Hemolytic Anemia--Causes

Hereditary
• RBC Membrane
  – HS, HE, pyropoikilocytosis
• RBC Metabolic Defects
  – EM pathway
  – HMP shunt
  – Nucleotide synthesis
• Hemoglobin Defects
  – Thalassemia
  – Abnormal variants

Acquired
• Immune
  – Autoimmune
  – Isoimmune
  – Drug
• RBC fragmentation syndromes
• PNH
• Secondary
  – Renal and liver disease
• Misc
  – Drugs, infections, chemicals, toxins, physical agents
Laboratory approach to the patient with suspected hemolytic anemia

- Laboratory
  - CBC
  - Reticulocyte Count
  - Blood smear
  - LDH
  - Bilirubin-direct and indirect
  - Haptoglobin
  - Urinalysis
Laboratory evaluation of hemolytic anemia

- Direct Coombs
  - Positive
  - Negative
Coombs +

- Warm autoimmune hemolytic anemia
- Cold agglutinin disease
- Drug induced
- Paroxysmal Cold Hemoglobinuria
Coombs -

- Hemoglobinopathies
- Enzymopathies
- Membrane Defects
- Microangiopathic
- Drugs
- Toxins/Wilson’s disease
- PNH
- Etc....
Autoimmune Hemolytic Anemia (AIHA)-Classification

**Warm-reacting antibodies**

- Optimally bind red blood cells at 37°C
- Idiopathic
- Secondary
  - Autoimmune disorders
  - Immunodeficiencies
  - Lymphoproliferative disorders
  - Nonlymphoid malignancies
  - Viral infections
- Mixed warm and cold antibodies
- Drug Induced

**Cold-reacting antibodies**

- Optimally bind red blood cells <37°C
- Cold agglutinins
  - Idiopathic
  - Secondary (associated clinical conditions)
    - Infections
    - Lymphoproliferative disorders
    - Nonlymphoid malignancies
  - Paroxysmal cold hemoglobinuria (Donath-Landsteiner antibodies)
    - Syphilis
    - Viral infections
Audience Response Question: Coombs test

Which of the following statements about the Coombs test and the blood bank evaluation of autoimmune hemolytic anemia is most accurate?

A.) A positive Coombs test implies at least low grade hemolysis

B.) Cold agglutinins are typically IgG positive on the Coombs test

C.) The thermal amplitude of a cold agglutinin is the best predictor of clinical significance

D.) Autoadsorption of autoantibodies are useful to define the specificity of the antibody

E.) The eluate of concentrated IgG from patients with warm autoimmune hemolytic anemia is used to rule out alloantibodies
Direct Coombs test

• Detects antibody coating the RBC surface

• Positive test isn’t necessarily diagnostic of hemolysis
  – As many as 0.1% of healthy blood donors are positive
  – 1-2% of hospitalized patients are positive

• Degree of hemolysis doesn’t always correlate with degree of positivity
Direct Coombs Test

30-40% IgG only
10% C3 only
40-50% mixed
<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warm AIHA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(67%)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>(20%)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>(13%)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cold agglutinin disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paroxysmal Cold</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobinuria</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Elution: Detection of specificity of the autoantibody

- Elute off IgG from patient RBCs
  - Incubate with reagent RBCs to test for activity and specificity of the antibody
  - Antibody most commonly reacts to a full RBC panel with similar agglutination strengths
  - Less commonly may show a relative specificity within the Rh system such as the e antigen (WAHIA) or I (cold agglutinin)
Difficulty performing a Type and Screen

| Cell# | Donor Number | Rh-hr | Donor | D | E | c | e | i* | Cw | V | K | Kp | Kp | Js | Js | Fy | Fy | Jk | Jk | Xg | Le | Le | S | s | M | N | P1 | Lu | Lu |
|-------|--------------|-------|-------|---|---|---|---|----|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1     | 110454       | R1R1  |       | + | + | 0 | 0 | 0 | + | 0 | 0 | + | 0 | + | + | + | 0 | 0 | 0 | + | 0 | + | 0 |
| 2     | 108830       | R2R2  |       | + | 0 | + | 0 | 0 | 0 | 0 | 0 | + | 0 | + | + | + | 0 | 0 | + | + | + | + | + | + | S | 0 | + |
| 3     | 113073       | rr    |       | 0 | 0 | 0 | + | + | + | 0 | 0 | + | 0 | + | + | 0 | + | 0 | + | + | 0 | + | 0 | + | + |

