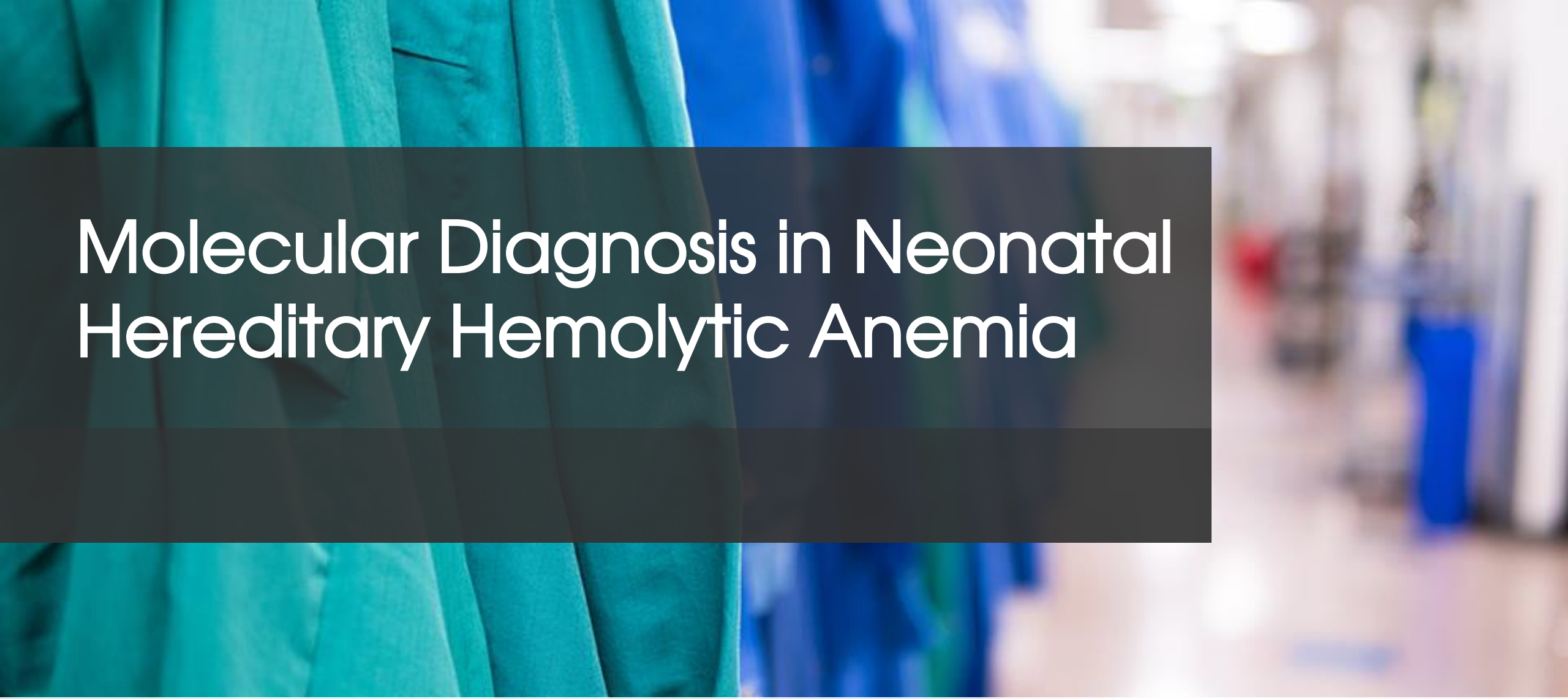

# Molecular Diagnosis in Neonatal Hereditary Hemolytic Anemia

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580
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# Learning Objectives

List the causes of neonatal hereditary hemolytic anemia (HHA)

Identify the limitations of routine diagnostic tests in these disorders

Discuss the association of HHA with neonatal hyperbilirubinemia/acute bilirubin encephalopathy

Recognize the advantages of molecular diagnostic tests in these disorders

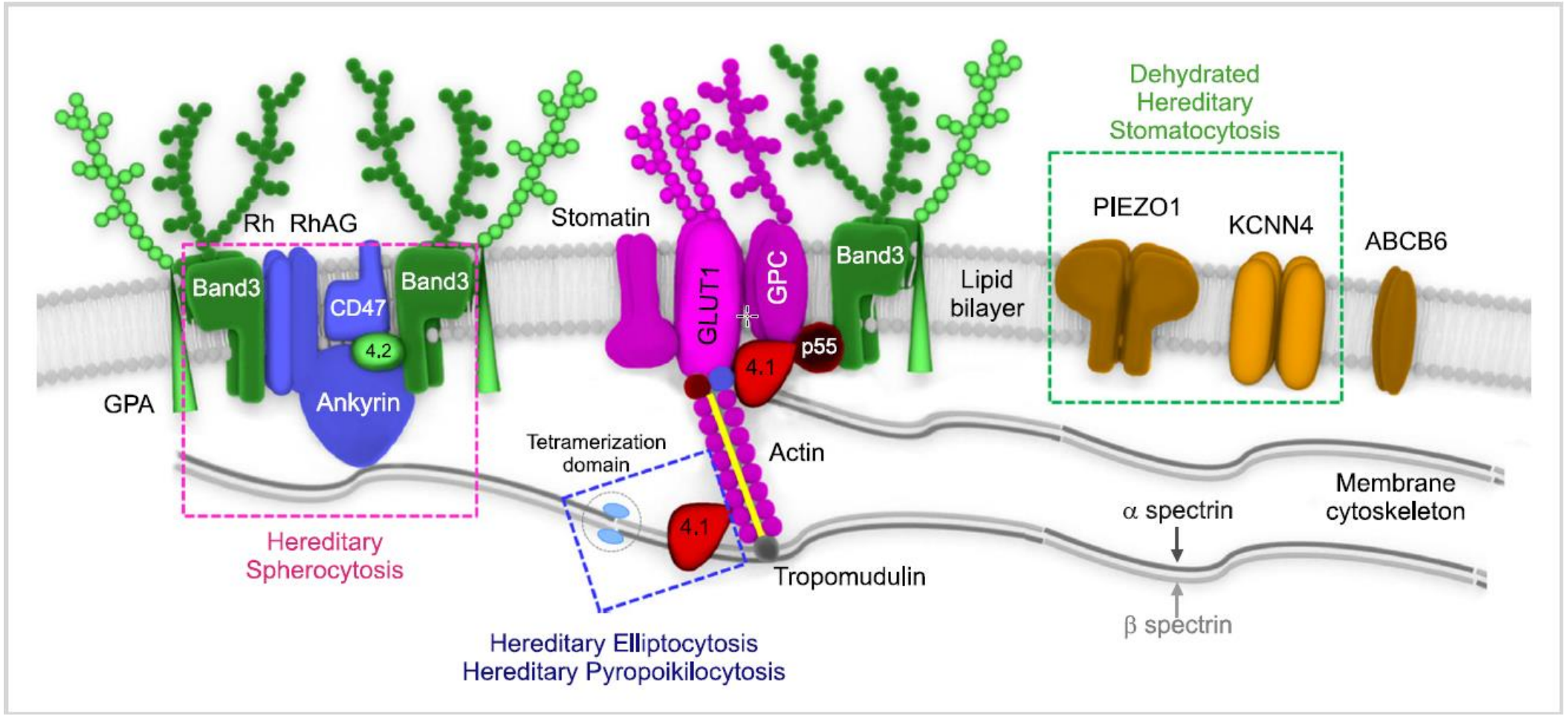
# What is Hemolytic Anemia?

