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INHERITED ANEMIAS

Red cell membrane disorders: structure meets function

Mary Risinger[1] and Theodosia A. Kalfa[2,3]

[1]College of Nursing, University of Cincinnati, Cincinnati, OH; [2]Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH; and [3]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH

The mature red blood cell (RBC) lacks a nucleus and organelles characteristic of most cells, but it is elegantly structured to perform the essential function of delivering oxygen and removing carbon dioxide from all other cells while enduring the shear stress imposed by navigating small vessels and sinusoids. Over the past several decades, the efforts of biochemists, cell and molecular biologists, and hematologists have provided an appreciation of the complexity of RBC membrane structure, while studies of the RBC membrane disorders have offered valuable insights into structure–function relationships. Within the last decade, advances in genetic testing and its increased availability have made it possible to substantially build upon this foundational knowledge. Although disorders of the RBC membrane due to altered structural organization or altered transport function are heterogeneous, they often present with common clinical findings of hemolytic anemia. However, they may require substantially different management depending on the underlying pathophysiology. Accurate diagnosis is essential to avoid emergence of complications or inappropriate interventions. We propose an algorithm for laboratory evaluation of patients presenting with symptoms and signs of hemolytic anemia with a focus on RBC membrane disorders. Here, we review the genotypic and phenotypic variability of the RBC membrane disorders in order to raise the index of suspicion and highlight the need for correct and timely diagnosis. (*Blood*. 2020;136(11):1250-1261)

Introduction

The mammalian erythrocyte has evolved as a highly differentiated cell to deliver oxygen throughout the circulation, with a structure optimized to maintain survival under continuous shear stress. The lipid bilayer of the cell membrane is lined on its cytoplasmic surface by the erythrocyte cytoskeleton, and together they compose the engineering marvel we call the red blood cell (RBC) membrane (Figure 1).[1,2] The scaffolding network of the cytoskeleton has a design of triangles weaved in a hexagonal network. Two α- and β-spectrin heterodimers are organized in an antiparallel fashion into a tetramer making up each side of the triangle. Each heterodimer is built from a series of 3-helix bundles (spectrin repeats) able to coil and uncoil, providing elasticity. The junctional complex, composed of an F-actin protofilament with its capping proteins adducin and tropomodulin, and protein 4.1R that enables the actin-spectrin association,[3] is positioned at each corner of the triangle. The length of the actin filaments is maintained fairly stable at 12 to 14 actin monomers per oligomer,[1,4] indicating the existence of strict mechanisms that control the filament length.[5-7] Work by multiple research teams over decades has determined the stoichiometry of the various proteins in the cytoskeleton.[1,8-10] Based on recent proteomic quantification of highly purified populations of reticulocytes and mature RBCs, actin monomers are calculated to be >1 million copies per cell and β-spectrin ~0.5 million copies per cell.[10] Consequently, assuming 12 actin monomers per protofilament, the quasihexagonal lattice of the RBC cytoskeleton is estimated to have ~80 000 junctional complexes linked with ~250 000 spectrin tetramers.[11] Band 3, at ~1.2 million copies per cell,[8] arranges in tetramers and dimers and creates >300 000 vertical linkages between the cytoskeleton and the lipid bilayer. PIEZO1, the mechanosensitive cation channel, is present only at a few hundred copies per cell[10] but functions as a major determinant of the RBC hydration status. The extensive horizontal protein–protein interactions in the cytoskeleton with perpendicular transmembrane channels, which serve as vertical linkages between the cytoskeleton and the cell membrane, maintain the cell's biconcave disk shape, securing an increased surface area-to-volume ratio and allow the cell to reversibly deform while traversing capillaries with cross section as small as one-third the RBC diameter or passing through the interendothelial slits of the splenic red-pulp sinusoids.[2]

Patients with RBC cytoskeleton or hydration disorders due to altered structural organization or abnormal transporter function, respectively, present with the common clinical findings of hemolytic anemia, including pallor, jaundice, fatigue, splenomegaly, gallstones, and cholecystitis. However, they may require different management approaches depending on the cause of the disease. Therefore, accurate diagnosis is critical. A proposed algorithm for laboratory evaluation of a patient presenting with symptoms and signs of hemolysis with or without anemia with a focus on RBC membrane disorders is shown in Figure 2.

Extensive studies on RBC membrane physiology and pathology in humans and mouse models for many decades have provided a comprehensive understanding of the most common erythrocyte membrane disorders, such as hereditary spherocytosis (HS),

