Update in Red Blood Cell Membrane Disorders

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Program Objectives

- List different types of RBC membrane defects
- To be able to suggest appropriate tests and to correlate results of laboratory testing with specific RBC membrane defects
- Describe the principles of different technologies used for the diagnosis of RBC membrane defects
- Discuss the utility of NGS in diagnosis of these disorders

Definition of Anemia

- From Greek meaning “without blood”
- Condition where capacity of blood to transport oxygen to tissues is reduced
  - Decreased hemoglobin, RBC count, and hematocrit
- Anemia is not a disease but a *manifestation* of disease
- Treatment depends on discovering underlying cause

What is Hemolytic Anemia?

- Characterized by premature destruction of red blood cells
- Anemia develops when the bone marrow cannot adequately compensate for the shortened life span of the red blood cells in the circulation
- Laboratory confirmation of hemolysis
  - Increased reticulocyte count
  - Signs of RBC destruction e.g. increased lactate dehydrogenase, low haptoglobin, increased unconjugated bilirubin

Classification of Hemolytic Anemia

- Intrinsic
  - Red blood cell membrane defects
  - Red blood cell enzyme defects
  - Hemoglobinopathies
- Extrinsic
  - Immune
    - Autoimmune
    - Alloimmune
  - Drugs and toxins
  - Physical damage to red blood cells like toxins, thermal injury and mechanical disruption

Practical Diagnosis of Hematologic Disorders (Kjeldsberg, Practical Diagnosis of Hematologic Disorders)

Classification of Hemolytic Anemia

- Hereditary
  - Membrane defects
  - Red cell enzyme def
  - Hemoglobin synthesis abnormality
    - Thalassemia
    - Sickle cell disease
- Acquired
  - Immune
    - Infections
    - Alloantibodies
    - Autoantibodies
  - Non-immune
    - Mechanical damage
    - Physiochemical damage

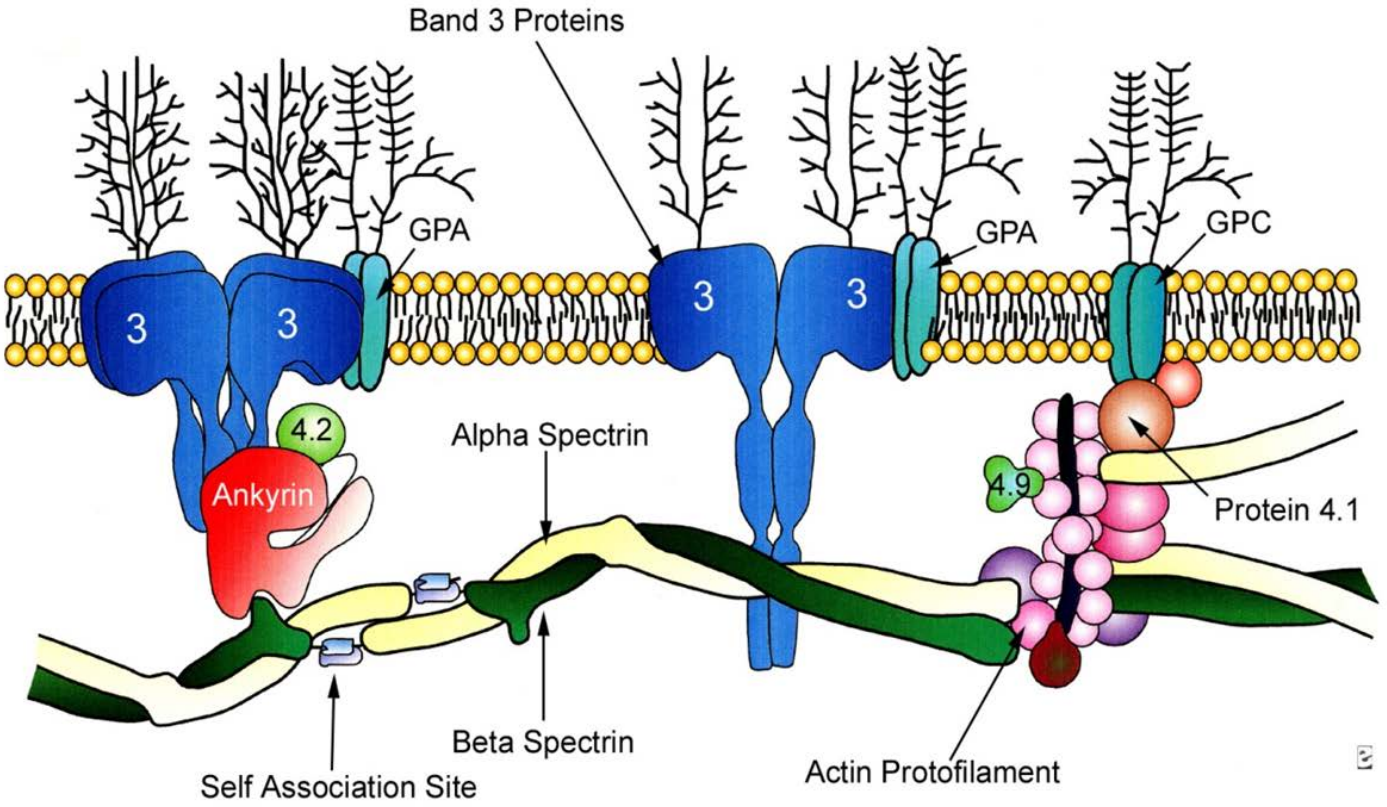
Let's Elaborate on the RBC Membrane Defect