- Characterized by premature destruction of red blood cells (RBC's) within the circulatory system
  - » RBC'S normally survive 60-120 days
  - » Bone marrow has the capacity to increase the production of RBCs 6-8 times than normal
- Anemia develops when the bone marrow cannot adequately compensate for the shortened life span of the red blood cells in the circulation

# Causes of Hereditary Hemolytic Anemia (HHA)

- RBC membrane defects
  - Hereditary spherocytosis
  - Hereditary elliptocytosis/Hereditary pyropoikilocytosis
  - Hereditary stomatocytosis
- Red cell enzyme deficiencies
  - G6PD deficiency
  - Pyruvate kinase deficiency
- Hemoglobin synthesis abnormality
  - Thalassemias
  - Hemoglobin S, C and E disorders

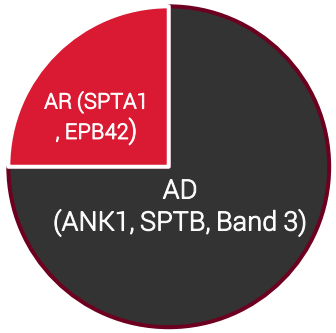
# Red Cell Membrane Cytoskeleton

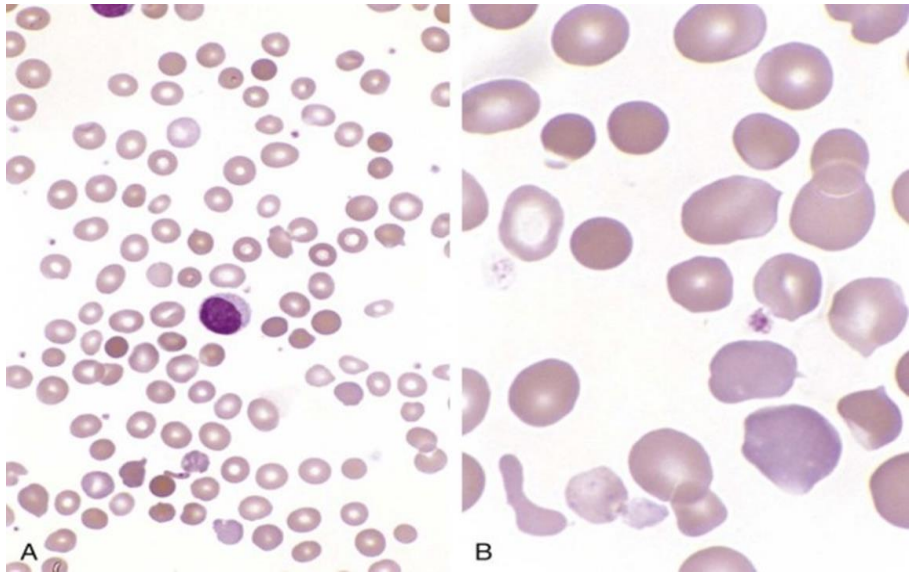



PMID: 28698843 Slide courtesy Dr Coumarane, ARUP

# Hereditary Spherocytosis (HS)

- Common inherited hemolytic anemia (1/1000 -1/3000)
- All ethnic groups; common in Northern Europeans
- AD (75%) and AR (25%)




- The disease is diagnosed in only one third of affected infants during the first year of life

# HS: Clinical Presentation

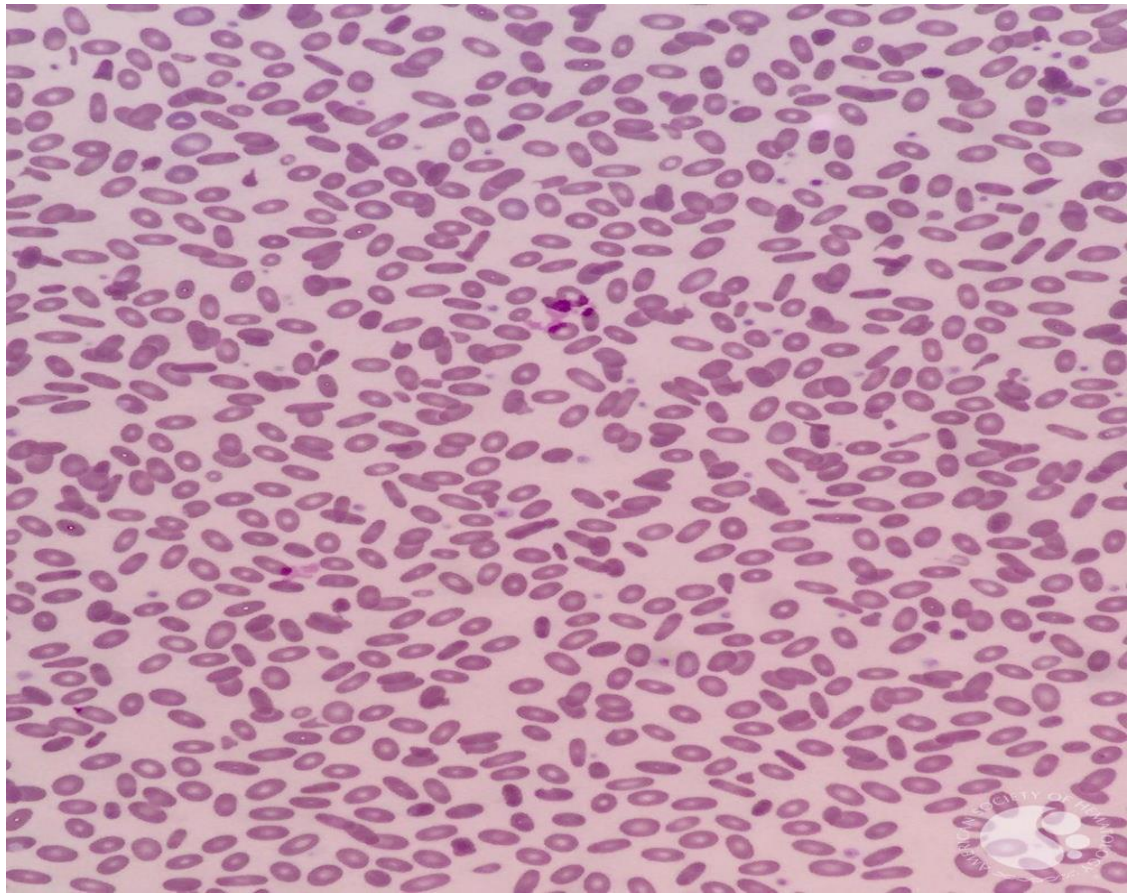
- Highly variable depending on the underlying defect
- 60%- intermediate-severity phenotype
  - » Moderate hemolysis (Hb 8-11 g/dL)
  - » Reticulocyte count > 8%
  - » Normal hemoglobin at birth that may sharply and transiently decline in the first 3 weeks of life
- 20% -mildly affected/asymptomatic
  - » May not be identified until there is a hemolytic crisis in childhood
  - » Often triggered by viral infection
  - » May be diagnosed due to aplastic crisis with parvovirus B19
- 20% -have severe disease
  - » Usually, AR inheritance due to abnormality of α-spectrin