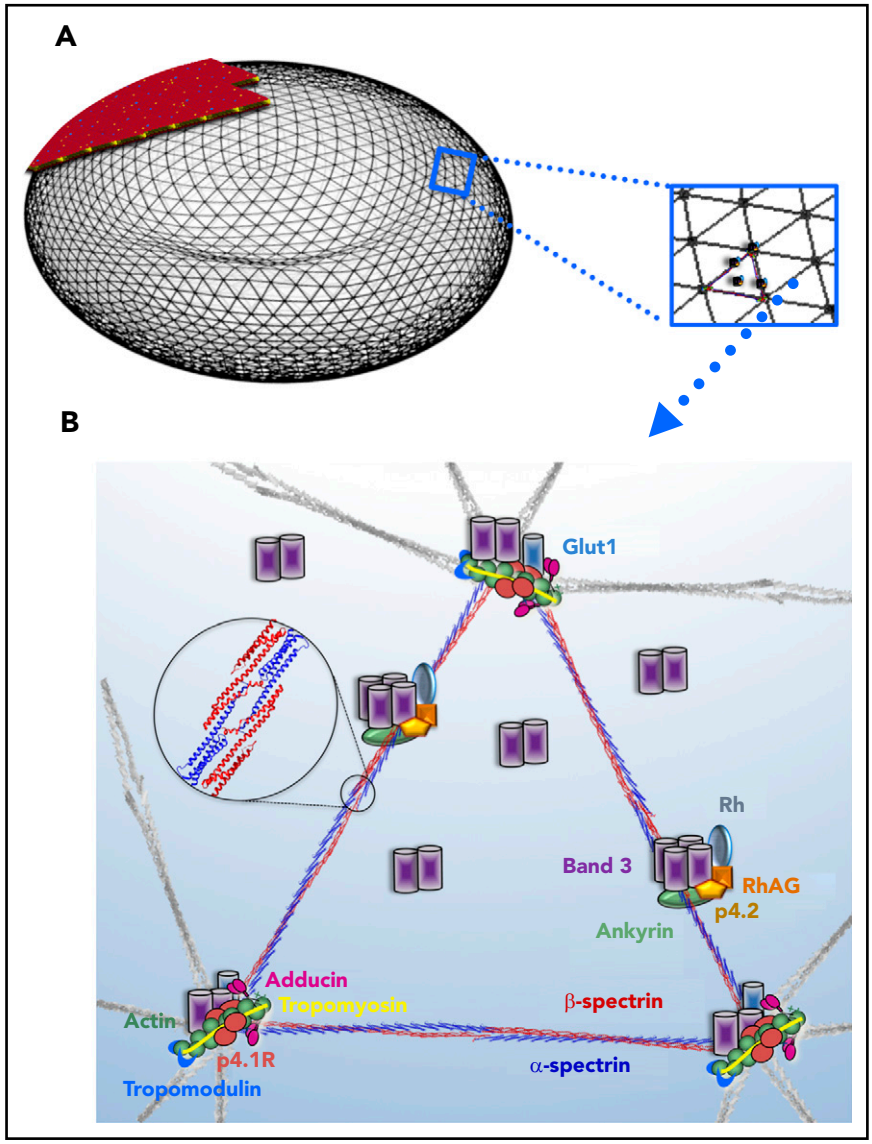

Figure 1. RBC membrane model depicting the structural proteins that, when abnormal, cause RBC membrane disorders. (A) Model of the RBC cytoskeleton quasihexagonal lattice forming a biconcave disc shape, supporting the lipid bilayer. An area of the cytoskeleton surface is shown "magnified" to demonstrate the arrangement of proteins within the hexagonal structure, focusing particularly on the proteins that, when defective, cause RBC membrane disorders. Illustration by Anastasios Manganaris (created using "blender" software, version 2.8). (B) α- and β-spectrin heterodimers associate head to head, as shown in the magnified circle, to form the spectrin tetramers that make the sides of each triangular unit in the hexagon. Each dimer head is composed of the N-terminal region of α-spectrin and the C-terminal region of β-spectrin. The junctional complex, at the corner of each triangle, is formed by an actin oligomer, with a length guided by tropomyosin, and capped by adducin and tropomodulin. Protein 4.1R enables the actin-spectrin association. The transmembrane protein complexes containing the integral membrane proteins band 3 and Rh-associated glycoprotein (RhAG) and the peripheral membrane proteins ankyrin and band 4.2 provide "vertical" linkages between the cytoskeleton and the lipid bilayer.

hereditary elliptocytosis (HE), and hereditary pyropoikilocytosis (HPP). The broader availability of genetic testing for clinical diagnosis (searchable in the National Center for Biotechnology Information Genetic Testing Registry: https://www.ncbi.nlm.nih.gov/gtr/) and the discovery of the molecular causes for hereditary xerocytosis (HX) have been major advances in the field over the last decade, improving feasibility of accurate diagnosis and revealing that the incidence of less common RBC membrane disorders may be underestimated. We will review here the genotypic and phenotypic variability of the RBC membrane disorders with the goal to raise the index of suspicion and emphasize the need for correct, timely diagnosis.

RBC cytoskeleton disorders

HS

Pathophysiology and genetics HS is the most common RBC membrane disorder worldwide and the most common hereditary hemolytic anemia (HHA) in people of Northern European ancestry, with prevalence of 1 in 1000 to 2500.[12-14] Spherocytes are formed due to loss of RBC membrane because of inadequate vertical linkages between the cytoskeleton and the lipid bilayer.[13] Specifically, the molecular mechanism causing HS is quantitative deficiency in α- or β-spectrin, ankyrin, protein 4.2, or band 3, coded by the genes *SPTA1*, *SPTB*, *ANK1*, *EPB42*, and *SLC4A1*, respectively.[3] An abundance of family-private mutations have been found in each of these genes to cause HS. Loss of membrane leads to decreased cell surface area to volume ratio, mandating a spherical shape that is associated with increased osmotic fragility and decreased deformability. The result is extravascular hemolysis due to increased destruction of RBCs as they pass through the spleen.

Heterozygous mutations of *ANK1*, *SLC4A1*, and *SPTB* genes cause autosomal dominant (AD) HS, which accounts for approximately two-thirds of the cases.[13,15] 10% to 15% of HS cases are inherited in an autosomal recessive (AR) fashion, caused by biallelic defects in *EPB42, SPTA1, or ANK1*.[12,13,16] De novo mutations have also been described, most commonly for the *ANK1* gene, likely due to high G+C content predisposing to slipped strand mispairing during DNA replication.[17]