- RBC Membrane Defect
  - Defects intrinsic to RBC membrane
    - Hereditary spherocytosis
    - Hereditary elliptocytosis/Hereditary Pyropoikilocytosis
  - RBC volume defects
    - Overhydrated and dehydrated stomatocytosis

Hereditary Spherocytosis (HS)

- Most common hemolytic anemia due to red cell membrane defect
- Alteration of one of the five genes which encode for proteins involved in the vertical association
- Occurs in all racial groups and is particularly common in individuals of northern European ancestry, affecting approximately one person in 3000

RBC membrane structure

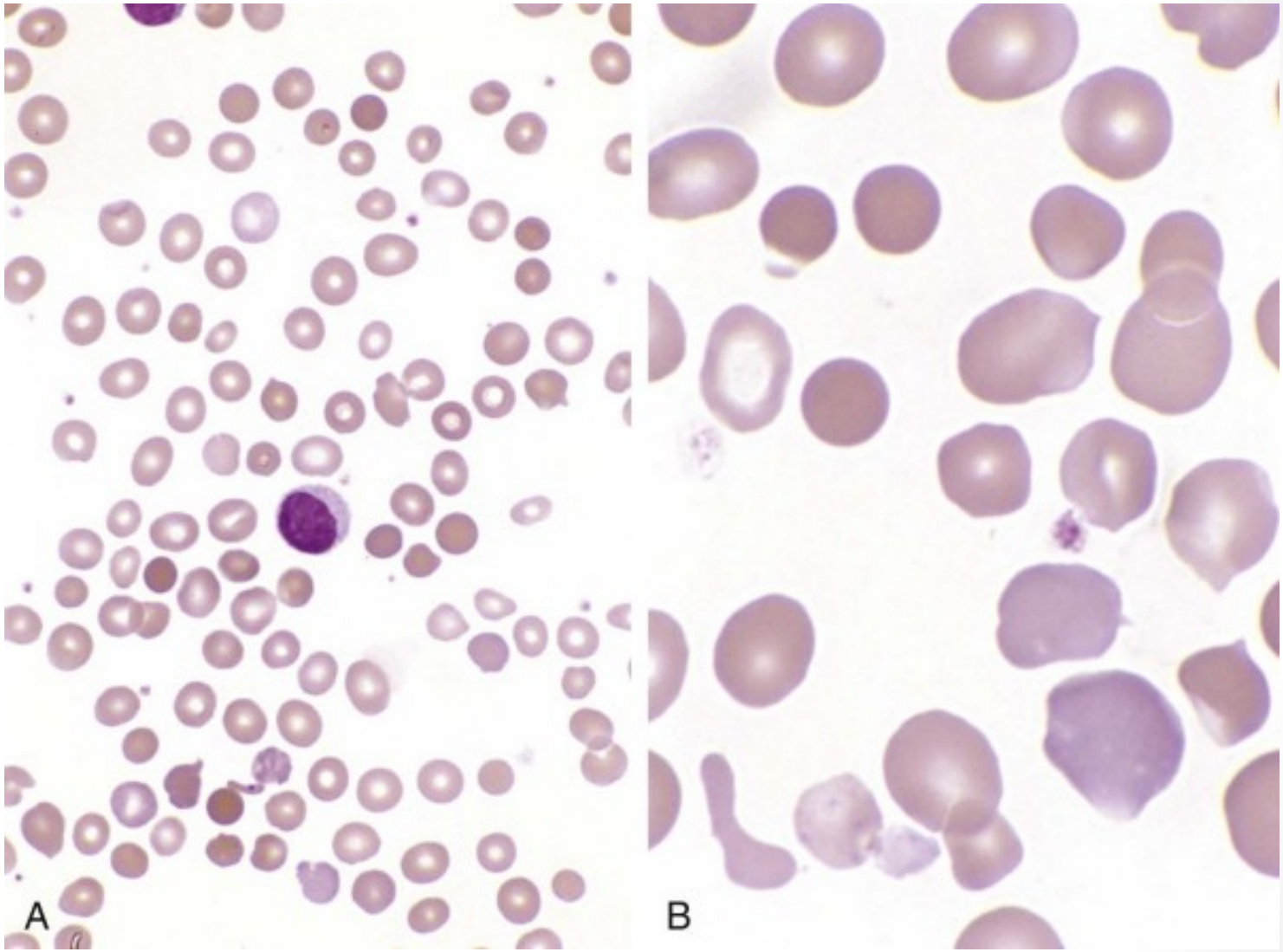


Vertically positioned transmembrane proteins

Horizontally positioned skeletal proteins (maintain shape, deformability)

http://www6.ufrgs.br/favet/imunovet/molecular_immunology/blood.html

Peripheral Smear of Hereditary Spherocytosis