- Hereditary elliptocytosis (HE)/ Hereditary pyropoikilocytosis(HPP)

# HE/HPP

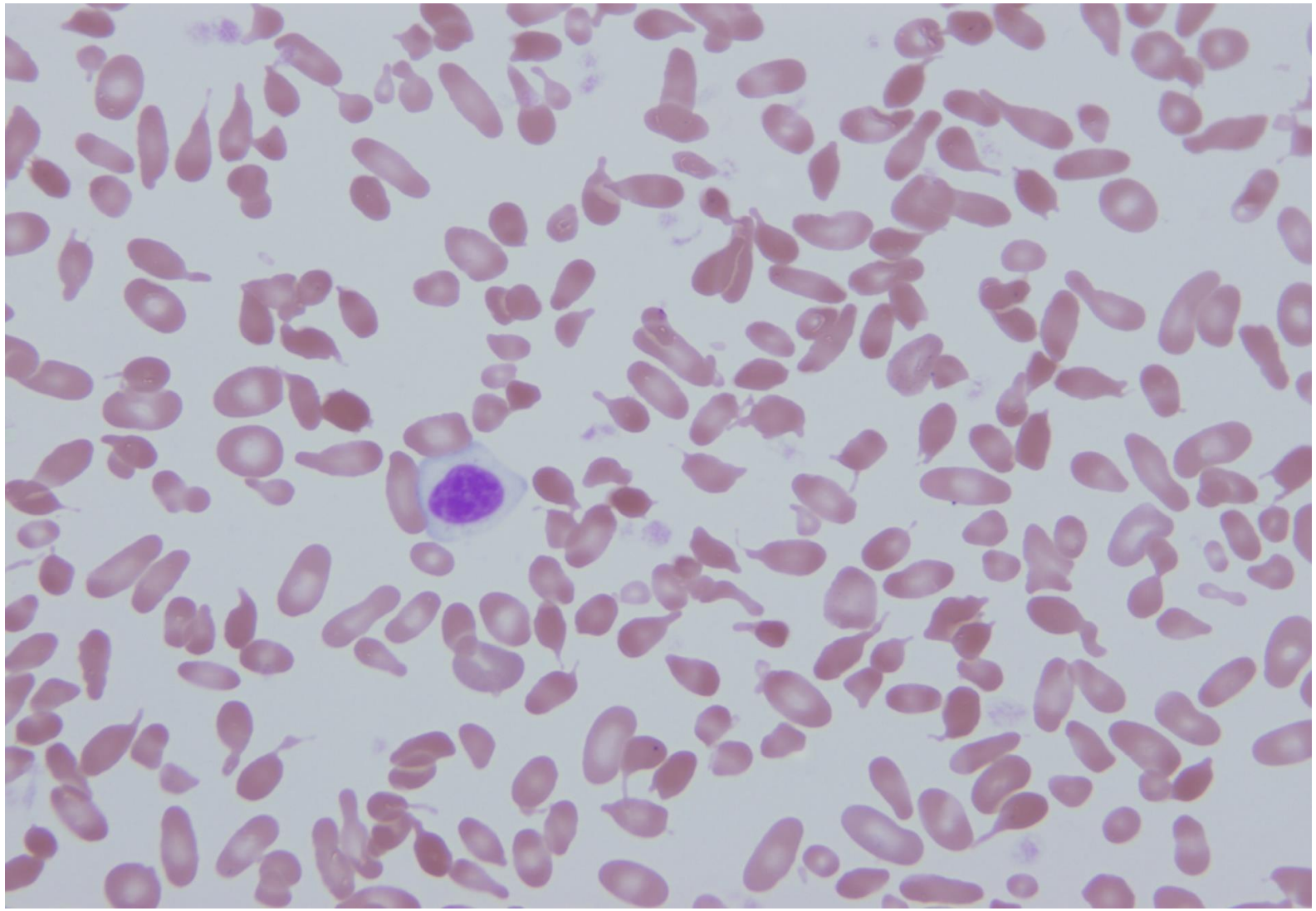
- HE /HPP- heterogenous clinical presentations
- HE- mostly asymptomatic, but HPP is a severe form of HE that presents with hemolytic anemia and jaundice during the infantile period
- Erythrocyte morphology in HPP resembles that of blood smears in thermal burns with poikilocytes, red blood cell fragments, microspherocytes, and elliptocyte
- HE - AD while HPP is an AR  disorder

# Hereditary Elliptocytosis



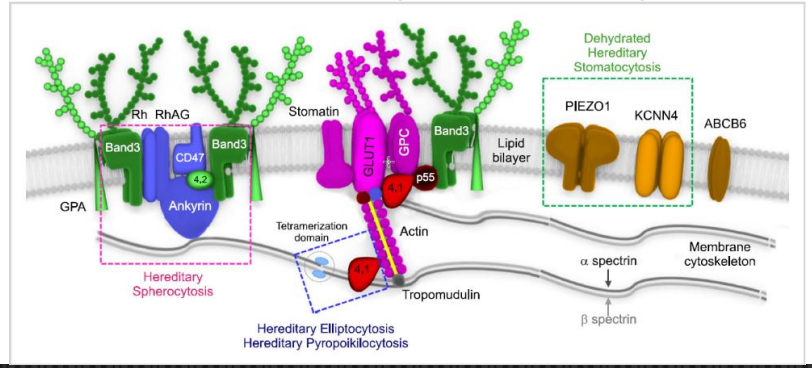
https://imagebank.hematology.org/image/61156/hereditary-elliptocytosis