Patient Cells

<table>
<thead>
<tr>
<th>Test Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell#</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
</tbody>
</table>
Autoadsorption: Detection of Alloantibodies

• Goal is remove the autoantibody from the serum to allow the detection of alloantibodies
  – 32% of patients with AIHA have alloantibodies
  – 1 ml of packed autologous RBCs treated to remove the Ab and then incubate with patient’s serum at 37°C

Antibody Screen Results for Patients With Autoimmune Hemolytic Anemia Without (Example A) or With (Example B) an RBC Alloantibody Before and After Autoadsorption*

<table>
<thead>
<tr>
<th>Screening Cell</th>
<th>Example A</th>
<th>Example B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadsorbed Serum (Autoantibody Only)</td>
<td>Adsorbed Serum</td>
</tr>
<tr>
<td>I</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>2+</td>
<td>0</td>
</tr>
</tbody>
</table>

*Agglutination is read from 0, which is a negative result, to 4+, which is the strongest possible agglutination.
Transfusion in AIHA

• Proceed with caution-consider risk/benefit ratio
  – Transfused blood often has a short half-life
  – Use phenotype matched blood if available
    • Rh groups, Kell, Duffy, Kidd antigens
    • If antibody shows specificity for a given antigen use antigen negative blood
  – If cold agglutininin disease transfuse through a blood warmer

Reardon AM J Clin Pathol 2006;125(Suppl 1):S71-77
Case 1

A 63 year-old previously healthy man is admitted with angina and dyspnea on exertion. He has had a lack of energy for 2 months and has also complained of some intermittent yellowing of his eyes.

No prior blood counts are available. He is taking no medications and does not recall any recent illness. He denies alcohol consumption, tobacco, or illicit drug use.

Laboratory data and blood smear are as follows:

- Hct: 26%
- MCV: 103 fL
- WBC count: 5900/ul
- Retic count: 7.1 %
- LDH: 949 IU/L
- Coombs: IgG++, C3+
Warm Autoimmune Hemolytic Anemia (W-AIHA)

- IgG panagglutinating antibody directed against “public” epitope often on the Rh system
  - Optimally bind red cells at 37°C
  - Primarily removed by Fc receptor macrophages in the reticuloendothelial system
    - Partial phagocytosis leads to spherocytes removed by the spleen
  - Less commonly or weakly fixes complement

spherocyte

Positive Coombs Test
Diagnosis: Warm Autoimmune hemolytic Anemia

• Investigation
  – Rule out lymphoproliferative disorder
    • Consider bone marrow aspirate and biopsy
  
  – Rule out autoimmune disorder
    • ANA, etc…
  
  – Rule out immunodeficiency
    • Quantitative immunoglobulins
  
  – Rule out drugs
Warm autoimmune hemolytic anemia-Treatment

- Treat underlying disease if identified

- Steroids first line (1mg/kg) for one to three weeks
  - Interfere with ability of macrophages to clear IgG coated RBC’s
  - Decreases antibody production
  - 60-85% initial response (20% CR), but frequent relapses
  - Initial quick taper, than slowly when down to 20mg (for 2-3 months)
  - Pulses of high dose glucocorticoids may be useful in some who fail

Warm autoimmune hemolytic anemia -

Treatment

• Splenectomy
  – Consider in two-three weeks if no response to steroids
  – 2/3 respond, but relapses occur
  – Ensure immunizations and education about infectious risk
    – Pneumococcus, meningococcus, and hemophilis influenza type B
    – ??? Prophylactic antibiotics
  – Higher incidence of post-splenectomy venous thrombembolism and pulmonary hypertension
    • High incidence of antiphospholipid antibodies