Jaffe et al., Hematopathology. Elsevier, 2011

Hereditary Spherocytosis, Continued

- Dominant inheritance (75%)
- Recessive inheritance (25%) and de novo have also been described
- The clinical manifestations of HS vary widely
- Mild, moderate, moderately severe

Hereditary Spherocytosis, Continued

- Spectrin deficiency is often present in HS
- Ankyrin mutation is the most common cause of HS in Northern European populations accounting for approximately 50–60% of cases but it is found in only 5–10% of HS cases in Japan
- Even in those conditions where primary mutation is in non-spectrin protein as alteration in these proteins adversely affect the assembly of spectrin onto the membrane protein
- The clinical severity is correlated well with the spectrin deficiency

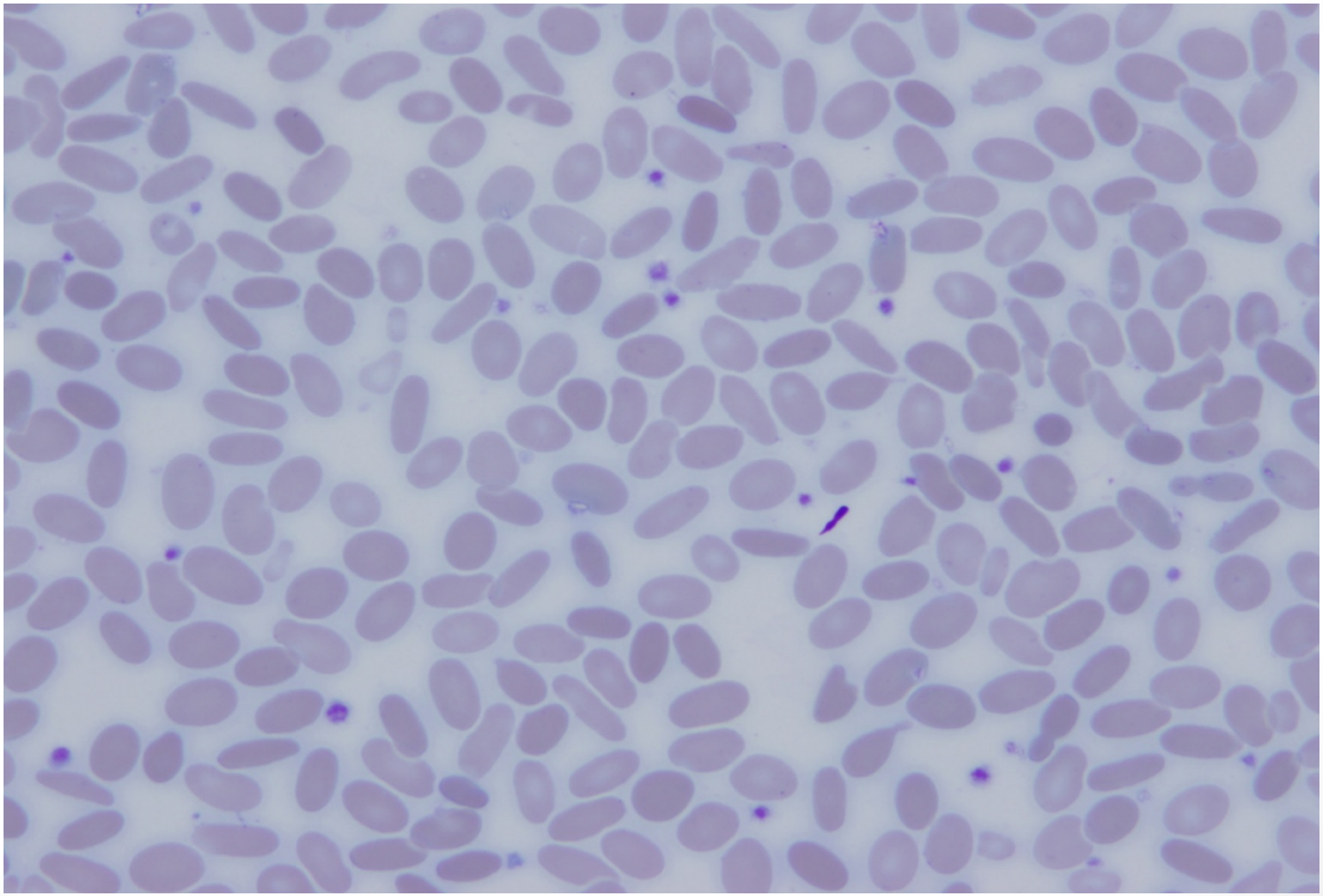
Hereditary Elliptocytosis/Hereditary Pyropoikilocytosis