# Hereditary Pyropoikilocytosis

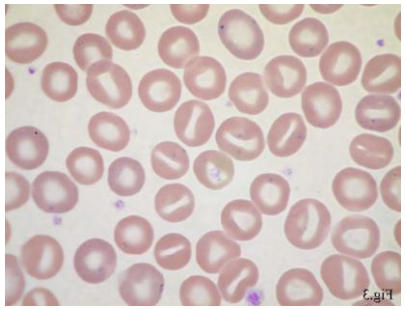


# HE/HPP Pathophysiology

- Principal defect is mechanical weakness or fragility of the erythrocyte membrane skeleton
- Due to defects in various membrane proteins, including α- and β-spectrin, protein 4.1, and glycophorin C
- Spectrin integrity is dependent on the self-association of heterodimers of α- and β-spectrin into mature spectrin molecules that are critical for membrane stability and erythrocyte shape and function



# Hereditary Stomatocytosis



- Stomatocytosis is associated with abnormalities in red cell cation permeability
- A mouthlike or slitlike pattern replaces the normal central zone of pallor
  - » Only seen in a subset
- Changes in red cell volume, which may be either increase (overhydrated) or decreased (xerocytosis), or, in some cases, near normal
- The pathobiology of the stomatocytic shape is poorly understood

# Dehydrated Hereditary Stomatocytosis (DHST)

- Also known as hereditary xerocytosis - most common inherited red cell cation permeability disorders
- Characterized by a mild increase in potassium permeability that is sufficient to lead to the gradual loss of red cell $K^+$ and water, and to red cell dehydration, stiffness, and hemolysis
- High MCHC, and **resistant** osmotic fragility are characteristics
- Hemoglobin and hematocrit values are often normal (compensated hemolysis) and patient blood smears are surprisingly normal, featuring mostly a few target
- Unexplained iron overload

Albuisson J, et al. Dehydrated hereditary stomatocytosis linked to gain-of-function mutations in mechanically activated PIEZO1 ion channels. Nat Commun. 2013;4:1884.

# Hereditary Stomatocytosis: Genetic Etiology

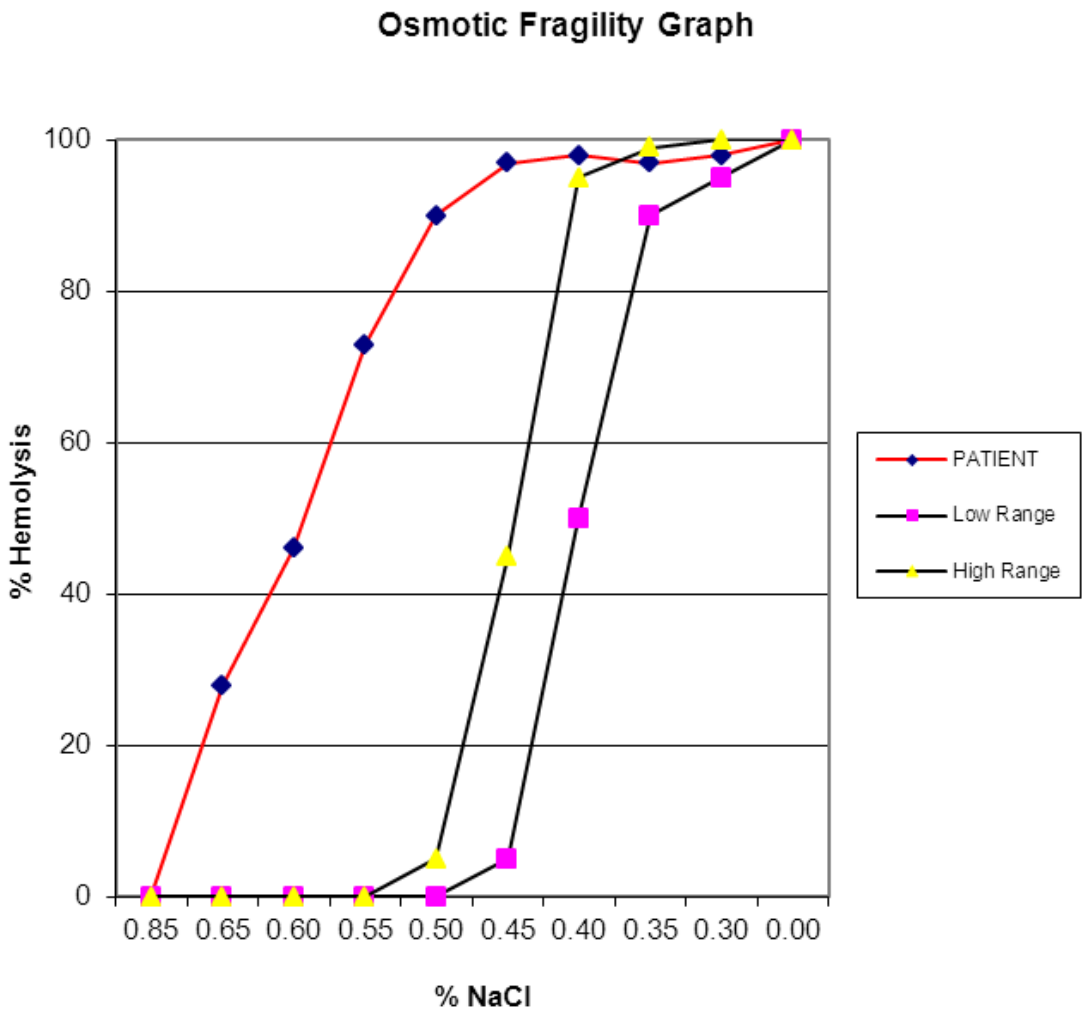
- Most reported DHS cases are caused by gain-of-function mutations in the gene *PIEZO1* (16q24.3) which encodes part of a mechanosensitive ion channel

- Rarely *KCNN4* gene that encodes a Gardos channel

# Diagnosis of HS, HE/HPP and Stomatocytosis

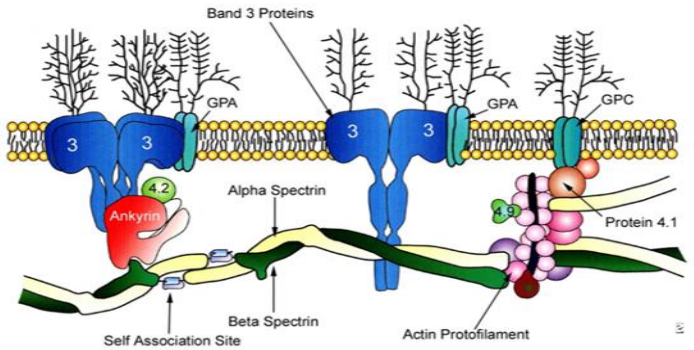
- Peripheral blood smear- elliptocytes, spherocytes, stomatocytes and fragmented cells (in HPP) are seen
- Osmotic fragility
  - » A laboratory test used in the diagnosis of HS, is sensitive but not specific. The test measures the *in vitro* lysis of RBCs suspended in solutions of decreasing osmolarity
  - » Spherocytes are characterized by membrane loss and less redundancy
  - » Not very specific for any of these disorders