Crary. Blood. 2009 Oct 1;114(14):2861-8
Rituximab

- Chimeric monoclonal antibody targeting CD-20 on mature B Cells

Median time to response 6 weeks (2-16)

Treatment-Other

10% refractory to both steroids and splenectomy

• Other immunosuppressants
  – Immuran
  – Cyclophosphamide
  – Cyclosporine
  – Danazol
  – IVIG
  – Not as effective as in ITP

• Paucity of randomized trials to suggest which agent is superior

• Responses may be delayed several months

### Case 2

A 63 year-old previously healthy male complains of fatigue and lack of energy for 2 months. He denies a history of fever, chills, night sweats or weight loss, but reports painful blue digits in the cold.

A routine CBC drawn 1 year ago was normal. He is taking no new medications and does not recall any recent illness. He denies alcohol consumption, tobacco, or illicit drug use.

Laboratory data and blood smear are as follows:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct</td>
<td>26%</td>
</tr>
<tr>
<td>MCV</td>
<td>133 fL</td>
</tr>
<tr>
<td>WBC count</td>
<td>5900/ul</td>
</tr>
<tr>
<td>Retic count</td>
<td>7.1 %</td>
</tr>
<tr>
<td>LDH</td>
<td>949 IU/L</td>
</tr>
<tr>
<td>Coombs</td>
<td>IgG-, C3++</td>
</tr>
</tbody>
</table>
Cold agglutinin Disease

- IgM antibody optimally binds to RBCs at lower temperatures
  - Fixes complement
    - Direct lysis of red blood cells
    - Removal of C3b-coated RBC’s by the liver

- Titers <1:64 usually not clinically significant
  - Titer should increase at lower temperatures

- Most commonly with I (or i in infections) specificity

Berntsten. Hematology. 2007; 12(5): 361-70
Blood Bank evaluation-Thermal Amplitude Test

• Keep blood at 37°C from point of bedside collection to testing
  – initial screening is often at 20°C or room temperature

<p>| Example of Thermal Amplitude Results for a Patient With Cold Agglutinin Disease* |
|---------------------------------|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Serum Dilution</th>
<th>4°C</th>
<th>22°C</th>
<th>30°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>4+</td>
<td>3+</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>2</td>
<td>4+</td>
<td>3+</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>4</td>
<td>3+</td>
<td>2+</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>8</td>
<td>3+</td>
<td>2+</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>16</td>
<td>2+</td>
<td>1+</td>
<td>1+</td>
<td>0</td>
</tr>
<tr>
<td>32</td>
<td>2+</td>
<td>1+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>...</td>
<td>2+/I+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1,024</td>
<td>1+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2,048</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Titer anti-I</td>
<td>1,024</td>
<td>32</td>
<td>16</td>
<td>8</td>
</tr>
</tbody>
</table>

*Agglutination is read from 0, which is a negative result, to 4+, which is the strongest possible agglutination.
Cold Agglutinin Disease: Diagnostic Criteria

• 1.) Clinical evidence of an acquired hemolytic anemia
• 2.) Positive Coombs test using anti-C3
• 3.) Negative Coombs test using anti-IgG
• 4.) Presence of cold agglutinin with reactivity up to 30°C
• 5.) Cold agglutinin titer at 4°C ≥ 256
Cooling of blood during passage through acral parts of the body allows cold agglutinins to bind to the RBC leading to agglutination, complement binding, and hemolysis.

