th>Hb g/L</th>
<th>MCV fL</th>
<th>MCH pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3</td>
<td>114</td>
<td>69</td>
<td>22</td>
</tr>
</tbody>
</table>

Biorad VII HPLC

<table>
<thead>
<tr>
<th>Peak Name</th>
<th>Calibrated Area %</th>
<th>Area %</th>
<th>Retention Time (min)</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown</td>
<td>0.1</td>
<td>0.60</td>
<td>1.07</td>
<td>1887</td>
</tr>
<tr>
<td>F</td>
<td>0.2</td>
<td>0.5</td>
<td>1.22</td>
<td>3063</td>
</tr>
<tr>
<td>P2</td>
<td>4.7</td>
<td>1.30</td>
<td>1.72</td>
<td>5972</td>
</tr>
<tr>
<td>P3</td>
<td>3.8</td>
<td>3.68</td>
<td>3.21</td>
<td>7973</td>
</tr>
<tr>
<td>A2</td>
<td>98.1</td>
<td>2.48</td>
<td>2.48</td>
<td>102419</td>
</tr>
<tr>
<td>A2</td>
<td>2.9</td>
<td>3.68</td>
<td>3.21</td>
<td>53908</td>
</tr>
</tbody>
</table>

Total Area: 2070663

F Concentration = 0.2 %
A2 Concentration = 2.9 %

Analysis comments:

- Alkaline Gel

  - A2
  - FA
Hb Q/Hb Setif

<table>
<thead>
<tr>
<th>Fractions</th>
<th>%</th>
<th>Ref. %</th>
</tr>
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<tbody>
<tr>
<td>Hb A</td>
<td>79.0</td>
<td></td>
</tr>
<tr>
<td>Hb Setif/Q Iran18.6</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>Hb A2</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
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CBC

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Unit</th>
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<tbody>
<tr>
<td>WBC</td>
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<tr>
<td>RBC</td>
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<td>ml/mm3</td>
</tr>
<tr>
<td>Hb</td>
<td>13.2</td>
<td>g/dl</td>
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<tr>
<td>Hct</td>
<td>37.7</td>
<td>%</td>
</tr>
<tr>
<td>MCV</td>
<td>82.7</td>
<td>FL</td>
</tr>
<tr>
<td>MCH</td>
<td>28.9</td>
<td>Pg</td>
</tr>
<tr>
<td>MCHC</td>
<td>35.0</td>
<td>g/dl</td>
</tr>
<tr>
<td>RDW</td>
<td>11.1</td>
<td>%</td>
</tr>
</tbody>
</table>

Comment: Hb F =<0.1  (Ref=<0.5)
Heterozygous Hb A/Hb Setif should be considered.
Molecular Diagnosis for definitive evaluation of presence of Hb A & Hb Setif should be considered.

Dr. B. Poopak

Web site: www.payvandlab.com  Email: info@payvandlab.com
**Hb Q/Hb Setif**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>%</th>
<th>Ref. %</th>
</tr>
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<tbody>
<tr>
<td>Hb A</td>
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<tr>
<td>Hb Q/Hb setif</td>
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<tr>
<td>Hb A2</td>
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<tr>
<td>Hb A2 Variant</td>
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**CBC**

<table>
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<th>Value</th>
<th>Unit</th>
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</thead>
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<td>x 1000/mm3</td>
</tr>
<tr>
<td>RBC</td>
<td>4.35</td>
<td>mil/mm3</td>
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<tr>
<td>Hb</td>
<td>11.4</td>
<td>g/dl</td>
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<tr>
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<tr>
<td>MCHC</td>
<td>32.9</td>
<td>g/dl</td>
</tr>
<tr>
<td>RDW</td>
<td>15.3</td>
<td>%</td>
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</tbody>
</table>

Comment: **Hb F <= 0.1** (Ref: <= 0.5)
HbQ

- Single nucleotide mutations in α1 or α2 genes produce abnormal α-chain hemoglobins (Hbs).
- Hb Q disorders including Hb Q-Iran [α75 (EF4) Asp→His], Hb Q-Thailand [α74 (EF3) Asp→His], and Hb Q-India [α64 (E13) Asp→His] are important Hb variants.
- All three of these hemoglobins are slow-moving variants with an Asp→His substitution that migrates at the electrophoretic position of Hb S at alkaline pH
HbQ

- Heterozygous individuals for Hb Q-Thailand generally present with moderate red cell microcytosis due to the association of the mutation with the deletion -α4.2 kb,

- but those carrying Hb Q-Iran or Hb Q-India are hematologically normal and no association with an α-thalassemic phenotype has been reported.