# Osmotic Fragility Test



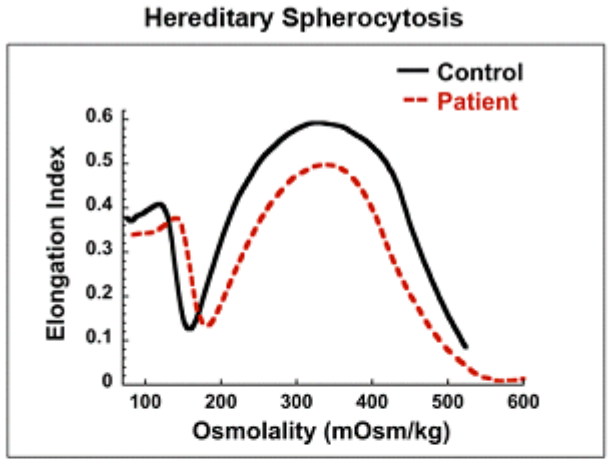
# Diagnosis Continued!

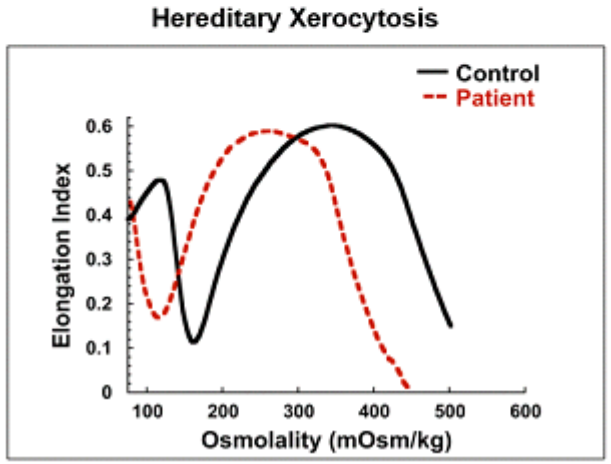
- Flow cytometry
  - » Measures the fluorescent intensity of intact red blood cells labelled with EMA (eosin-5-maleimide)
  - » EMA binds specifically with band 3 protein
  - » EMA binding is affected by all sorts of membrane protein abnormalities, not just band 3 deficiency
  - » Greater than 95% sensitive and specific for HS



# Ektacytometry

- Briefly, RBC are subjected to a defined value of shear stress and an osmotic gradient, and the laser diffraction pattern generated by the RBC suspension is recorded




Bryce Canyon

# Enzyme deficiency

# Enzymopathies

**Table 1.** Clinical phenotypes and associated genes in inherited hemolytic anemia.

| Clinical phenotypes | Genes | Location | Inheritance |
|---|---|---|---|
| RBC enzymopathies | | | |
| G6PD deficiency | *G6PD* | Xq28 | XR |
| Pyruvate kinase deficiency | *PKLR* | 1q22 | AR |
| Enolase deficiency | *ENO1* | 1p36.23 | AD |
| Adenylate kinase deficiency | *AK1* | 9q34.11 | AR |
| Glucose phosphate isomerase deficiency | *GPI* | 19q13.11 | AR |
| Pyrimidine 5′ nucleotidase (UMPH1) deficiency | *NT5C3A* | 7p14.3 | AR |
| Gamma-glutamylcysteine synthetase deficiency | *GCLC* | 6p12.1 | AR |
| Glutathione peroxidase deficiency | *GPX1* | 3p21.31 | AR |
| Glutathione reductase deficiency | *GSR* | 8p12 | AR |
| Glutathione synthetase deficiency | *GSS* | 20q11.22 | AR |
| Hexokinase deficiency | *HK1* | 10q22.1 | AR |
| Bisphophoglycerate mutase deficiency | *BPGM* | 7q33 | AR |
| Phosphoglycerate kinase 1 deficiency | *PGK1* | Xq21.1 | XR |
| Triosephosphate isomerase deficiency | *TPI1* | 12p13.31 | AR |

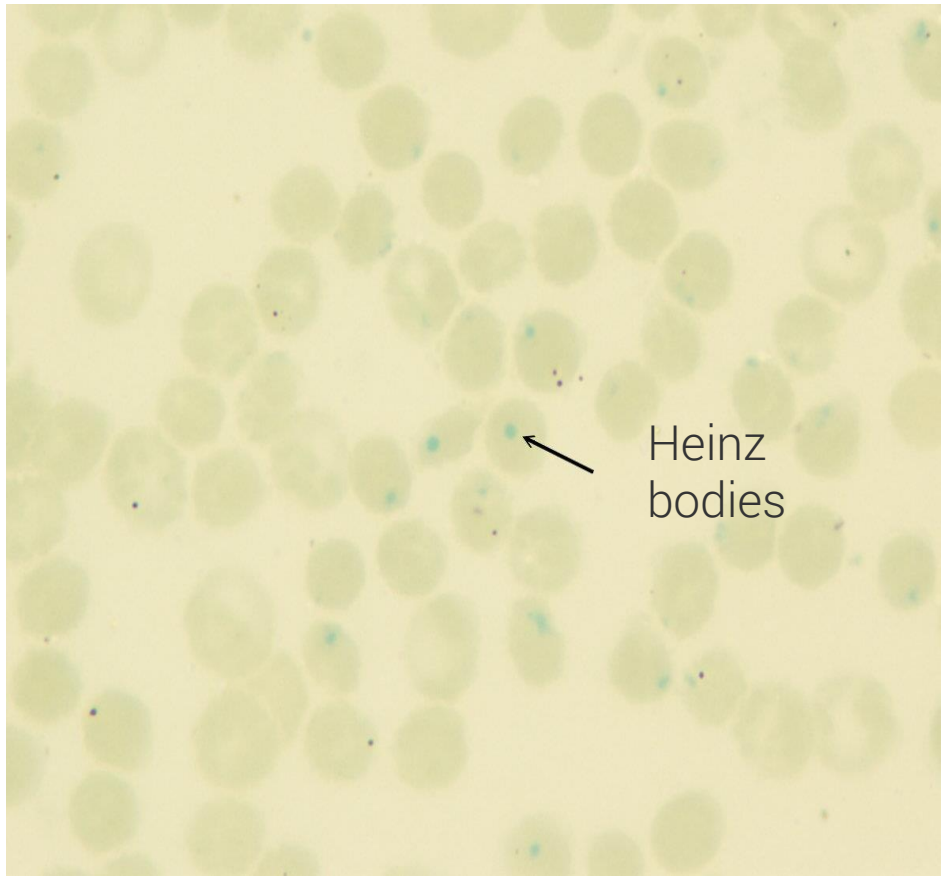
Abbreviations: AD, autosomal dominant; AR, autosomal recessive; XR, X-linked recessive.

