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TITLE: Paroxysmal Nocturnal Hemoglobinuria

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Slide 1:

Hello, my name is Nahla Heikal. I am an Assistant Professor of Clinical Pathology. University of Utah School of Medicine. Welcome to this Pearl of Laboratory Medicine on Paroxysmal Nocturnal Hemoglobinuria

Slide 2:

Paroxysmal nocturnal hemoglobinuria or PNH is a rare benign clonal acquired hematopoietic stem-cell (HSC) disorder that results from somatic mutation of the X-linked phosphatidylinositol glycan class A gene known as the *PIGA* gene. Mutations can arise de novo or in the setting of acquired bone marrow (BM) failure syndromes. The product of the *PIGA* gene is required for the synthesis of anchor protein known as GPI-anchor that ties other proteins to the cell surface. Hematopoietic cells contain more than a dozen different GPI-anchored proteins including adhesion molecules, enzymes, and receptors. Two GPI-anchored proteins (CD55&CD59) normally function as complement regulatory proteins. CD59 also called membrane inhibitor of reactive lysis (MIRL) forms defensive shield for red blood cells to inhibit the assembly of the membrane attack complex. CD55 also called the decay accelerating factor (DAF) prevents the formation and augments instability of C3 convertase essential for complement activation. In PNH patients these two complement regulatory proteins are absent or partially expressed on red blood cells.

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According to the type of mutation, deficiency of GPI-anchored protein can be partial or complete. This deficiency is seen in white blood cells and red blood cells. As a result, PNH is characterized by continuous destruction of PNH red blood cells due to vulnerability to complement mediated lysis.

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This figure illustrates the defect in PNH red blood cells. In normal red blood cells, the small blue arrows attached to the red blood cells surface represent the GPI-anchor needed for the expression of CD59 complement regulatory protein. GPI-anchor is missing in PNH red blood cells and as a result CD59 is not expressed. The alternate complement pathway is under continuous state of activation. Normal red blood cells can resist the effect of complement activation by the expression of CD59. PNH red blood cells clone lacks CD59 will undergo hemolysis and release of free hemoglobin in plasma.

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The chronic complement-mediated hemolysis is the underlying cause of progressive morbidities and mortality in PNH. Clinical manifestations with decreasing prevalence include:

- Fatigue, impaired quality of life
- Anemia
- Dyspnea
- Chronic kidney disease
- Abdominal pain
- Pulmonary hypertension
- Erectile dysfunction
- Dysphagia
- Thrombosis

- Hemoglobinuria
- Bone marrow failure

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Although the disease is called paroxysmal, there is ongoing destructive progressive hemolysis even in the absence of symptoms. The disease is described as nocturnal, although hemolysis is subtle and constant 24 hours a day. Hemoglobinuria is part of the name but it is a less commonly seen complication, and approximately 75% of patients present without hemoglobinuria.

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Pathophysiology of PNH

As we mentioned, hemolysis is due to complement activation of vulnerable red blood cells. Chronic kidney disease is a major cause of death occurs in 67% of patients due to toxicity of free hemoglobin and iron with extensive hemoglobin deposition. Esophageal spasm, abdominal pain, pulmonary hypertension, fatigue and smooth muscle dystonia are all attributed to nitric oxide (NO) scavenging. Nitric oxide is a major regulator of vascular physiology to maintain normal tone and regulate smooth muscles. Free hemoglobin has enormous affinity to nitric oxide. Thrombosis is the leading cause of death and affects 40% of PNH patients. It has been described as the most vicious acquired thrombotic state known with 5-10 fold increase in mortality and is characterized by affecting unusual sites like cerebral, portal, and mesenteric veins. The pathophysiology for thrombosis in PNH is multifactorial. It is due to platelet activation through nitric oxide depletion and complement mediated activation through loss of CD59. It is also due to disrupted fibrinolysis and tissue factor inhibitor pathway because of the lack of GPI-anchored receptors and cofactors. Many patients with PNH concomitantly present with cytopenia or bone marrow failure. The mutant hematopoietic stem-cell exhibit a survival advantage over normal cells and tend to expand leading to hemolysis. The mechanism is unknown but one hypothesis that this is due to immune mechanism for selection that occurs in unfavorable microenvironment.