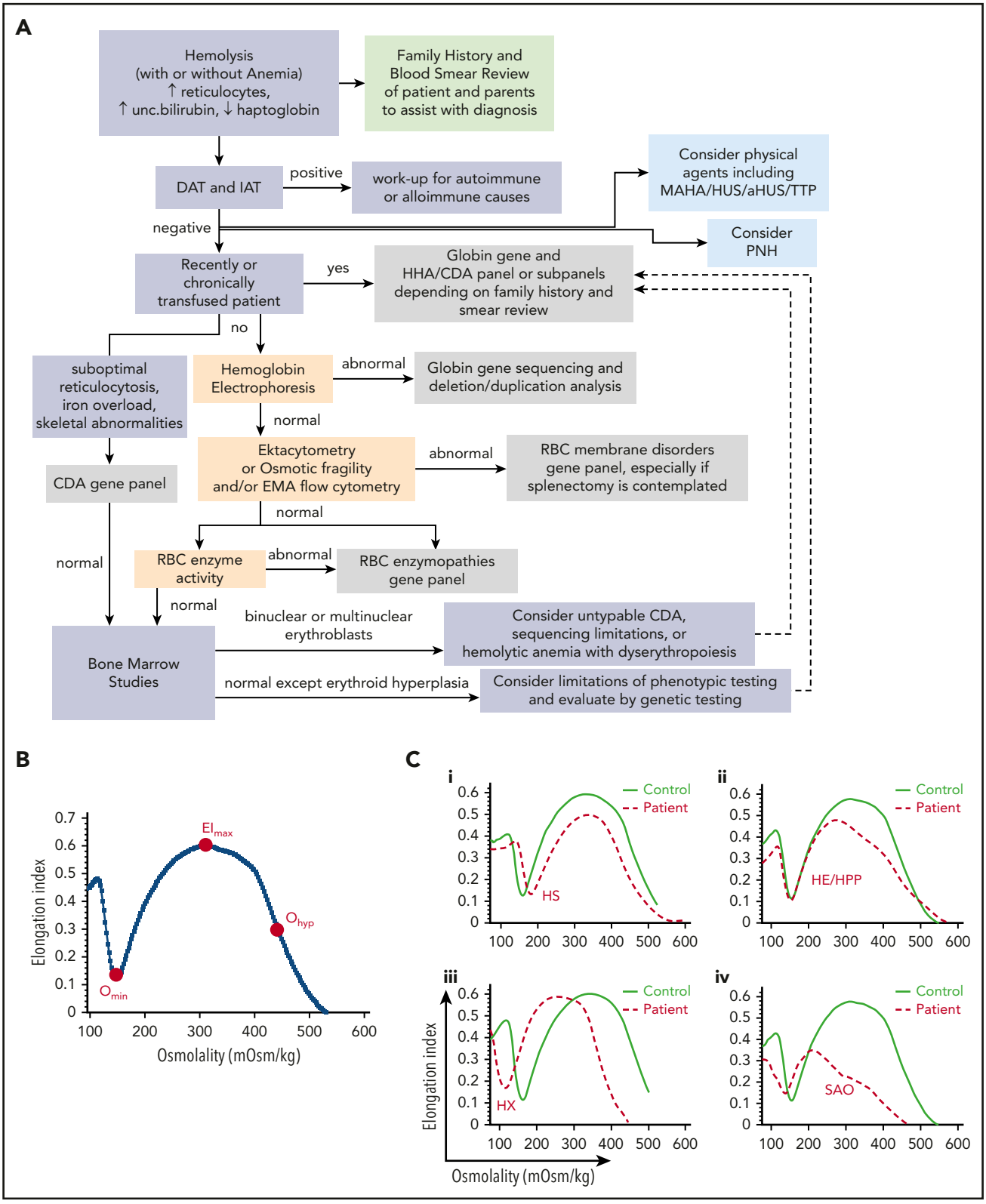

Figure 2. Evaluation workflow of patients with hemolytic anemia. (A) Proposed algorithm for the laboratory evaluation of a patient presenting with symptoms and signs of hemolysis. Of note, anemia may be well compensated, as in many cases of mild HS and most cases of PIEZO1-associated HX. Evaluation for autoimmune or, especially in an infant, alloimmune hemolytic anemia with direct and indirect antiglobulin test (DAT and IAT) is the first priority, since such a diagnosis typically requires immediate action. Consideration of the possibility of microangiopathic hemolytic anemia (MAHA) and paroxysmal nocturnal hemoglobinuria (PNH) may also be necessary. Blood smear review of the patient and parents, with attention to the RBC indices, including MCV, MCHC, and red blood cell distribution width (RDW), along with hemolytic markers (bilirubin, lactate dehydrogenase, and haptoglobin [the last one reliable after 6 months of life, since earlier, it may be low due to decreased production by the infant's liver]), ferritin, and transferrin saturation to consider iron-loading inefficient erythropoiesis, can guide the differential diagnosis. Flow cytometry with eosin-5′-maleimide (EMA) binding of band 3 and Rh-related proteins is a rapid screening test for RBC membrane disorders that are characterized by membrane loss.[96,97] Osmotic fragility is increased in HS and often decreased in

AR *EPB42*-associated HS is due to complete or near-complete absence of protein 4.2 leading to band 3 decrease in the RBC membrane; heterozygous carriers of *EPB42*-HS are asymptomatic. This type of HS is usually of mild or moderate severity and accounts for 40% to 50% of HS in Japan but <5% of all cases in other populations.[18,19] Patients with AR HS due to α-spectrin deficiency typically have a null (eg, nonsense or frameshift) *SPTA1* mutation in *trans* to a low-expression allele with an intronic variant that activates alternative splicing, most commonly the variant c.4339-99C>T called α^{LEPRA} (low-expression PRAgue).[20-22] α^{LEPRA} has a minor allele frequency (MAF) of 0.49% (gnomad.broadinstitute.org) and produces only ~16% of full-length spectrin as compared with the normal *SPTA1* allele or ~8% in comparison with 2 normal *SPTA1* alleles.[20] This rather low amount of α-spectrin allows for a normalized RBC survival in patients with AR *SPTA1*-associated HS after splenectomy, pointing also to the fact that 2 normal *SPTA1* alleles produce redundant levels of protein (specifically, α-spectrin is produced in threefold excess of β-spectrin).[23] Complete deficiency of α-spectrin, due to biallelic null *SPTA1* mutations, leads to the most severe form of HS presenting as fatal hydrops fetalis in a term pregnancy.[22] Babies with such genotype may survive if they are delivered prematurely, allowing for early transfusion support, or receive intrauterine transfusions. Reticulocytopenia is characteristic of this rare type of HS; splenectomy reveals reticulocytosis but fails to significantly improve the extremely decreased survival of the spectrin-deficient RBCs.[22] The parents of patients with *SPTA1*-associated HS, even when carrying 1 null *SPTA1* allele, are asymptomatic[22,24]; some may have slight reticulocytosis or increased osmotic fragility in incubated blood.[13] AR *ANK1*-associated HS is usually due to a null mutation in *trans* to a promoter or intronic variant affecting levels of expression or a missense mutation decreasing, but not obliterating, the incorporation of ankyrin into the cytoskeleton; these patients have typically severe, transfusion-dependent disease responding to splenectomy.[15] A handful of cases with homozygous *SLC4A1*[25,26] variants presenting with life-threatening hydrops fetalis and remaining transfusion dependent even after splenectomy have also been reported. Homozygous, truly null *SPTB* mutations have not been described, indicating that complete deficiency of β-spectrin may be incompatible with life.