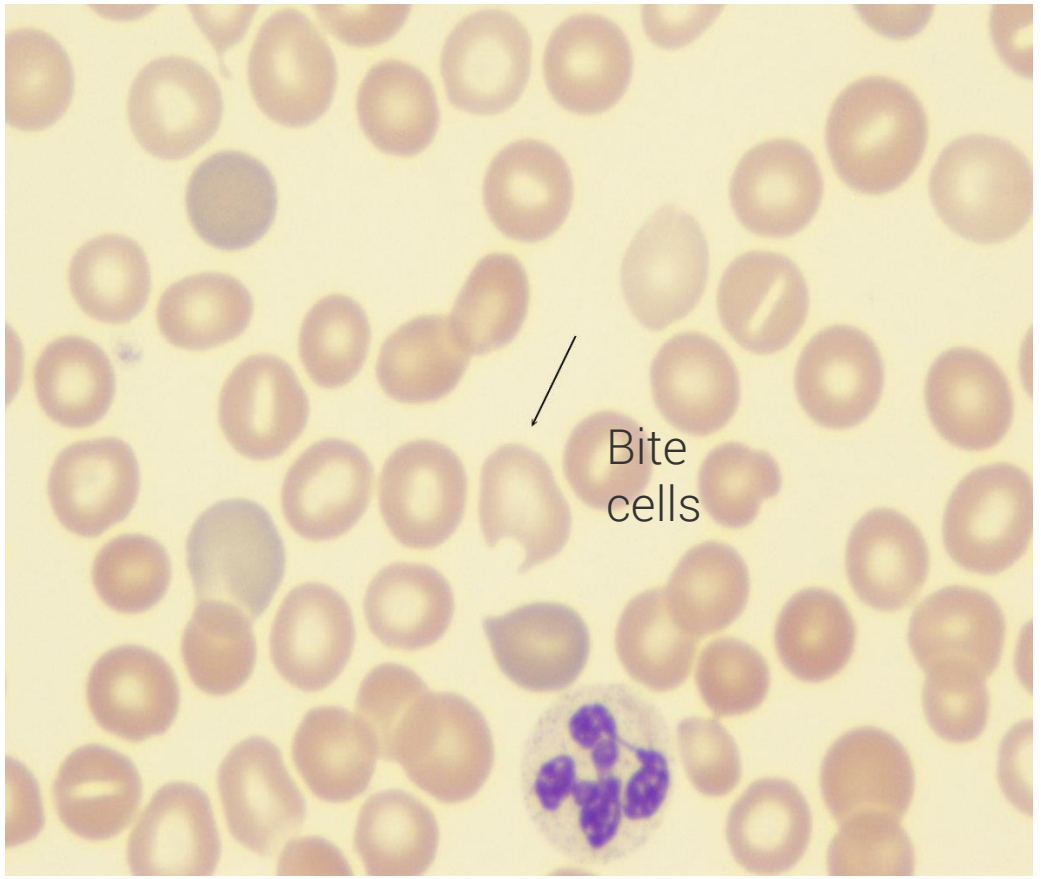
PMID: 28698843

# Glucose 6 phosphate Dehydrogenase Deficiency

- The most common human enzyme defect! >400-600 million people
- X linked
- Most prevalent in populations with endemic malaria
- Enzyme deficiency leading to ↓ NADPH-glutathione (GSH) depletion
  - » ↑ Oxidative stress, ↑ Hemolysis
- ***Diagnosis***
  - » ↓ G6PD (false negative with ↑reticulocytes)
  - » Heinz bodies (denatured Hb) and Bite cells in PB smear

# Heinz (denatured Hb) and bite cells



# Pyruvate Kinase Deficiency

- Common cause of congenital non-spherocytic chronic hemolytic anemia
- Autosomal recessive disorder
- Found in north Europeans; high frequency in Pennsylvania Amish population
- Diagnosis
  - » Direct measurement of enzyme activity in RBCs-↓ PK activity

- Severe disease may require frequent red cell transfusion throughout infancy and into adulthood.
- Splenectomy ameliorates the severity of hemolysis

# PK Deficiency

- Treatment - supportive
- Data from a phase II study in PK deficiency patients treated with AG-348 showed that approximately half of treated subjects experienced a rise in hemoglobin (Hb)

RF, Rose C, Layton M. Safety and efficacy of Mitapivat in pyruvate kinase deficiency. *N Engl J Med.* 2019; 381(10):933-944tained hemoglobin increase in adults

# Arches National Park, Utah

# Clinical Features of HHA

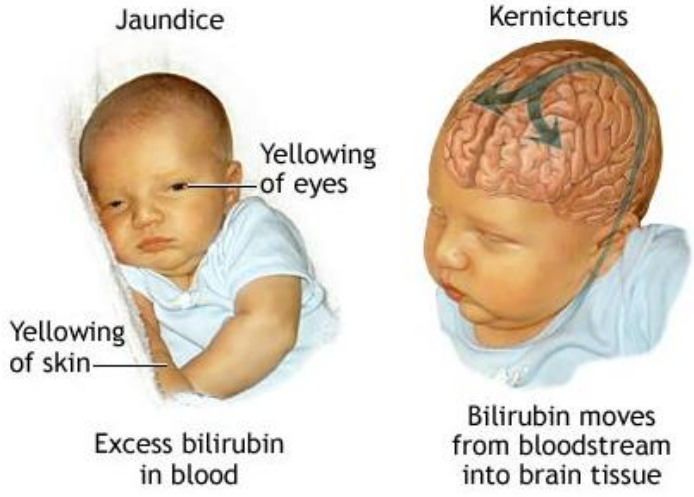
- Onset may be acute or chronic
- Symptoms and signs of anemia-pallor, fatigue
- RBC break down-increased bilirubin (hyperbilirubinemia)
  - » Jaundice and gall stones
  - » Neonatal toxicity
- Crises (chronic hemolytic disease)
  - » Hemolytic (increased splenic activity)
  - » Aplastic  (B19 Parvo-virus)
  - » Megaloblastic

# Hyperbilirubinemia and Its Consequences

- Bilirubin needs to be conjugated and excreted in bile
- Conjugation in the liver happens by the enzyme **uridine diphosphogluconurate (UDP) glucuronyltransferase**
- Gene involved in *UGT1A1*
  - » *Gilbert (usually due to additional TA repeats) and Crigler-Najjar syndrome (due to AR mutations)*
- Solute carrier organic anion transporter family members 1B1 and 1B3 (*SLCO1B1* and *SLCO1B3*)
  - » Rotor syndrome