- A level of 14.2 – 29.3% for Hb Q-Iran in heterozygous individuals
the coinheritance of Hb Q-Iran and the -α3.7 deletion in 17% of carriers of Hb Q-Iran (three out of 18) that resulted in increased expression of Hb Q-Iran (26.7%) compared to those with normal α-chains (18.7%).
Hb Setif

Hb Setif [α94 (G1) Asp→Tyr] is an α-chain Hb variant with electrophoretic mobility similar to Hb S at alkaline pH.

Hb Setif is much less soluble than Hb A and induces pseudosickling of the red cells in vitro.

Substitution of Asp by Tyr produces an unstable Hb with decreased O2 affinity and cooperativity present with a low percent (12 – 17%) expression.

Hb Setif has been found in Algeria, Iran, Lebanon, Saudi Arabia, Turkey, Italy, Malta, and Cyprus
HbQ / Hb Setif

The high prevalence of Hb Q-Iran and Hb Setif encountered in Kermanshah Province, western Iran, suggests that this region could be a hotspot for these mutations, differing from other regions of Iran, such as Hormozgan and Tehran where other large deletions and point mutations responsible for α-thalassemia have been found.
Hb-J

<table>
<thead>
<tr>
<th>Fractions</th>
<th>%</th>
<th>Ref. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb j</td>
<td>23.7</td>
<td></td>
</tr>
<tr>
<td>Hb Denatured</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Hb A</td>
<td>71.2</td>
<td></td>
</tr>
<tr>
<td>Hb F</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Hb A2</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

x 1000/mm³
mil/mm³
HbJ

- Hb J-Iran (β77 His → Asp) is one of the many informative Hb variants that have been discovered in the ethnically diverse population of Iran.
- Subsequently this variant was reported in Turkey and in a Russian-Armenian family.
- The concentration of this variant in heterozygotes was always found to be nearby equal to that of Hb A.
HbD-Iran / Punjab

Capillary Zone Electrophoresis

<table>
<thead>
<tr>
<th>Fractions</th>
<th>%</th>
<th>Ref. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A</td>
<td>58.8</td>
<td></td>
</tr>
<tr>
<td>Hb D-punjab/D-Iran</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb A2</td>
<td>3.0</td>
<td></td>
</tr>
</tbody>
</table>

CBC

<table>
<thead>
<tr>
<th>Name</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>8.7</td>
<td>x 1000/mm3</td>
</tr>
<tr>
<td>RBC</td>
<td>4.42</td>
<td>mil/mm3</td>
</tr>
<tr>
<td>Hb</td>
<td>12.7</td>
<td>g/dl</td>
</tr>
<tr>
<td>Hct</td>
<td>37.3</td>
<td>%</td>
</tr>
<tr>
<td>MCV</td>
<td>84.4</td>
<td>FL</td>
</tr>
<tr>
<td>MCH</td>
<td>28.7</td>
<td>Pg</td>
</tr>
<tr>
<td>MCHC</td>
<td>34.0</td>
<td>g/dl</td>
</tr>
<tr>
<td>RDW</td>
<td>12.7</td>
<td>%</td>
</tr>
</tbody>
</table>

Comment: Hb F =<0.1 (Ref=<0.5)

For definitive Dx of HbD-Iran / HbD Punjab molecular Dx should be considered.

Dr. B. Poopak

Web site: www.payvandlab.com      Email: info@payvandlab.com
HPLC system
The typical baseline to baseline resolution of a hemoglobin mixture containing Hb F, A, A 2 , S and C is shown.
Hb D-Iran

$\alpha_2\beta_2$ 22Glu $\rightarrow$ Gln

HbD-Iran & HbA2 both exit at the same time from column Hb D punjab in which glutamine replaces the normal 121 glutamic acid
Homozygous Hb Constant-Spring

<table>
<thead>
<tr>
<th>Fractions</th>
<th>%</th>
<th>Ref. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb Bart's</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Hb A</td>
<td>90.8</td>
<td></td>
</tr>
<tr>
<td>Hb F</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Hb A2</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Hb Constant spring</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>Hb alpha A2</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

CBC

<table>
<thead>
<tr>
<th>Name</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>6.9 x 10^6/mm^3</td>
<td></td>
</tr>
<tr>
<td>RBC</td>
<td>4.13 mil/mm^3</td>
<td></td>
</tr>
<tr>
<td>Hb</td>
<td>10.8 g/dl</td>
<td></td>
</tr>
<tr>
<td>Hct</td>
<td>35.5 %</td>
<td></td>
</tr>
<tr>
<td>MCV</td>
<td>86 FL</td>
<td></td>
</tr>
<tr>
<td>MCH</td>
<td>26.2 Pg</td>
<td></td>
</tr>
<tr>
<td>MCHC</td>
<td>30.4 g/dl</td>
<td></td>
</tr>
<tr>
<td>RDW</td>
<td>15.3 %</td>
<td></td>
</tr>
</tbody>
</table>

Comment: The picture is highly suggestive of homozygous Hb Constant-Spring state. The homozygous state appears as an asymptomatic mild hemolytic anemia with a Hb level of 9-11 g/dl, normal mean cell volume (MCV), and a small peak of Hb Bart's. Dr. B. Poopak

Web site: www.payvandlab.com
Email: info@payvandlab.com
Hemoglobin Constant Spring

It was first observed in Constant Spring, Jamaica, in a Chinese family.