Clinical picture and management The phenotypic variability of HS matches the variability in the associated causative genetic variants. Interestingly, patients with the same genotype may also have differences in their phenotype. Heterogeneity in the level of expression from splicing variants, variability in erythropoiesis response, and sometimes a different tolerance of anemia by patients and/or parents may contribute to this phenomenon of apparent incomplete penetrance. Patients with AD *SLC4A1*, *SPTB*, *ANK1*, and AR *EPB42*-associated HS present with a range of well-compensated hemolysis up to a moderately severe anemia (hemoglobin <8 g/dL with brisk reticulocytosis >10%).[13,19] AR *ANK1*- or *SPTA1*-associated HS is most frequently a transfusion-dependent disease since early infancy.[22] All types of HS are likely to present with neonatal jaundice within the first 24 hours of life, posing risk of kernicterus without appropriate monitoring and treatment with phototherapy; rarely, exchange transfusion is also needed.[27] After 2 to 3 weeks, when the normal neonatal hyposplenism resolves, anemia ensues and may be severe enough to require transfusion. Transfusion dependency during the first 9 months of life is not rare with any type of HS, due to the delayed erythropoietin response, and is not prognostic of the severity of the disease.[28] Infants with frequent transfusion requirement despite evidence by family history or genetic evaluation that they have an AD form of HS may benefit from erythropoietin administration.[29]

Patients with compensated hemolysis may present at a time of hemolytic or aplastic crisis due to a concurrent viral infection (eg, Epstein-Barr virus or parvovirus B19, respectively) or with gallstones at a relatively young age. Jaundice and gallstones may be exacerbated with coinheritance of a bilirubin metabolism defect, such as Gilbert syndrome.[30] Examples of red cell morphology per gene affected[13,31] and associated ektacytometry are depicted in Figure 3. Mean cellular volume (MCV) is usually normal, while mean corpuscular hemoglobin concentration (MCHC) is increased at high-normal or above-normal (>36g/dL) values. Since decreased deformability of spherocytes leads to their increased destruction in the spleen, splenectomy improves anemia. Notable exceptions are the rare cases due to near-complete deficiency of α-spectrin or band 3, which with the advent of intrauterine transfusions can survive.[22,26] These patients require either a lifelong program of transfusions and iron chelation or stem cell transplant. Splenectomy is indicated for HS patients with moderate and severe anemia requiring frequent transfusions and/or having compromised quality of life. Due to the lifelong risk of sepsis after splenectomy, the patient should be immunized against encapsulated bacteria and receive immediate medical attention with blood culture and IV antibiotics for fever. Partial splenectomy (removal of 80%-90% of the spleen) is also effective to mitigate hemolysis and offers the advantage of maintaining splenic immune function, which is especially important for cases in which splenectomy is performed at age <5 years.[12,32-34]

Figure 2 (continued) HX. Osmotic gradient ektacytometry, which evaluates the deformability of RBCs as they are subjected to constant shear stress in a medium of increasing osmolality in a laser diffraction viscometer, is the reference technique for differential diagnosis of erythrocyte membrane and hydration disorders when a recent transfusion does not interfere with phenotypic evaluation of the patient.[98-100] When the patient is recently or chronically transfused, as it is typically the case in young children with congenital severe hemolytic anemia, options for phenotypic evaluation are limited. In such cases, genetic evaluation with clinically available next-generation sequencing panels may provide an accurate diagnosis necessary for appropriate management decisions.[12,101,102] aHUS, atypical hemolytic uremic syndrome; HUS, hemolytic uremic syndrome; TTP, thrombotic thrombocytopenic purpura. (B) Osmotic gradient ektacytometry. The ektacytometry curve is determined by the RBC structural features.[99] The points indicated in red are the following: O_{min} corresponds to the value of the hypotonic osmolality at which 50% of the cells hemolyze in an osmotic fragility assay and provides information on the initial surface to volume ratio of the RBCs. A shift to the right reflects a decrease in the surface area to volume ratio (ie, increased osmotic fragility). EI_{max} corresponds to the maximum deformability of the RBC, and its value is affected by the cytoskeleton mechanics. The hypertonic descending part of the curve is represented by O_{hyp}, the osmolality value at which the cells' average maximum diameter is half of EI_{max}. The value of O_{hyp} correlates with the initial intracellular viscosity of the cell sample. A shift to the left reflects increased intracellular viscosity of the erythrocyte caused by increased intracellular concentration of hemoglobin, typically due to dehydration of the cell; a shift to the right may represent an overhydration state of the cell in overhydrated stomatocytosis or, most commonly, a decreased intracellular concentration of hemoglobin, such as in iron deficiency. (C) Typical osmotic gradient ektacytometry curves for various RBC membrane disorders (in red) in comparison with a normal control curve run concurrently (in green). (i) HS characterized by increased O_{min} and decreased EI_{max}. (ii) HE/HPP characterized by decreased EI_{max} and a trapezoid shape of the curve. (iii) HX with decreased O_{min} and decreased O_{hyp}. (iv) SAO with severely decreased deformability and decreased O_{min}.