- Spurious marked elevation of MCV, MCHC may occur
- Agglutination will abate with warming

Berntsten. Hematology. 2007; 12(5): 361-70
Investigation of Cold Agglutinin Disease

• Consider infectious etiologies such as Mycoplasma or Epstein-Barr virus

• Consider lymphoma or other lymphoproliferative disorders
  – Serum protein electrophoresis (SPEP)
  – Bone marrow biopsy if no obvious infection
  – Consider radiologic imaging

Berntsten. Hematology. 2007; 12(5): 361-70
Cold Agglutininin Disease

- Monoclonal band can be detected in the majority of patients on serum protein electrophoresis/immunofixation
  - Majority IGM κ
    - Kappa 94% in one study
    - Most encoded by IGHV4-34 gene

- Clonal B lymphocytes often found by flow even if bone marrow morphology is negative and no other evidence of lymphoma
Clinical

• Moderate chronic hemolytic anemia
  – Hb usually 9-12 g/dL

• Most lack physical findings
  – Acrocyanosis
  – Raynaud’s phenomenon
  – Gangrene

Cold agglutinin Disease
Treatment

• Avoid cold environments (temperatures reach <30° in exposed skin vessels)
  – Wear socks, mittens, ear muffs in cold temperature
  – Transfuse through a blood warmer when necessary
  – Caution with bypass surgery or hypothermic surgeries

• Treat underlying disease
  – Lymphoma
  – If secondary to mycoplasma (anti-I), mono (anti-i) or other virus treatment is supportive and hemolysis transient

• Steroids and splenectomy are not generally effective
  – Unless secondary to steroid responsive disease
    • lymphoma

Cold agglutinin Disease
Treatment

• Cytotoxic therapy
  – Chlorambucil
    – Begin with low daily doses of 2-4 mg/day
    – Follow counts closely
  – Cyclophosphamide
  – Favorable responses in the minority of cases
  – Risk of suppressing bone marrow and reticuloctye response

Cold Agglutininin Disease: Rituximab

- Gaining popularity with recognition of most cases of CAD as a clonal B cell disorder

- Biggest series
  - 27 patients with 37 courses
  - OR response rate of 54%, mostly partial
  - Responders had a median increase in Hb of 4g/dL and decrease in IgM by 54%
  - Median response time was 1.5 months

Cold Agglutinin Disease: Plasma exchange

• IgM is primarily intravascular

• Used with life-threatening hemolysis or acrocyanosis
  – Rapid, but transient responses
  – Need to use a blood warmer
  – Consider prior to procedures requiring hypothermia

Which of the following statements is true concerning the treatment of autoimmune hemolytic anemia?

A.) Splenectomy is an effective second line treatment in cold agglutinin disease
B.) Rituximab has proven effective in trials of cold agglutinin disease, but not warm autoimmune hemolytic anemia
C.) Steroids usually lead to complete, but transient responses in warm autoimmune hemolytic anemia
D.) Cytotoxic therapy is used more frequently in cold agglutinin disease as compared to warm autoimmune hemolytic anemia
# Treatment comparison between IgG and IgM mediated hemolysis

<table>
<thead>
<tr>
<th></th>
<th>IgG (WAIHA)</th>
<th>IgM (cold aggl.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Severity</strong></td>
<td>May be severe</td>
<td>moderate</td>
</tr>
<tr>
<td><strong>Mechanism of hemolysis</strong></td>
<td>Fc mediated</td>
<td>Complement mediated</td>
</tr>
<tr>
<td><strong>Blood smear</strong></td>
<td>Spherocytes</td>
<td>Agglutination</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>Typically warm</td>
<td>cold</td>
</tr>
<tr>
<td><strong>Steroids</strong></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Rituximab</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Splenectomy</strong></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Cytotoxic</strong></td>
<td>Rarely</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Plasmapheresis</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Case 3

A 43 year-old male is referred with a 3 month history of transfusion dependant anemia. Prior history 1 year ago is significant for an unprovoked DVT. His only medication is warfarin. Evaluation has been negative for GI bleeding. Blood counts 3 years ago were normal

- WBC count: 3900/ul
- Hb: 9.3 g/dL
- Hct: 29%
- Plt: 127k/cc mm
- MCV: 107 fL
- Retic count: 5.1 %
- LDH: 849 IU/L
- Haptoglobin: undetectable
- Coombs: negative
- Peripheral smear: normal morphology
Case