- Not uncommon, clinically and genetically heterogeneous disorder
  - Elliptically-shaped red cells on peripheral blood smear to marked anisopoikilocytosis in HPP
- HE has a worldwide distribution but is more common in malaria endemic regions with prevalence approaching 2% in West Africa
- Inheritance of HE is autosomal dominant and AR in HPP

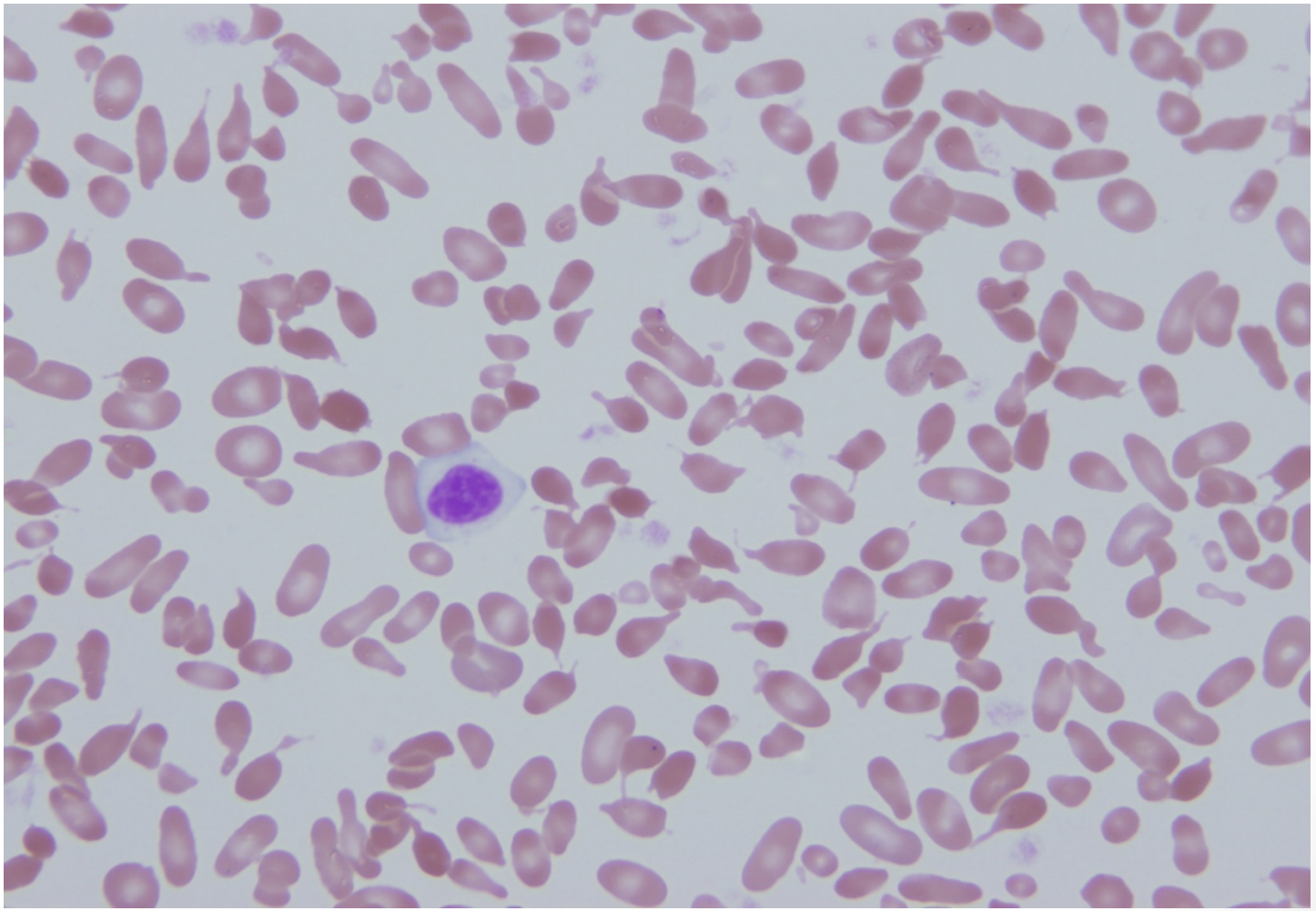
Hereditary Elliptocytosis/Hereditary Pyropoikilocytosis