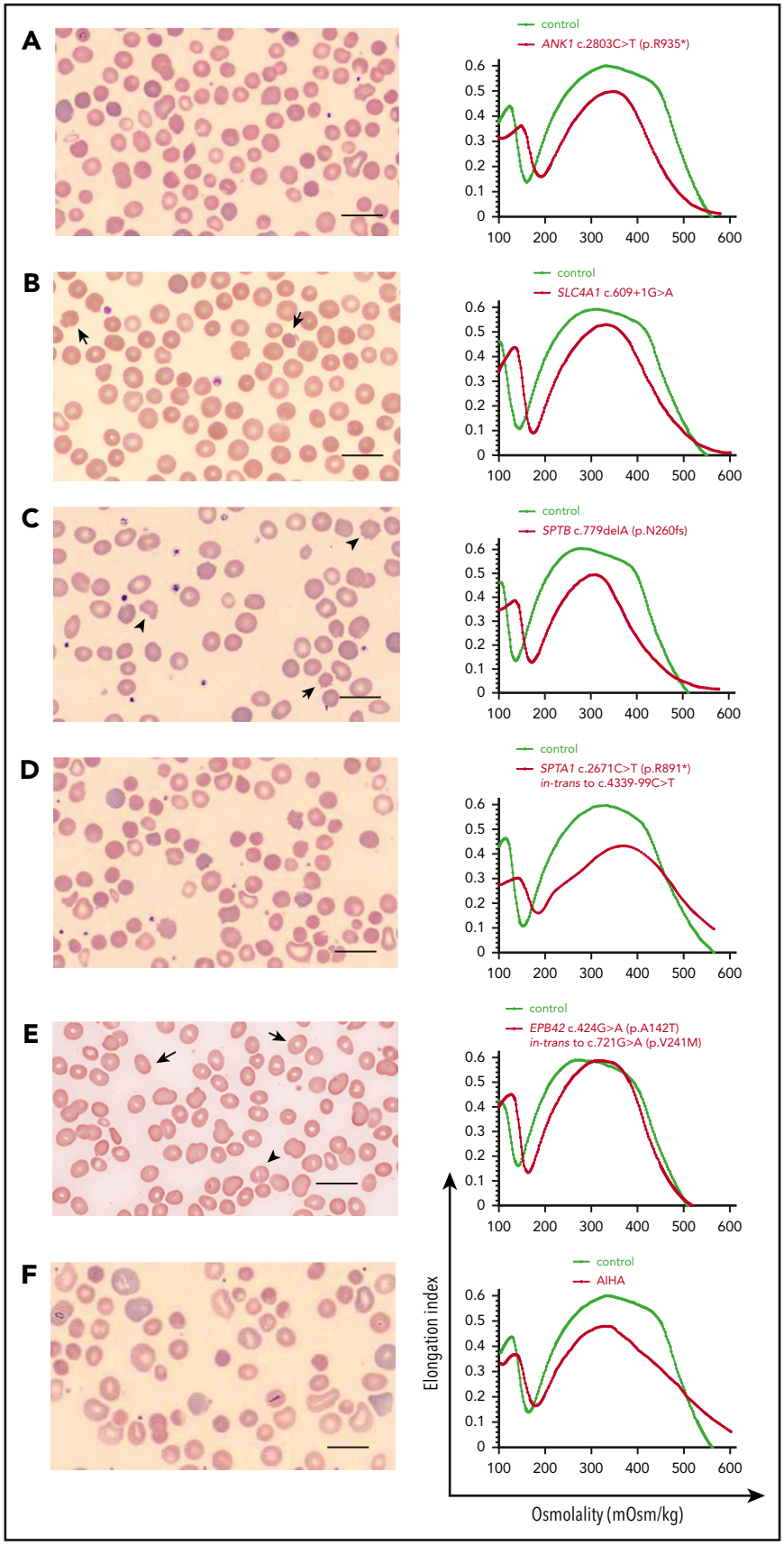

Figure 3. HS. Examples of red cell morphology in HS due to different gene mutations and associated osmotic gradient ektacytometry curves. Spherocytes (ie, RBCs with no or decreased central pallor) predominate, but additional RBC morphology characteristics may provide hints to the gene/protein defect causing HS, as Palek and Sahr[31] and Eber and Lux[13] have described very astutely in the past. (A-E, left) Blood smear of a patient with AD *ANK1*-HS showing anisocytosis (A). Occasional mushroom-shaped red cells (arrows) are characteristic of HS due to deficiency of band 3 (*SLC4A1*) (B). Acanthocytes (arrowheads) and echinocytes (arrow) are noted along with spherocytes in *SPTB*-associated HS (C). Blood smear of a not-recently transfused patient with AR *SPTA1*-HS demonstrates remarkable anisocytosis and poikilocytosis with contracted dense cells (D). Ovalocytes (arrows) and few ovalostomatocytes (arrowheads) are noted in AR *EPB42*-HS (E). Nevertheless, the above described changes in RBC morphology are not always specific for the gene affected in HS.[102] Scale bars, 14 μm; Wright-Giemsa stain. (A-E, right) The ektacytometry curve in HS is characterized by increased O_{min}, which corresponds to the increased osmotic fragility of the spherocytes. In almost all cases, decreased maximum deformability indicated by low EI_{max} is also noted, as well as decreased O_{hyp}. The decrease in EI_{max} tends to correlate with the degree of hemolysis and severity of anemia. (F) Important for differential diagnosis is that not all spherocytosis is hereditary. Autoimmune hemolytic anemia due to warm-reacting immunoglobulin G causes RBC membrane loss and acquired spherocytosis, with erythrocyte morphology and ektacytometry resembling HS. It is advisable to initiate the workup by first considering the possibility of an immune-mediated cause in every hemolytic anemia, since this radically alters management. In addition, blood smear and ektacytometry in congenital dyserythropoietic anemia type II also resemble HS; higher MCV, suboptimal reticulocytosis, and ferritin values disproportionally high for the number of transfusions will provide a clue to this possibility if bone marrow studies have not yet been performed. AIHA, autoimmune hemolytic anemia.