- Given Coombs negative, non spherocytic hemolytic anemia, recent DVT, and pancytopenia, PNH is suspected and peripheral blood is sent for CD-55 and CD-59 on RBCs

**Diagnosis: PNH**

Acquired clonal hematopoietic stem cell disorder
The glycosyl phosphatidylinositol (GPI) anchor

Due to a mutation in the phosphatidylinositol glycan complementation class A gene (PIGA)

Needed to make glycosyl phosphatidylinositol (GPI), a molecule that anchors specific proteins to the cell membrane

Lack of this anchor leads to a variety of clinical sequelae

PIG A gene
Xp22.1


©2002 by BMJ Publishing Group Ltd and Association of Clinical Pathologists
GPI-anchored surface proteins on human hematopoietic cells

Hematology 2008;2008:491-506
Overview of the complement cascade

Brodsky, R. A. Blood 2009;113:6522-6527
Parker. Lancet. 2009 Feb 28;373(9665):759-67
PNH: Selective Survival Advantage

Figure showing the process of clonal selection and expansion, leading to the selective survival advantage of PIGA mutant hematopoietic stem cells. The diagram illustrates the immune attack and subsequent clonal expansion of these cells.
PNH: Clinical

• Median survival is 10-15 years

• No current cure other than bone marrow transplantation

• 10-15% have spontaneous remission
Audience Response Question: Clinical Manifestations

Which of the following is not a well described clinical sequela of PNH

- A.) Thrombosis of the hepatic vessels
- B.) Transfusion dependent anemia
- C.) Esophageal spasm
- D.) Pancytopenia
- E.) Pulmonary fibrosis
- F.) Renal dysfunction
- G.) Erectile dysfunction
Clinical Manifestations

- Impaired quality of life
  - Disabling fatigue
  - Poor physical functioning
  - Pain
  - Dyspnea
  - Renal impairment

- Anemia
  - Transfusions
  - Fatigue
  - Dyspnea
  - Angina

- Thrombosis
  - Venous
  - Liver, mesenteric, dermal, cerebral
  - Arterial
  - Myocardial infarction, cerebral vascular accident

- Smooth muscle dystonia
  - Abdominal pain
  - Dysphagia
  - Erectile dysfunction

Nature Biotechnology 25, 1256 - 1264 (2007) Published online: 7 November 2007
PNH: Consequences of chronic hemolysis

The pathophysiology of disease in patients with paroxysmal nocturnal hemoglobinuria (PNH)
PNH: Thrombosis

- Increased propensity for life threatening thrombosis
  - Cerebral, hepatic, portal, mesenteric, splanchnic, and renal veins
  - Approximately 40% of patients have a thrombotic event during their illness
  * Higher risk with larger PNH clone (>60%)
  * Venous greater than arterial
  * Main cause of death

PNH: Bone Marrow Failure

• Small to moderate PNH clones found in up to 70% of patients with aplastic anemia
  – Usually less than 30% PNH granulocytes
  – May evolve into clinical PNH
  – 20% of MDS patients have small clones

• Small populations of PNH cells can be found in virtually all healthy controls
  – Approx 1 in 50,000 granulocytes
  – Arise from a more differentiated colony forming cell
  – No self renewal capacity

PNH: Bone Marrow Failure

• Most patients with clinical manifestations of PNH who do not have overt signs of marrow aplasia have evidence of diminished hematopoiesis
  – Two-thirds exhibit granulocytopenia and/or thrombocytopenia at some time during the course of their disease

• Leukemia develops in up to 5% of patients
  – The average onset occurs at about five years (range is from a few months to 22 years)

Rosse. Up-To-Date. Clinical manifestations of paroxysmal nocturnal hemoglobinuria. January 2010 last update
### PNH: Classifications