- HE is a relatively common disorder (1:1000-1:3000)- mild clinical phenotype
- HPP- usually presents with moderate to marked hemolytic anemia and significant anisopoikilocytosis
- Neonates are commonly affected

Hereditary Elliptocytosis (HE)



Hereditary Pyropoikilocytosis (HPP)

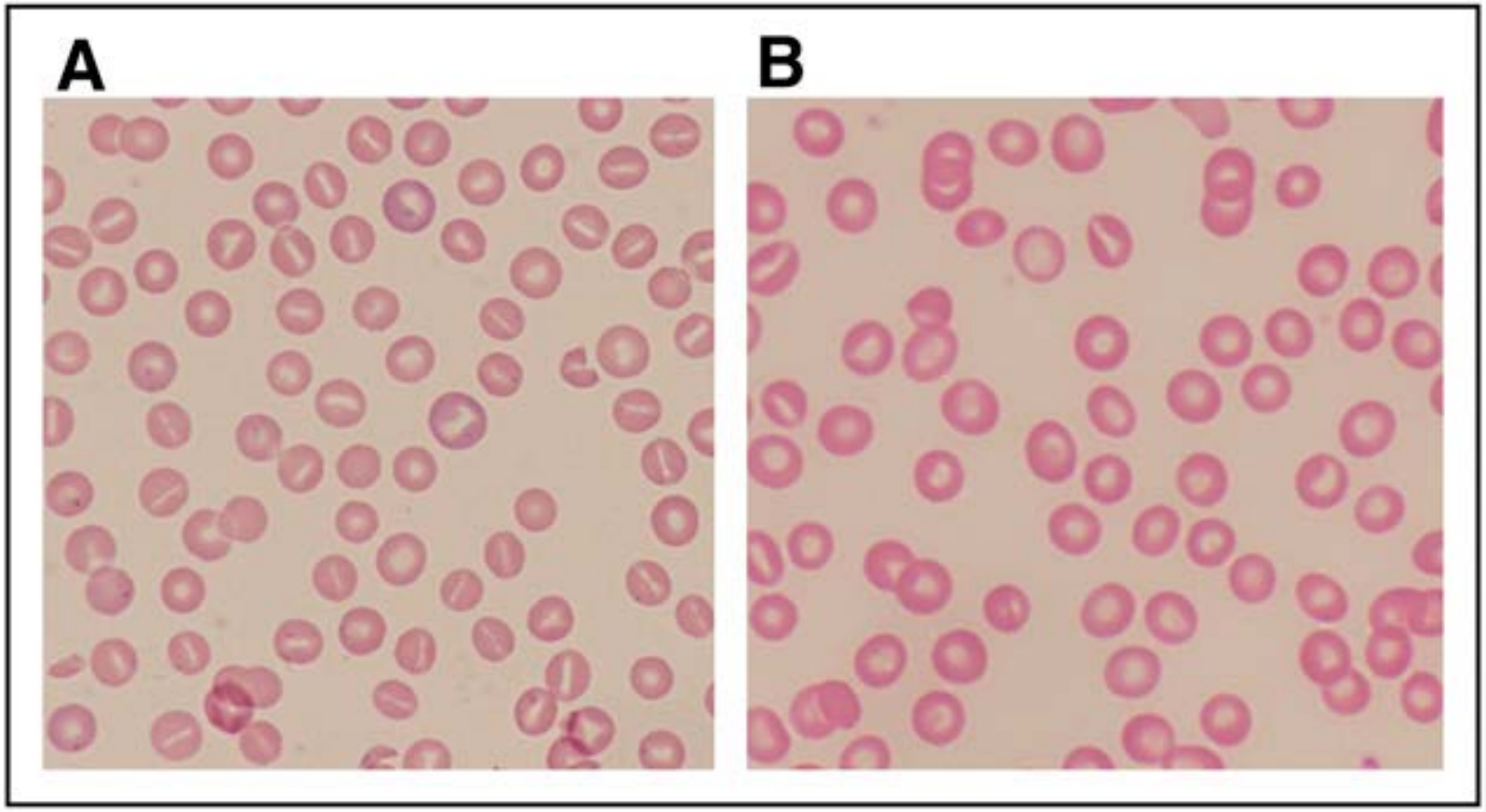


Hereditary Stomatocytosis

- Overhydrated and dehydrated stomatocytosis/xerocytosis
- **Dehydrated stomatocytosis/xerocytosis**
  - **High MCHC, and resistant osmotic fragility**
  - Hemoglobin and hematocrit are often normal
  - Iron overload
  - Splenectomy contraindicated due to an increased risk of thromboembolic complications
  - Most of them are due to mutations in *PIEZO1*
  - PIEZO1 proteins are mechanically activated cation channels