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There are three clinical categories for PNH. First is the classical PNH which includes patients with hemolytic and thrombotic events. This class is characterized by marked hemolysis, hemoglobinuria, elevated lactate dehydrogenase (LDH) as a biochemical marker for hemolysis, normal bone marrow with erythroid hyperplasia, and PNH clone >50%. The second category is PNH in the setting of bone marrow failure syndromes like aplastic anemia (AA) and myelodysplastic syndrome (MDS). This class is characterized by mild hemolysis, minimal abnormality in biochemical markers of hemolysis, bone marrow examination shows the concomitant bone marrow failure, and PNH clone is usually <10%. The third class is the subclinical PNH. Patients in this clinical category are characterized by having no clinical or biochemical evidence of intravascular hemolysis, bone marrow examination shows the concomitant bone marrow failure, and very small PNH clone <1%.

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Early diagnosis is essential for improved patient management and prognosis and for that the International Clinical Cytometry Society (ICCS) Guidelines and international PNH Interest Group (IPIG) recommend evaluation of high risk patients which include patients with:

- Coombs negative hemolytic anemia (non-autoimmune hemolytic anemia)
- Hemoglobinuria
- Aplastic anemia
- Refractory anemia-myelodysplastic syndrome
- Unexplained venous or arterial thrombosis
- Unexplained cytopenia

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Let’s talk briefly about PNH clone in patients with bone marrow failure syndromes. Aplastic anemia is a disease of the bone marrow where it stops making enough red blood cells, white blood cells, and platelets but what is produced is functioning normally.

Studies have shown that PNH clone is present in 40-50% of patients with severe aplastic anemia where bone marrow cellularity is less than 30%. The PNH clone size in patients with aplastic anemia may increase rapidly and unpredictably. This is why these patients need to be screened and monitored regularly. Studies have also shown that the presence of PNH clone in severe aplastic anemia is associated with low morbidity and mortality, and reported to be predictive of response to immunosuppressive therapy. Clone size often decrease after immunosuppressive therapy.

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Another bone marrow failure syndrome that may be associated with a PNH clone is MDS. Myelodysplastic syndrome is a group of diverse bone marrow disorders in which the bone marrow does not produce enough healthy blood cells. Studies have reported more than 1 out of 18 patients with MDS have PNH clone and most studies showed that PNH clones were only present in patients with refractory anemia which is a clinical category of MDS. Refractory anemia patients with detectable PNH clone have more indolent clinical course. While PNH clones in other categories of MDS have been reported in limited number of studies.

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Flow-cytometry performed on peripheral blood is the established method of choice for the diagnosis and monitoring of PNH. It is recommended that both red blood cells and white blood cells be tested because sometimes a white blood cells PNH clone may be present in the absence of a red blood cells clone. However, a significant red blood cell PNH clone is always associated with white blood cell clone. This is explained by the fact that red blood cell clone size may be affected by hemolysis or transfusion.

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In red blood cells analysis we need to Identify and quantify cells that are lacking the expression of CD59 or CD55 (Type III cells). It’s also important to identify and quantify deficient red blood cells that partially express CD59 or CD55 (Type II cells) if present.

Type II PNH red blood cells don't undergo hemolysis as they are less sensitive to complement activation.
Flow testing for red blood cells starts by using glycophorin -A (CD235a) to gate on Red blood cells followed by CD59 as the GPI-anchor protein which is superior over CD55.

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In this slide, the first blot on the left shows gating on red blood cells using glycophorin -A.
A illustrates a histogram and a dot plot for normal sample with100% normal (type I) red blood cells. Looking at the upper left quadrant of the dot blot there is 0% PNH cells that are identified as glycophorin positive and lacking the expression of CD59. **B** represents a histogram and a dot plot for a positive PNH sample with 41% type III clone. **C** represents a histogram and a dot plot for a positive PNH sample with 19% type III clone and 13% type II clone.

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In order to identify small subclinical PNH red blood cells clone a high sensitivity red blood cells analysis should be used. This is performed by counting more red blood cells events (500,000 or one million) where sensitivity of 0.005% or less is achievable. The figure shows an example of a subclinical 0.037% type III PNH red blood cells clone.

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For white blood cell analysis both granulocytes (PMN) and monocytes are tested. Granulocyte population is most typically used to assess the PNH clone size and occasionally Type II granulocytes can be detected. If present, Type II is more readily detected in red blood cells assay. Monocytes are often analyzed to confirm the granulocyte PNH clone. Granulocytes and monocytes PNH clone size should match but monocytes clone is often higher for unknown reasons which may be attributed to the smaller number of monocytes in general. Sensitivity and precision are also lower due to

lower cell number. As with granulocytes, occasionally Type II PNH cells can be detected.