# Hyperbilirubinemia: Neonates

- Increased unconjugated bilirubin can pass blood brain barrier- neurotoxicity in neonates
- Pathophysiology
  - » Increased bilirubin production (twice/kg body weight as adults)
  - » Delayed UGT1A1 induction
- Risk factors:




| Genetic factors | Maternal/Perinatal/Other neonatal factors |
|---|---|
| G6PD deficiency | Diabetes, Rh incompatibility, Age, Race |
| RBC membrane defects (HS, HE) | Mode of delivery, birth trauma, delayed cord clamping |
| Hemoglobinopathies | Congenital infections |
| | Nutritional deficiencies |
| Gilbert's syndrome (UGT1A1 deficiency) | |
| Crigler-Najjar syndrome (UGT1A1 deficiency) | |

# Park City, Utah

# 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
- G6PD/PK deficiency of mild-to-moderate severity can be missed in neonates when reticulocyte counts are high
- To diagnose complex interactions (HHA +Bilibrubin metabolism disorders)

# Molecular Approach

- Focused targeted Next-generation sequencing (NGS) provides a cost-effective and relatively rapid approach
- Evaluated since 2012 with gene counts on these panels varying from 28-70
- Diagnostic yield - 30-75%



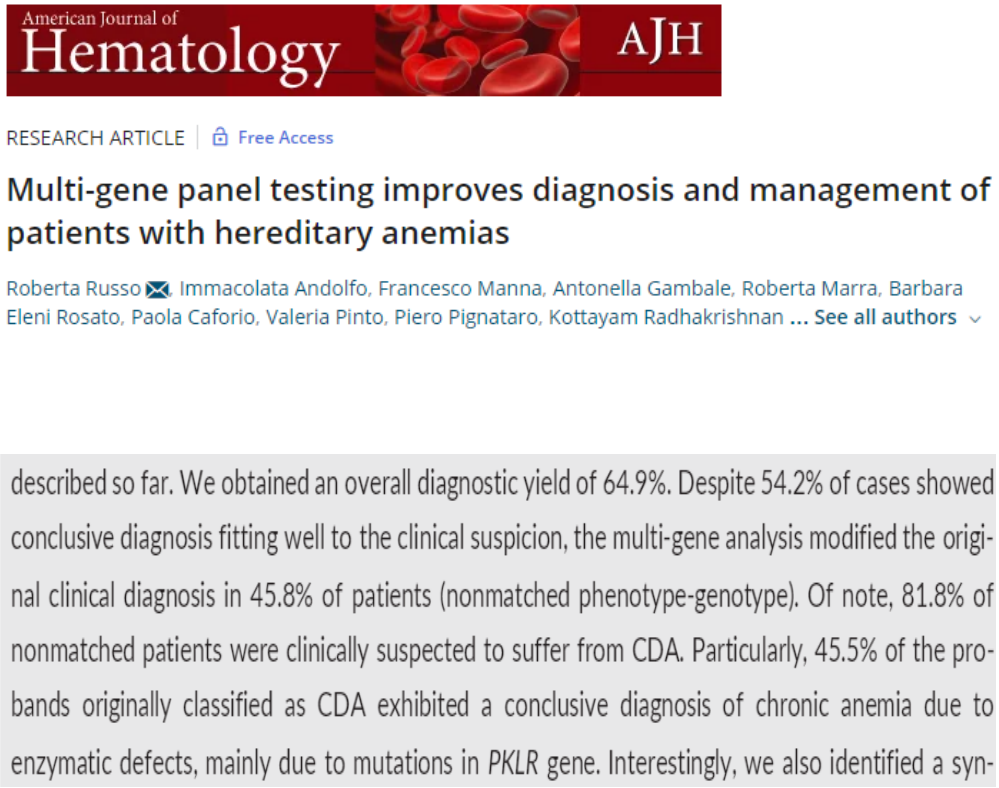
**Table 1** Recent studies performed by targeted NGS panels in patients with hemolytic anemias. Number of the genes included in the panel, patients studied, overall sensitivity and, when available, sensitivity of panels in patient with undefined hemolytic anemia are reported

| Studies | No. of genes analysed | No. of cases | Overall sensitivity | Sensitivity in hemolytic patients with no previous diagnosis |
|---|---|---|---|---|
| Chonat *et al*., 2019 (21) | 32 (membrane defects) | 11 (HS) | 100% | Not studied |
| van Vuren *et al*., 2019 (16) | 7 (membrane defects) | 95 (HS) | 89% | Not studied |
| Xue *et al*., 2019 (1) | 10 (membrane defects) | 10 (HS) | 90% | Not studied |
| Peng *et al*., 2018 (20) | n.a. | 51 (HS) | 72% | Not studied |
| Li *et al*., 2018 (17) | 217 | 46 (CHA) | 60.9% | n.a. |
| Russo *et al*., 2018 (14) | 34 and 71 | 74 (CHA) | 64.9% | 45.8% |
| Agarwal *et al*., 2016 (18) | 28 | 17 (CHA) | 70% | 70% |
| Roy *et al*., 2016 (13) | 33 | 57 (CHA) | 38.6% | 11% |

CHA, chronic hemolytic anemias; HS, hereditary spherocytosis; n.a., not available.

# Targeted NGS Panels: Importance

- Russo et al. - 74 patients with HHA with an emphasis on congenital dyserythropoietic anemia (CDA)
- One of their interesting findings was CDA misdiagnosed as enzyme deficiency particularly PK deficiency
  - » Out of 22 patients initially diagnosed as CDA, 10/22 were later diagnosed with enzyme deficiencies



American Journal of Hematology AJH

RESEARCH ARTICLE | Free Access

**Multi-gene panel testing improves diagnosis and management of patients with hereditary anemias**

Roberta Russo, Immacolata Andolfo, Francesco Manna, Antonella Gambale, Roberta Marra, Barbara Eleni Rosato, Paola Caforio, Valeria Pinto, Piero Pignataro, Kottayam Radhakrishnan ... See all authors

described so far. We obtained an overall diagnostic yield of 64.9%. Despite 54.2% of cases showed conclusive diagnosis fitting well to the clinical suspicion, the multi-gene analysis modified the original clinical diagnosis in 45.8% of patients (nonmatched phenotype-genotype). Of note, 81.8% of nonmatched patients were clinically suspected to suffer from CDA. Particularly, 45.5% of the probands originally classified as CDA exhibited a conclusive diagnosis of chronic anemia due to enzymatic defects, mainly due to mutations in *PKLR* gene. Interestingly, we also identified a syn-