- **Overhydrated is very rare and due to mutation in Rh-associated glycoproteins.**

Hereditary Stomatocytosis



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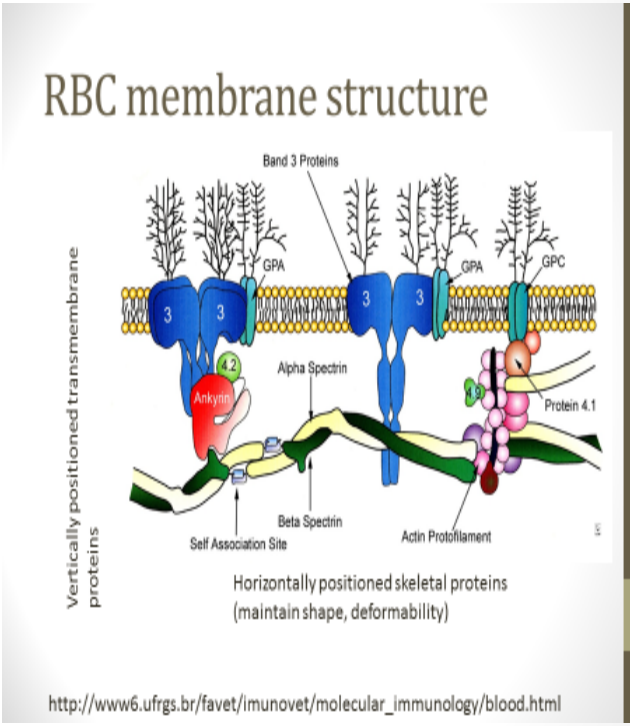
Genetic Etiology

Table 1. Molecular characteristics of RBC membrane and volume disorders associated with HAA.
AR, autosomal recessive; AD, autosomal dominant

RBC Disorders	Gene Symbol	Inheritance Pattern	Percentage of Mutations
Hereditary spherocytosis	*ANK1*	AD/AR (rarely)	40-65
	SLC4A1	AD	20-35
	SPTB	AD	15-30
	SPTA1	AR	5
	EP4B2	AR	5
Hereditary elliptocytosis/Hereditary pyropoikilocytosis	*SPTA1*	AD/AR	65
	SPTB	AD/AR	30
	EPB41	AD/AR	5
Dehydrated stomatocytosis	*PIEZO1*	AD	Majority
	KCNN4	AD	Few cases
Overhydrated stomatocytosis	*RHAG*	AD	Very few cases