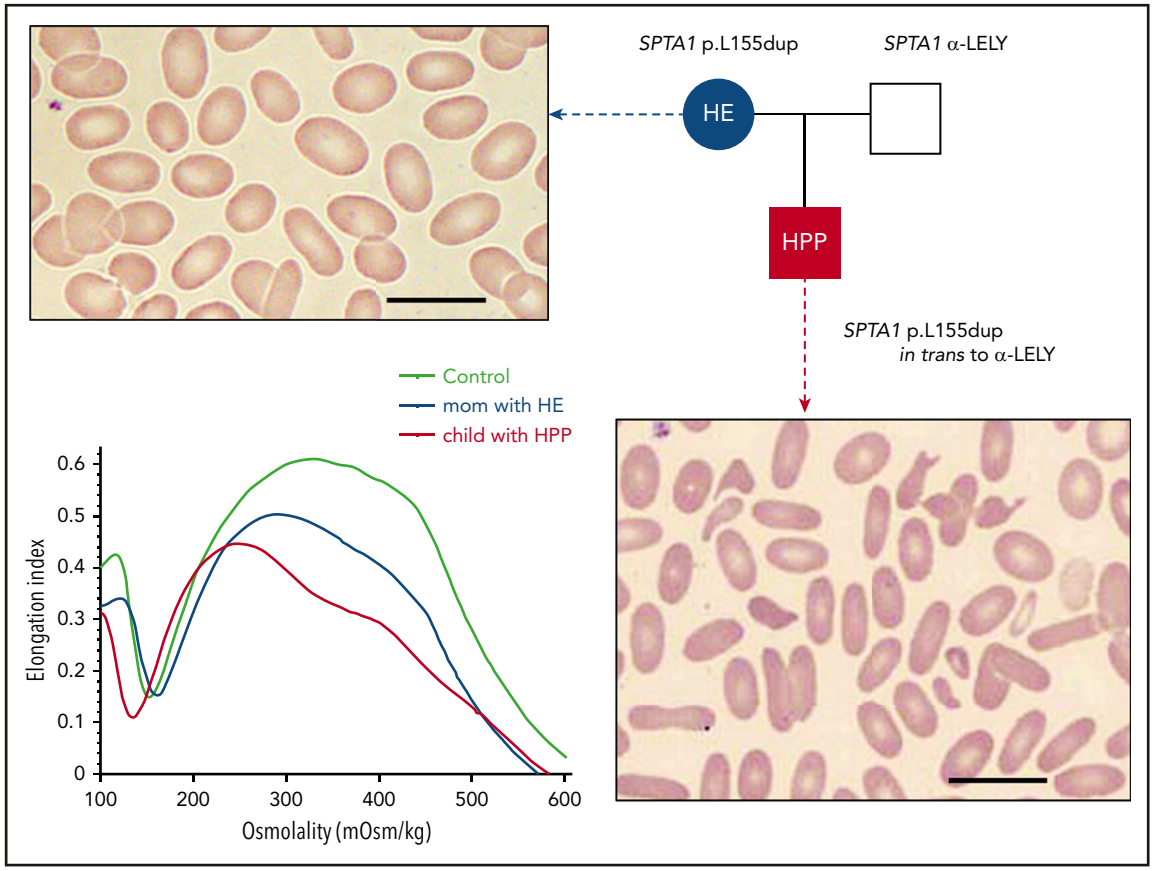

Figure 4. HE and HPP. The most common form of HPP, diagnosed in infants with neonatal hyperbilirubinemia, is due to an *SPTA1* HE-causing mutation in *trans* to the intronic *SPTA1* variant c.6531-12C>T known as α^{LELY} (low-expression LYon). Familial studies at the time reveal that 1 parent carries the *SPTA1* HE-causing mutation and has elliptocytosis (smear characterized by elliptically shaped red blood cells), while the other parent carries α^{LELY} with a normal erythrocyte phenotype. The blood smear in HPP is characterized by marked anisocytosis and poikilocytosis with bizarre microcytes and fragmented cells along with elliptocytes (scale bars, 14 μm; Wright-Giemsa stain). HPP RBCs were found early on to have increased thermal sensitivity; this is maybe the source of the name pyro (coming from the Greek word "πυρ" meaning fire)-poikilocytosis. Another possibility for the origin of the term is that the cells resemble the morphology of the blood smear in patients with the microangiopathic hemolytic anemia of patients with extensive burns.