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White blood cells analysis uses lineage specific markers to gate on granulocytes and monocytes for higher sensitivity and cleaner assay. CD15 is used to identify and gate on granulocytes. CD64 or CD33 is used to gate on monocytes. CD64 is superior over CD33 in identifying monocytes because CD33 poorly differentiates basophils from monocytes with basophils appear like monocytes PNH clone. Also, CD33 expression sometimes is very low or negative. After identifying granulocytes and monocytes populations, two GPI-linked proteins are assessed on each cell population. The most commonly used and accurate markers evaluated are CD24 or CD157 and FLAER on granulocytes and CD14 or CD157 and FLAER on monocytes. The use of CD157 has the advantage of being expressed on both granulocytes and monocytes which can be used instead of using two markers (CD24 for granulocytes and CD14 for monocytes). FLAER or fluorescein-labeled pro-aerolysin is a flourochrome conjugated inactive bacterially derived channel forming protein that binds specifically to GPI anchors.

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A high sensitivity white blood cell analysis is useful for the diagnosis of subclinical PNH associated with bone marrow failure disorders and is not needed for the diagnosis of classic PNH. Sensitivity of 0.01% or even less is achievable given the acquisition of sufficient number of events (100, 000) for granulocytes and (20,000) for monocytes which is recommended by the practical guidelines for high sensitivity detection of PNH. In addition to the acquisition of enough events, the evaluation of multiple parameters and assessment of PNH cell frequency in normal samples are critical to limit false positive events.

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This slide shows the staining strategy for white blood cell analysis. **A**: display of the initial gate is set on CD45/SSC to include both granulocytes and monocytes for pattern recognition and debris exclusion. **B**: display of gating on monocytes using CD64. **C**: display of FLAER and CD157 (the two GPI-anchored proteins) with 0% PNH monocytes. **D**: display gating on granulocytes using CD15 and **E**: display of FLAER and CD157 with 0% PNH PMN.

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In this slide **A**: represents a positive clinical white blood cell PNH sample with PNH clone of 97.7% in monocytes and 96.9% in granulocytes shown in the lower left quadrant of the dot plots identified by lacking the expression of both FLAER and CD157. **B**: represents a subclinical sample with 0.34% monocytes PNH clone and 0.25% granulocyte PNH clone.

Slide 21:

This slide shows a positive white blood cell PNH sample using different antibodies that are commonly used. **A**: display of CD33 vs. CD15 for gating on monocytes and granulocytes respectively. **B**: display FLAER and CD24 as the two GPI-anchored proteins expressed on granulocytes with 8% PNH clone lacking both proteins. **C**: display of FLAER and CD14 as the two GPI-anchored proteins expressed on monocytes with 7.7% PNH clone lacking both proteins.

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Treatment: Folic acid supplementation and supportive care for patients with minimal symptoms, blood transfusion, and steroids can be helpful but prolonged use should be avoided, prophylactic anticoagulation has not been proven to decrease the risk of thrombosis, allogenic bone marrow transplantation may be the cure but with associated

high morbidity and mortality and difficulty in finding suitable donor. In 2007, the FDA approved eculizamab for treatment of PNH.

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Eculizamab is a humanized monoclonal antibody that effectively blocks complement activation at C5 that inhibits terminal complement activation. It stops hemolysis and all related effects with better quality of life, less thrombotic events, and higher survival rate. Patients with subclinical clones are not candidates for treatment with eculizamab since they have no intravascular hemolysis. The downside of eculizamab is that blocking the terminal portion of complement predisposes to *Neisseria;* therefore patients should be immunized two weeks before treatment. In addition, the drug is very expensive and must be given intravenously every 12-14 days.

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We will end with this slide showing a PNH red blood cells analysis in a patient with PNH treated with eculizumab. **A:** shows the red blood cells histogram results prior to therapy with 29% PNH clone and a dot plot slowing 97% white blood cell clone. The discrepancy is explained by hemolysis of PNH red blood cells. Following successful therapy with eculizamab the patient's red blood cells clone matched the white cell clone because type III PNH cells were protected from hemolysis.

Slide 25: References

Slode 26: Disclosures

Slide 27: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on "**Paroxysmal Nocturnal Hemoglobinuria"**