Diagnosis of HS, HE and HPP

- Family history
- Peripheral blood smear- Spherocytes, elliptocytes and fragmented cells (in HPP), ovalocytes are seen
- Complete blood count (CBC), retic count and RBC indices

- **HS - MCHC ≥ 36.** normal to slightly low MCV

- **HE/HPP-**Profound microcytosis (MCV 30 to 50 fL), high MCHC, and/or abundant microspherocytes with RBC fragmentation are consistent with HPP or severe (hemolytic) HE

Diagnosis of HS:Confirmatory test

- Flow cytometric analysis of eosin-5'-maleimide-labeled intact RBC
  - Preferred test

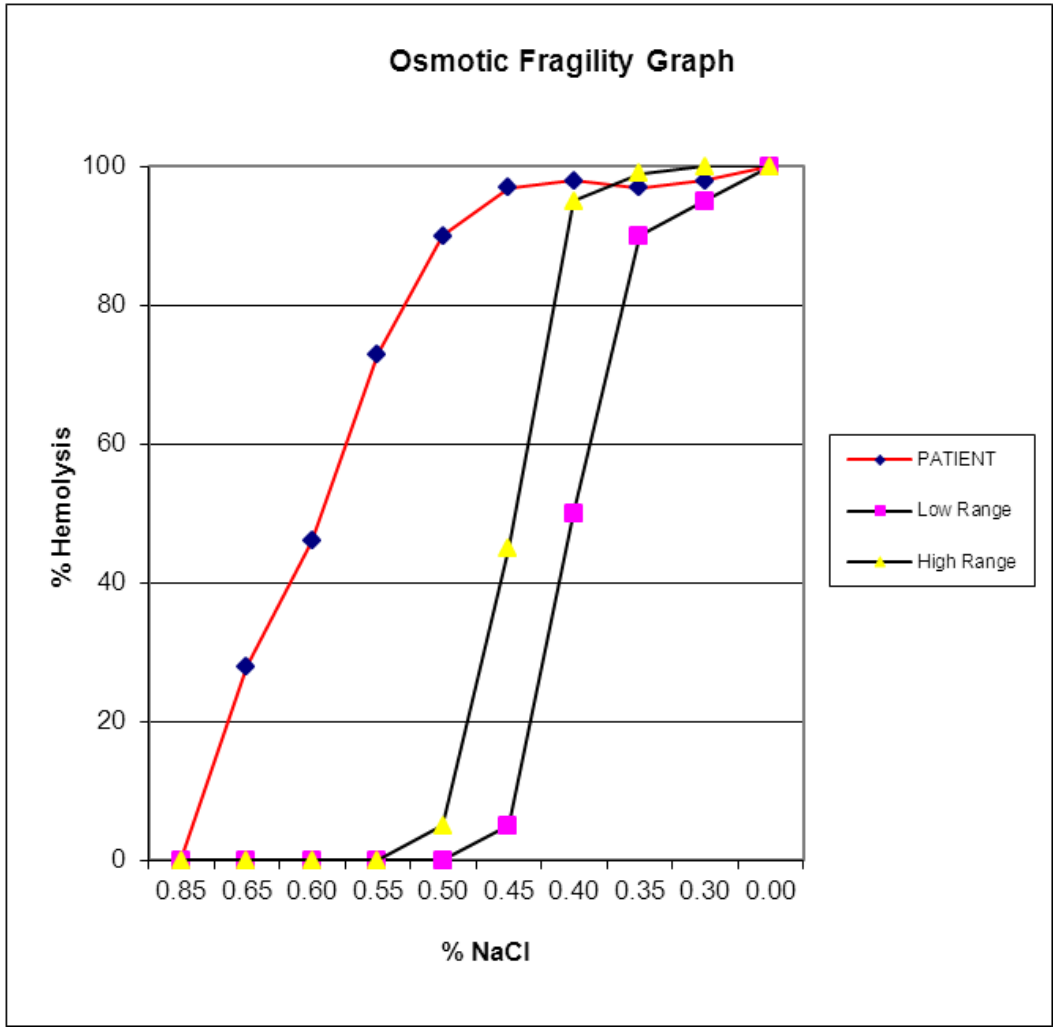
- Osmotic fragility
  - a laboratory test used in the diagnosis of HS, but not specific
  - The test measures the *in vitro* lysis of RBCs suspended in solutions of decreasing osmolarity
  - 60-80% sensitive

Diagnosis Continued: Flow

- EMA binds specifically with band 3 protein
- Reduction in fluorescence
- EMA binding is affected by all sorts of membrane protein abnormalities, not just band 3 deficiency