**Table 1 -- Classification of PNH**

<table>
<thead>
<tr>
<th>Category</th>
<th>Rate of Intravascular Hemolysis [b]</th>
<th>Bone Marrow</th>
<th>Analysis of Glycosyl Phosphatidylinositol–Anchored Protein Expression by Flow Cytometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classic</td>
<td>Florid (macroscopic hemoglobinuria is frequent or persistent)</td>
<td>Cellular marrow with erythroid hyperplasia and normal or near-normal morphology [c]</td>
<td>Large population (&gt; 50%) of GPI-AP–deficient PMNs [e]</td>
</tr>
<tr>
<td>PNH in the setting of another bone marrow failure syndrome [d]</td>
<td>Mild to moderate (macroscopic hemoglobinuria is intermittent or absent)</td>
<td>Evidence of a concomitant bone marrow failure syndrome [d]</td>
<td>Although variable, the percentage of GPI-AP–deficient PMNs [e] usually is relatively small (&lt; 30%)</td>
</tr>
<tr>
<td>Subclinical</td>
<td>No clinical or biochemical evidence of intravascular hemolysis</td>
<td>Evidence of a concomitant bone marrow failure syndrome [d]</td>
<td>Small (&lt; 1%) population of GPI-AP–deficient PMNs detected by high-resolution flow cytometry</td>
</tr>
</tbody>
</table>

Who should be tested for PNH

- All patients with unexplained:
  - hemoglobinuria
  - Coombs negative nonspherocytic hemolytic anemia
  - visceral or cerebral vein thrombosis
  - cytopenias
  - unexplained iron deficiency
  - PNH symptoms—fatigue, esophageal spasms, erectile dysfunction

Audience Response Question: Diagnostic Testing

Which of the following is a true statement regarding diagnostic testing in PNH?

A.) Screening of only red blood cells can lead to falsely negative tests
B.) FLAER is the most sensitive reagent to detect PNH on red blood cells
C.) The percentage of PNH red blood cells more accurately reflects the size of the clone as compared to white blood cells
D.) CD 55 and CD 4 are the most common GPI anchored protein antigens evaluated on RBCs of PNH patients
## Laboratory Testing

### Table 1 Laboratory tests for the diagnosis of PNH

<table>
<thead>
<tr>
<th>Laboratory tests</th>
<th>Principle</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ham test</td>
<td>The lysis of PNH red blood cells exposed to activated complement</td>
<td>Cheap and simple to perform</td>
<td>Labor intensive, low sensitivity and specificity, not quantitative</td>
</tr>
<tr>
<td>Sucrose lysis test</td>
<td></td>
<td>Cheap and simple to perform</td>
<td>Low sensitivity and not quantitative</td>
</tr>
<tr>
<td>Sephacryl gel card test</td>
<td>Haemagglutinin test using the gel microtyping system</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow cytometric analysis CD 59 and CD55 on RBC</td>
<td>Study expression of GPI-AP using monoclonal antibodies by flowcytometry</td>
<td>Rapid, sensitive, and quantitative</td>
<td>Lower estimate of clone size because of shorter life span of RBC</td>
</tr>
<tr>
<td>CD 59, CD55, CD24, CD66b, and CD16 for granulocytes</td>
<td></td>
<td>Better estimate of clone size</td>
<td>Require fresh blood sample</td>
</tr>
<tr>
<td>FLAER for granulocytes</td>
<td>Lack of FLAER binding to PNH granulocytes</td>
<td>Highly sensitive as single reagent for diagnosis</td>
<td>Difficult in patients with AA with neutropenia</td>
</tr>
</tbody>
</table>

PNH, paroxysmal nocturnal haemoglobinuria; RBC, red blood cells; GPI-AP, GPI-anchored proteins; AA, aplastic anemia; FLAER.