HE and HPP

Pathophysiology and genetics HE-causing mutations are common in malaria-endemic regions, with a prevalence of 0.6% to 3% in West Africa, supporting the hypothesis that elliptocytosis confers survival advantage to malaria. In the United States, the incidence of HE is 1 in 2000 to 4000 people, although this may be underestimated, since HE is frequently asymptomatic, with no or mild hemolytic anemia. It is caused by monoallelic (heterozygous) mutations in the genes encoding α-spectrin (*SPTA1*), β- spectrin (*SPTB*), and protein 4.1R (*EPB41*). HE-causing *SPTA1* and *SPTB* variants are located in the spectrin tetramerization domain (Figure 1B) or distally and produce altered spectrin chains that incorporate into the cytoskeleton and weaken the spectrin tetramer.[35] *EPB41* mutations lead to altered or deficient protein 4.1R that compromises the spectrin-4.1R-actin association at the junctions.[12,36] Rare biallelic mutations of *GYPC* causing complete deficiency of glycophorin C (Leach phenotype) also cause HE due to concurrent partial deficiency of 4.1R, with no significant hemolysis.[37] The defective "horizontal" linkages in the cytoskeleton, caused by HE mutations, affect RBC membrane deformability and shape, allowing permanent deformation under shear stress into elliptocytes.[36] The most common form of HPP, frequently diagnosed in infants of African ancestry presenting with neonatal hyperbilirubinemia (Figure 4), is due to an *SPTA1* HE-causing mutation in *trans* to the intronic *SPTA1* variant c.6531-12C>T known as α^{LELY} (low-expression LYon).[38,39] α^{LELY} is a common variant (MAF 25.5%) (gnomad.broadinstitute.org), leading to a 50% decrease in α-spectrin expression. α^{LELY} is clinically silent in normal individuals, even in the homozygous state, since α-spectrin is produced in excess, but in *trans* to an HE-causing SPTA1 mutation leads to aggravation of the phenotype to HPP, because it allows for increased relative incorporation of the abnormal spectrin chain into the cytoskeleton. HPP can also be caused by biallelic HE-causing mutations, homozygous or compound heterozygous variants of *SPTA1*, *SPTB*, *EPB41*, or an allele each of *SPTA1* and *SPTB* carrying HE mutations.[36,38,40]

Clinical picture and management HE is associated with low rate of hemolysis, with normal hemoglobin values and borderline high reticulocyte count. It is frequently diagnosed during investigation for gallstones or splenomegaly or in family work-up in a parent of an infant with neonatal jaundice diagnosed with HPP. Infants with the common form of HPP (*SPTA1* HE-variant in *trans* to α^{LELY}) may have moderate to severe hemolytic anemia requiring frequent transfusions early in life, but the phenotype improves after the first 1 to 2 years and evolves into typical HE.[12] HPP due to biallelic HE-causing mutations may present with mild to severe chronic anemia. Most of the cases with severe