Haemoglobin Constant Spring (HbCS) involves a TAA→CAA base pair substitution in the termination codon of the α2-globin gene.

Hb CS, α-chain variant with 31 extra AA.

It is synthesized slowly (poor output, 1% of normal) & results in thalassemia like picture.

HbCS is the most common α-globin structural variant in Malaysia and in other Southeast Asian countries.
Hb CS

Homozygote: Asymptomatic mild Hemolytic anemia, Hb: 9-11 g/dl, RBC indices are unusual for thalassemia: MCV nl (88 fl) & RBC is low (3.9 mil/cumm),

Hb CS: 5-8%, HbA2 nl, Hb Barts trace and the rest HbA

Heterozygote: silent carrier phenotype with no hematologic abnormality and about 1% Hb CS, more slowly migration than HbA2
Relative mobilities of some abnormal Hb. Cellulose acetate pH 8.5

<table>
<thead>
<tr>
<th>Anode (+)</th>
<th>Cathode (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Carbonic anhydrase</td>
</tr>
<tr>
<td></td>
<td>(A_2')</td>
</tr>
<tr>
<td>C</td>
<td>(A_2, E, C-Harlem, O-Arab)</td>
</tr>
<tr>
<td>S</td>
<td>(D, G, Q-India, Hasharon)</td>
</tr>
<tr>
<td></td>
<td>Lepore</td>
</tr>
<tr>
<td></td>
<td>F</td>
</tr>
<tr>
<td>A</td>
<td>K-Woolwich</td>
</tr>
<tr>
<td>J</td>
<td>Bart's</td>
</tr>
<tr>
<td>N</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>H</td>
</tr>
</tbody>
</table>
College of American Pathologists hemoglobinopathy survey showed

- A CV of **33.6%** for densitometric scanning of electrophoretic gels at a Hb A2 concentration of 2.41%.
- For column chromatography, the CV was **14.6%** at a mean Hb A2 concentration of 3.21%,
- and for HPLC, the CV was **4.3%** at a mean Hb A2 of 3.47%
III. Laboratory Test Findings Useful in Differential Diagnosis

2. The Antiglobulin (Coombs) Test

Positive test results indicate that the red cells are coated with IgG or complement components, especially C3. The test is usually satisfactory, but 2 to 5% of patients with immunohemolytic disease associated with warm complete agglutinins have negative test results because the amount of globulin on the cell surface is below the detection limits.

Positive tests are found in as many as 34% of patients with AIDS without other evidence of immunohemolytic disease.
Significance Positive DAT

- Incidence of positive DAT is hospitalized population is 7 – 8%
  - 80% due to C3d with no IgG
  - Most have no evidence of hemolysis even among patients with anemia
    - In most, eluates are non-reactive with RBCs and positive DAT associated with elevated plasma gamma globulins
  - Predictive value positive DAT in random patient: 1.4%
  - In patient with hemolytic anemia PPV positive DAT 83%; NPV negative DAT is 99%
Significance Positive DAT

- DAT-negative healthy individuals have 5 – 90 IgG and up to 560 C3d molecules per RBC
  - Distribution, physiological role?, RBC senescence?, Complement receptors

- 1:13,000 normal blood donors have positive DAT
  - IgG, increasing age, may persist for years, with sensitive testing possible to demonstrate increased RBC turnover but clinically significant hemolysis rare
DAT Immune Hemolysis

- **Warm antibody AIHA**
  - 67% positive IgG and C3d
  - 20% positive IgG and negative C3d
  - 13% Positive C3d and negative IgG

- **Cold Agglutinin Syndrome**
  - 100% positive C3d and negative IgG

- **Paroxysmal Cold Hemoglobinuria**
  - 100% positive C3d and negative IgG
3. The Osmotic Fragility Test

The osmotic fragility test is a measure of the resistance of erythrocytes to hemolysis by osmotic stress.