# Genes Involved in Hereditary Membrane Disorders

| Condition | Genes | Inheritance |
|---|---|---|
| Hereditary spherocytosis | *ANK1, SLC4A1, SPTB, EPB42, SPTA1* | AD/AR |
| Hereditary elliptocytosis/pyropoikilocytosis | *SPTA1, SPTB, EPB41* | AD/AR |
| Hereditary stomatocytosis | *PIEZO1, KCNN4* | AD |

| Liver Related | | |
|---|---|---|
| SLCO1B1 | Hyperbilirubinemia, rotor type | AR (digenic) |
| SLCO1B3 | Hyperbilirubinemia, rotor type | AR (digenic) |
| UGT1A1 | Gilbert syndrome | |
| | Crigler-Najjar syndrome, types I and II | |
| | Hyperbilirubinemia, transient familial neonatal | AR |
| UGT1A6 | | |
| UGT1A7 | | |

| Condition | Genes | Inheritance |
|---|---|---|
| Glucose 6 phosphate dehydrogenase deficiency | G6PD | XR |
| Pyruvate kinase deficiency | PKLR, | AR |
| Glucose phosphate isomerase deficiency | GP1 | AR |
| Glutathione reductase deficiency | GSR | AR |
| Phosphoglycerate kinase deficiency | PGK1 | XL |
| Triosephosphate isomerase def | TPI1 | AR |
| Adenylate kinase 1 | AK1 | AR |
| Pyrimidine 5' nucleotidase | NT5C3 | AR |
| Hexokinase 1 | HK1 | AR |

# Our Recent Findings:

https://doi.org/10.1182/blood-2018-99-112589

- 268 patients evaluated using targeted NGS panel with 28 genes
- Age - newborn to 62 years
- Mild lifelong anemia to severe hemolytic anemia with extreme hyperbilirubinemia

101. RED CELLS AND ERYTHROPOIESIS, STRUCTURE AND FUNCTION, METABOLISM, AND SURVIVAL, EXCLUDING IRON:
POSTER II | NOVEMBER 29, 2018

**Use of Next Generation Sequencing Panel for Routine Diagnosis of Hereditary Hemolytic Anemias**

Archana M Agarwal, MD, Jay L Patel, MD, Adam Clayton, PhD, Noel Scott Reading, PhD

# 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)
- Complex interactions (12/64 )
- 11%  (29/268)- *UGT1A1* gene

Received: 15 January 2019 | Revised: 27 February 2019 | Accepted: 1 March 2019

DOI: 10.1111/ijlh.13014

SUPPLEMENT ARTICLE

WILEY ISLH International Journal of Laboratory Hematology

## Molecular diagnostic update in hereditary hemolytic anemia and neonatal hyperbilirubinemia

Anton Rets[1,2] | Adam L. Clayton[2] | Robert D. Christensen[3] | Archana M. Agarwal[1,2]

Recently, our group evaluated 268 patients with HA and identified pathogenic and likely pathogenic variants in 64/268 (24%) patients that were clearly responsible for the disease phenotype (eg, moderate to severe HA). Interestingly, half of the variants were novel mutations.[41] Complex interactions between variants in the *SPTA1* gene and the common alpha-LELY and alpha-LEPRA alleles were predicted to be associated with HPP and AR HS in 12/64 patients. Overall, 29/268 (11%) of patients were homozygous for a promoter polymorphism in the *UGT1A1* gene A(TA)7TAA (*UGT1A1*28*), which leads to reduced expression of the *UGT1A1* gene and Gilbert syndrome. Moreover, 4/29 *UGT1A1* polymorphism cases were associated with hyperbilirubinemia along with pathogenic mutations in spectrin genes.

# Case Study

Journal of
eISSN: 2373-4426
Pediatrics & Neonatal Care

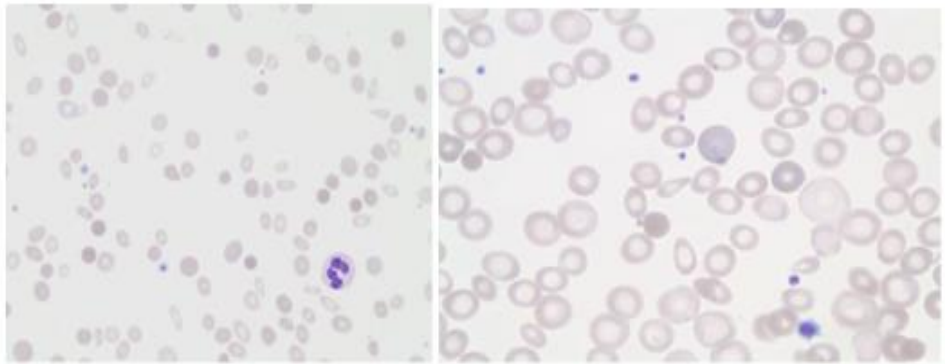
Check for updates

Research Article Volume 8 Issue 5

**Novel mutation in SPTA 1 gene associated with severe hemolytic anemia**

James Polega,[1] Jennifer Stumph,[2] Archana Agarwal,[3] Chi L Braunreiter[4]

- Caucasian ethnicity, lacking regular well child visits
- Presented at 3.5 yrs -with mild anemia and splenomegaly
- The peripheral blood smear—anemia with many spherocytes
- Past history-significant for hyperbilirubinemia in the newborn period
- Parents declined childhood immunizations and transfusions; the patient's anemia and splenomegaly worsened
- Genetic testing was permitted
  - » after dilated cardiomyopathy developed



# Case Study: Continued

- Two variants - *SPTA1* gene identified
  - » A novel mutation (c.7134+2T>G, p.?), predicted to cause abnormal splicing of *SPTA*1 gene, was identified in addition to heterozygous low expression variants $\alpha^{LEPRA}$
- $\alpha^{LEPRA}$ in *trans* to a pathogenic *SPTA1* variant- associated with autosomal recessive HS

.

- A third heterozygous variant in the *SPTB* gene (c.4564-4G>A)

  - » *SPTB* variant - one child with HS and reduced *SPTB* mRNA level.
  - » Previous computational study predicted this *SPTB* variant would result in abnormal splicing of *SPTB* gene; however its effect on splicing remains to be determined experimental

# Case Study: Continued

- Given the genetic testing results, the parents consented to immunizations, and splenectomy

- Four months after splenectomy, the patient's hemoglobin improved to 15.2g/dL and echocardiogram changes were resolving

# Summary

- Our results demonstrate that many patients with hemolytic anemia harbor complex combinations of known and novel mutations in RBC cytoskeleton/enzyme genes
- Their clinical significance can be further augmented by polymorphisms of UGT1A1 gene contributing to severe neonatal hyperbilirubinemia and its consequences in pediatric population
- Molecular diagnosis can be helpful to understand the pathophysiology

# Lake Powell, Utah

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