---

Flow Cytometry on RBCs: Three different phenotypes

Figure 1 Expression of the GPI-linked antigens on RBC in patient with paroxysmal nocturnal haemoglobinuria (PNH). (A) Gating of RBC using forward scatter (FSC) and sideways scatter (SSC) amplification in log mode. (B) Histogram showing expression of CD59 on RBC with clear separation of Type I, II and III cells. (C) Histogram showing expression of CD55 on same patient with poor separation between Type I, II, & III cells compared to CD59.
Figure 2 PNH clone size and the proportion of Type I, II, and III cells differs on neutrophils and RBC done simultaneously on the same patient. (A) Shows analysis of granulocyte with FLAER showing predominantly Type III cells. (B) Shows analysis on RBC with CD59 on the same sample showing markedly reduced Type III cells and higher proportion of Type II cells.
**Figure 3** Expression of CD55, CD59 and FLAER on neutrophils in a patient with PNH. (A) Shows gating of neutrophils using SSC/CD45 gating strategy. (B) Histogram showing expression of CD55 on neutrophils with Type I, II, and III cells. (C) Histogram shows expression of CD59 on same patient showing poor separation between Type I, II, & III cells compared to CD55. (D) Histogram shows FLAER expression with clear separation of Type I, II, & III cells.
### Table 2: Comparison between FLAER and Immunophenotyping for the Diagnosis of PNH

<table>
<thead>
<tr>
<th>FLAER</th>
<th>Immunophenotyping using monoclonal antibodies against GPI-AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive as a single agent and hence more economical as screening test.</td>
<td>At least two antibodies required</td>
</tr>
<tr>
<td>Detection of PNH clone only on leukocytes</td>
<td>Detection of PNH clone on all peripheral blood cells</td>
</tr>
<tr>
<td>Better separation Type I, II, and III cells on granulocytes</td>
<td>Separation of Type I, II, and III cells on granulocytes is not always clear</td>
</tr>
<tr>
<td>Better estimation of clone size on granulocytes and monocytes and hence useful for estimation of small clone on granulocyte in AA and MDS using multiparametric assay</td>
<td>Essential for estimation of clone size on RBCs and monitoring of RBC clone size in patients on Eculizumab therapy</td>
</tr>
<tr>
<td>More robust assay for detection of clone on granulocytes, can be performed on samples stored up to 48 h</td>
<td>Analysis on granulocyte needs to be performed within 8 h of collection, but analysis on RBCs can be done in samples stored up to 21–30 d</td>
</tr>
</tbody>
</table>

PNH, paroxysmal nocturnal haemoglobinuria; GPI-AP, GPI-anchored proteins; AA, aplastic anemia; MDS, myelodysplastic syndrome.
Historical Management of PNH

Palliative options do not impact progression and carry risk for severe morbidity and mortality

• Steroids/androgen hormones
  – No controlled clinical trials
• Red cell supplements
  – Folic acid, iron, erythropoiesis-stimulating agents
  – ESAs may expand clones and elevate hemolysis
• Transfusions
  – Transient treatment of anemia
  – Risk of iron overload
• Anticoagulants
  – Prophylaxis is debated, especially if >50% clone
  – Warfarin is recommended for all with VTE
  – Maybe ineffective in certain patients


ESA=erythropoietin stimulating agents
Historical Management of PNH

Bone Marrow Transplant

- Allogeneic bone marrow transplant
  - 44% mortality at 2 yrs with HLA-matched sibling donor
  - Acute GVHD in 34%; chronic GVHD in 33%
  - GVHD-free survival in 14% of patients

Audience Response Testing: Ecluzimab

Which of the following is the most likely consequence in a PNH patient treated with Ecluzimab?

A.) Increase in hemoglobin to normal
B.) Neisseria meningitides infection
C.) Increase in the percentage of Type III (totally deficient of GPI linked proteins) erythrocytes
D.) Deep vein thrombosis
E.) Stabilization of LDH
Structure of eculizumab

Brodsky, R. A. Blood 2009;113:6522-6527
Hillmen, P. Hematology 2008;2008:116-123
Eculizumab PNH Clinical Studies

**Pilot Study – NEJM. 2004**

N = 11

**TRIUMPH – NEJM. 2006**

Pivotal Phase III, Double-Blind, Placebo-Controlled Trial, N = 87

**SHEPHERD – Blood. 2008**

Broader patient population, including those receiving minimal transfusions or with thrombocytopenia, N = 97