- Advantages
  - High sensitivity and specificity >95%
  - Rapid turnaround time
  - Small amount of blood

Osmotic Fragility Test



Other tests for HS

- **Osmotic gradient ektacytometry** – generates a profile of osmotically induced RBC shape change (deformability) across an osmotic gradient
- **Glycerol lysis** – GLT and the acidified GLT (AGLT) are similar to osmotic fragility that add glycerol (in the GLT) or glycerol plus a sodium phosphate (to lower the pH to 6.85, in the AGLT) to the hypotonic buffered salt solutions in which the patient's RBCs are incubated
- **Cryohemolysis** –RBCs are suspended in a hypertonic solution, briefly heated to 37°C, then cooled to 4°C for 10 minutes

Confirmatory tests for HE/HPP and stomatocytosis

- HE/HPP are morphological diagnosis
- EMA binding could be markedly decreased
- **Osmotic gradient ektacytometry might be helpful**
- Molecular testing

Why Do We Need Molecular Testing?
Utility of this testing during neonatal period

- HS of AR variant without family history
- Screening tests not that useful during neonatal period
- Spherocytes on the blood smear are not specific for HS and peripheral smear findings are not informative in all cases
- Some patients may require transfusion, making the biochemical testing unreliable and uninformative
- To diagnose complex interactions (HHA +Bilibrubin metabolism disorders)

Targeted NGS Panels

- Evaluated since 2012 with gene counts on these panels varying from 28-70
- We also evaluated 268 patients using our 28 gene NGS panel (ARUP#2012052)
  - The age of the patients ranged from newborn to 62 years
  - These patients presented with symptoms ranging from mild lifelong anemia to severe hemolytic anemia with extreme hyperbilirubinemia

Continued!

- We identified pathogenic and likely pathogenic variants in 64/268 (24%) patients that were clearly responsible for the disease phenotype (eg, moderate to severe HA)

Inheritance Pattern	Homo/Compound heterozygous	Heterozygous	Hemizygous
SPTB (AD)		14	
SPTA1(AR)	12		
ANK1 (AD)		9	
SLC4A1 (AD)		5	
PIEZO1 (AD)		6	
PKLR (AR)	10		
G6PD(X-Linked)			7
GP1 (AR)	1		
UGT1A1 (AR)	29		

Case Study #1

- Caucasian ethnicity and lacking regular well child visits, initially presented at 3.5 years of age with mild anemia associated with splenomegaly
- PS - marked erythrocyte poikilocytosis
- Hyperbilirubinemia in the newborn period
- No family history
- Genetic testing was ordered after dilated cardiomyopathy developed

Case Study; Continued

- Three variants in *SPTA1* gene were identified by NGS including a novel mutation (c.7134+2T>G, p.?)
  - Consistent with HPP
- Four months after splenectomy, the patient's hemoglobin improved to 15.2 g/dL and echocardiogram changes are resolving

Case example #2

- 2year old female with congenital hemolytic anemia
- At baseline, HB <6gm/Dl
- Retic anywhere from 10-20
- Transfusion dependent with significant hepatosplenomegaly and iron overload

Further testing's

- R/O RBC membrane defect: osmotic fragility normal. MCHC's are normal. Peripheral smear shows normal RBC morphology
- R/O RBC enzyme defect: a full RBC enzyme panel was normal
- R/O unstable hemoglobinopathy: No Heinz bodies. Hb electrophoresis did not demonstrate evidence of unstable Hb's.
- R/O thalassemia: Alpha thal mutation analysis negative. Hb electrophoresis consistent only with sickle cell trait. Beta globin gene sequencing identified only sickle cell trait, otherwise negative
- Extensive evaluation had also ruled out occult bleeding (GI as well as pulmonary)

We reported these results

- EMA-Borderline
- NGS Panel picked up mutations:
  - Two mutations in ANK1

Follow up…

- She had a splenectomy performed a few months ago and since then her baseline hemoglobin is 10-11
- She has been doing ok for last 6 months

Summary

- Relatively common disorder with varied etiology
- Can cause significant morbidity and mortality if not diagnosed
- Can also cause significant neonatal hyperbilirubinemia
- Diagnosis requires
  - Family history, CBC and peripheral smear
  - EMA and osmotic fragility combination
- NGS can be used for the diagnosis of unexplained causes of hemolytic anemia


Lake Powell, Utah