Increased osmotic fragility is observed in conditions associated with spherocytosis.
OFT
# Osmotic Fragility Tests

<table>
<thead>
<tr>
<th>NaCl g/L</th>
<th>9</th>
<th>7.5</th>
<th>6.5</th>
<th>6</th>
<th>5.5</th>
<th>5</th>
<th>4</th>
<th>3.5</th>
<th>3</th>
<th>2</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>% hem cont-0</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0.70%</td>
<td>1.60%</td>
<td>64%</td>
<td>93%</td>
<td>94%</td>
<td>97%</td>
<td>99%</td>
</tr>
<tr>
<td>% hem cont-24</td>
<td>0%</td>
<td>0.30%</td>
<td>0.40%</td>
<td>0.70%</td>
<td>5%</td>
<td>45%</td>
<td>82%</td>
<td>95%</td>
<td>96%</td>
<td>98%</td>
<td>99%</td>
</tr>
<tr>
<td>% hem pt-0</td>
<td>0.30%</td>
<td>0.30%</td>
<td>0.40%</td>
<td>0.45%</td>
<td>2.10%</td>
<td>46%</td>
<td>91.80%</td>
<td>92.50%</td>
<td>95.60%</td>
<td>94.40%</td>
<td>99.40%</td>
</tr>
<tr>
<td>% hem pt-24</td>
<td>1.10%</td>
<td>5.80%</td>
<td>35.10%</td>
<td>61.90%</td>
<td>74.40%</td>
<td>80.60%</td>
<td>91.60%</td>
<td>94.10%</td>
<td>95.70%</td>
<td>96.70%</td>
<td>98.90%</td>
</tr>
</tbody>
</table>
Osmograph

MCF (50% lysis), time 0: 4.8 (RI: 4.0-4.45)
MCF (50% lysis), After 24 Hr.: 6.2 (RI: 4.65-5.9)
III. Laboratory Test Findings Useful in Differential Diagnosis

4. Tests for Hemolytic Disorders Associated with Heinz-body Formation
Heinz Body
History

- They are named after Robert Heinz (1865-1924), a German physician who in 1890 described these inclusions in connection with cases of hemolytic anemia.
Heinz Body - Definition

- Refractile red cell inclusions of **variable size (1-3μm)** and usually **eccentrically** located and adhered to the red cell membrane.
- Seen only with **supravital staining** with crystal violet, brilliant cresyl blue, methyl violet or on a fresh, wet preparation of blood.
- *Not seen on a Wright-Giemsa stain*, but **spherocytosis** of varying degree, depending on the severity of the hemolysis, is usually present, and **bite cells** may be seen.
Pathobiology

- The inclusions are composed of denatured hemoglobin that occurs as a result of oxidative injury to the red cell. Oxidative injury to the red cell membrane also occurs.
Differential diagnosis

- Red cell *enzymopathies* (usually a result of oxidant drug exposure or infection)
  - *acute Heinz body hemolytic anemia*, e.g. G-6PD deficiency
- chemical poisoning, drug intoxication,
- Unstable hemoglobinopathies, e.g. Hb Gun-Hill
- Thalassemias
Heinz Body
*Peripheral blood smear, BCB stain, 1000x*
Heinz bodies

Heinz Body

Peripheral blood smear, 1000x
Reticulocytosis- Heinz bodies
Alpha Chain Precipitation
Alpha Chain Precipitation

- In β-thalassaemia major, methyl violet staining of the BM will demonstrate precipitated α-chains.

- These appear as large irregular inclusions in late normoblasts, usually single and closely adhering to the nucleus.

- If such patients are splenectomized, inclusions are also found in reticulocytes and mature red blood cells.
Heinz body, wright stain
Hb H INCLUSION BODIES IN BCB STAIN

RETICULOCYTE: 5-10%
Golf Ball Appearing RBC
Hemoglobin H Inclusions

- Mix together in a small tube as for staining reticulocytes equal volumes of fresh blood or EDTA-blood and 10 g/l brilliant cresyl blue or 20 g/l New methylene blue in iso-osmotic phosphate buffer pH 7.4.

- Leave the preparation at 37°C for 1-3 hours, and make films **at intervals** during this time.

- Haemoglobin H precipitates as **multiple pale-staining greenish-blue**, almost spherical, bodies of varying size.
5. Other Tests

Other important procedures for detecting and differentiating the hemolytic anemias include methods for identifying enzyme deficiency, for detecting and defining abnormal hemoglobins, the other serologic techniques for evaluating immunohemolytic anemias, and tests for paroxysmal nocturnal hemoglobinuria.
Ham's Test

Sucrose Hemolysis Test
A final diagnosis of one of the hemolytic anemias is established by a two-step process:

- First, demonstrating that a hemolytic anemia is present and,
- Second, determining the specific cause of the condition.
Thank you for attention