**Long-Term Extension Trial**

Hillmen Blood. 2007

Evaluated long-term safety, efficacy and effect on thrombosis; Placebo patients switched to SOLIRIS®

N = 187

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Modified Alexion slide deck
Kaplan-Meier Curves for the Time to the First Transfusion during Treatment

No. at Risk
Placebo group
Eculizumab group

Week
0 2 4 6 8 10 12 14 16 18 20 22 24 26

Patients Remaining Transfusion Independent (%)
0 10 20 30 40 50 60 70 80 90 100

P = 0.001

Stabilization of Hemoglobin Levels and the Number of Units of Packed Red Cells Transfused during Treatment

<table>
<thead>
<tr>
<th>Primary End Point</th>
<th>Before Treatment</th>
<th>During Treatment</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo Group</td>
<td>Eculizumab Group</td>
<td>Placebo Group</td>
</tr>
<tr>
<td>Patients with stabilized hemoglobin levels (%)</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Packed red cells transfused (units/patient)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>8.5</td>
<td>9.0</td>
<td>10</td>
</tr>
<tr>
<td>Interquartile range</td>
<td>7–12.3</td>
<td>6–12</td>
<td>6–16</td>
</tr>
<tr>
<td>Mean</td>
<td>9.7±0.7</td>
<td>9.6±0.6</td>
<td>11.0±0.8</td>
</tr>
<tr>
<td>Total</td>
<td>417</td>
<td>413</td>
<td>482</td>
</tr>
</tbody>
</table>

* Plus–minus values are means ±SE. NA denotes not applicable.
† Transfusion data obtained during 12 months before treatment were normalized to a value equivalent to the value for a 6-month period.
‡ The P value is for the comparison between groups during treatment, calculated with the use of a two-tailed Fisher’s exact test.
§ The P value is for the comparison between groups during treatment, calculated with the use of the Wilcoxon rank-sum test.

Mean (SE) units packed red blood cells transfused by pre-treatment transfusion strata during the TRIUMPH and SHEPHERD studies

Hillmen, P. Hematology 2008;2008:116-123
Levels of lactate dehydrogenase during treatment with Eculizumab

Hillmen, P. Hematology 2008;2008:116-123
Parker. Lancet. 2009 Feb 28;373(9665):759-67
PNH RBC proportions (mean {+/-} SE) during Eculizumab treatment

Analysis of thrombosis before and during Eculizumab therapy

Hillmen, P. Hematology 2008;2008:116-123
## Ecluzimab: Expectations of therapy

### Table 2 -- Clinical activity of eculizumab

<table>
<thead>
<tr>
<th>What It Does</th>
<th>What It Does Not Do</th>
<th>What It May Do</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocks complement-mediated intravascular hemolysis</td>
<td>Eliminate anemia and reticulocytosis[^e]</td>
<td>Reduce the risk of thromboembolism[^f]</td>
</tr>
<tr>
<td>Reduces transfusion requirement[^a]</td>
<td>Affect the underlying marrow dysfunction</td>
<td>—</td>
</tr>
<tr>
<td>Improves quality of life (particularly fatigue)</td>
<td>Affect the PIGA-mutant stem cell clones</td>
<td>—</td>
</tr>
<tr>
<td>Increases the proportion of circulating type III PNH erythrocytes[^d]</td>
<td>Increase the risk for a catastrophic hemolytic crisis if the drug is discontinued[^c]</td>
<td>—</td>
</tr>
<tr>
<td>Increases the risk of infection with <em>Neisseria meningitides</em>[^b]</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
Safety

• In three major trials no patients withdrew due to an adverse event
  – 2/195 patients developed meningococcal sepsis
    • Vaccinations required
    • Alert cards suggested
  – Nasopharyngitis
  – Headache
  – Upper respirator tract infections
Future therapy?

• Inhibitor of complement C9
  – Block MAC and control hemolysis
  – Leave intact downstream functions of complement including the ability to generate C5a
    • Reduce risk of *Neisseria* sp infections