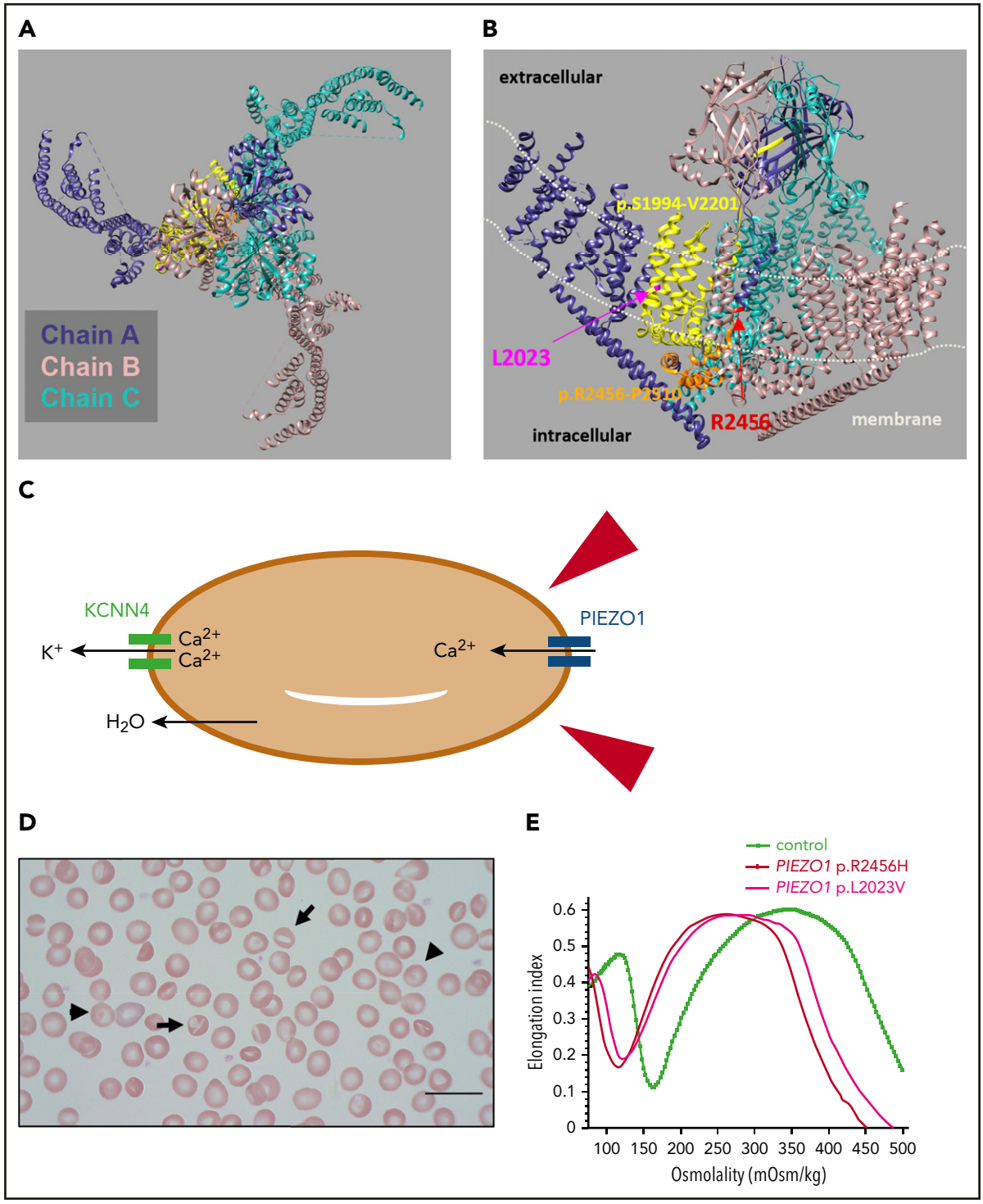

Figure 5. HX due to heterozygous *PIEZO1* mutation. Human PIEZO1 is a 2521 amino acid (287 kDa) protein with ~38 transmembrane helices. Cryoelectron microscopy studies of the highly homologous mouse Piezo1 reveal that PIEZO1 trimers form an elegant 3-bladed propeller structure with a curved transmembrane region creating an inverted membrane dome and a central ion pore formed by the C-terminal domains of the subunits, in which most of the disease-causing mutations are located.[103-106] This unique structure of PIEZO1 senses changes in membrane tension to alter gating of the ion channel.[106,107] (A) Top view of the homotrimeric PIEZO1 channel showing its 3-bladed, propeller-like architecture (from the extracellular space looking down through the central pore). The 3 subunits of the trimer are color coded. (B) Side view of the homotrimeric PIEZO1 channel showing the curved transmembrane region, which creates a membrane dome.[106,107] The position of the membrane is roughly indicated with white dotted lines. Two of the mutation "hot spot" areas described by Picard et al[56] are highlighted in chain A (dark blue); the sequence p.R2456-P2510 in the pore domain (coded within exon 51) is colored orange, and one of the most common HX mutations in this region (p.R2456) is labeled in red; the sequence p.S1994-V2201 (coded within exons 42-45) is colored yellow, and one of the mutations in this area (p.L2023V) is indicated in magenta. A third hot-spot region for mutations is located N-terminally in exons 14 to 18; the structure of this area has not yet been modeled. Patients demonstrating a more severe phenotype are more likely to have mutations in the PIEZO1 pore domain.[56] Images created using UCSF Chimera,[108] with the Protein Data Bank structure model 3JAC. (C) Sketch of interaction between PIEZO1 and KCNN4 as RBCs travel through the vasculature. In narrow capillaries and sinusoids, mechanical stress (represented by the red arrowheads) results in activation of PIEZO1 and Ca^{2+} entry. Increased intracellular Ca^{2+} leads to activation of KCNN4 (a calcium ion binds to each of the 4 calmodulin molecules tightly bound to the cytoplasmic domains of the 4 KCNN4 subunits),[109] and K^+ efflux ensues. Subsequent water loss

hemolysis and chronic transfusion requirement respond to splenectomy,[38,40] but rare cases have persistent severe hemolysis even after splenectomy, especially if there is a concurrent HS component of decreased protein expression. For example, splenectomy failed to improve transfusion dependency in a child with compound heterozygosity for a previously reported splice site *SPTA1* mutation (c.2806-13T>G) known as spectrin St. Claude[41] and a novel nonsense *SPTA1* mutation (c.352C>T; p.R118*) in *trans,* predicted to be associated with severe HPP due to incorporation in the membrane of only the truncated α-spectrin produced by the spectrin St. Claude allele (T.A.K., unpublished observation).

SAO

Southeast Asian ovalocytosis (SAO) is an AD HHA caused by deletion in *SLC4A1* of 27 base pairs encoding amino acids 400 to 408 of band 3 at the junction of the N-terminal cytoplasmic domain with the transmembrane domain, resulting in abnormal folding of the protein.[42,43] SAO confers resistance to cerebral malaria[44] and has been under selective advantage in the Pacific rim of Southeast Asia, with MAF in this population ≤1%. Newborns with SAO frequently present with neonatal hyperbilirubinemia.[45] Hemolysis resolves completely by 3 years of age, and SAO heterozygotes are asymptomatic, despite their large, oval-shaped RBCs, which are markedly rigid.[46] SAO homozygosity is lethal in utero with no intervention. Only 1 homozygous patient has been described presenting with hydrops fetalis at 22 weeks of gestation, salvaged with intrauterine transfusions, and born with transfusion-dependent, hemolytic, and dyserythropoietic anemia and distal renal tubular acidosis.[47]

RBC hydration disorders

Dehydrated hereditary stomatocytosis or HX

Pathophysiology and genetics HX is a clinically heterogeneous, AD HHA caused by mutations in PIEZO1,[48-50] a mechanosensitive nonselective cation channel (Figure 5A-B), or, less commonly, by mutations in the Ca^{2+}-activated K^+ channel KCNN4 (Gardos channel).[51-55] It is characterized by an abnormal RBC K^+ leak not compensated by a proportional intracellular Na^+ gain, leading to cellular dehydration. Although HX due to *PIEZO1* mutations and HX due to mutation in *KCNN4* share many characteristics, including a variable degree of RBC dehydration, hemolysis that persists after splenectomy, and iron overload disproportionate to the transfusion history,[56] there are differences in cellular pathophysiology, clinical presentation, and potential response to therapeutic measures, leading to suggestions to refer to *KCNN4*-associated HX with its own name as "Gardos channelopathy".[54,56] As RBCs navigate narrow capillaries and sinusoids, normal activation of PIEZO1 by pressure and shear stress increases intracellular Ca^{2+}, which activates KCNN4 and causes K^+ efflux and water loss, to attain a temporary decrease in cell volume and facilitate passage (Figure 5C).[57-59] Most of the gain-of-function PIEZO1 mutations causing HX are due to a slower rate of channel inactivation,[60,61] but other mechanisms of PIEZO1 dysfunction, including altered channel kinetics, response to osmotic stress, and membrane trafficking, have also been described and contribute to the phenotypic heterogeneity of the disease.[56,62,63]

Human KCNN4 has 6 transmembrane helices and forms a tetramer in the RBC membrane.[64] KCNN4 mutations affect channel kinetics displaying increased current magnitude, like PIEZO1 gain-of-function mutations. However, the cellular pathology may be more complex with compensatory effects by multiple ion-transport systems in patients with HX due to KCNN4 p.R352H[54] and paradoxically reduced activity of the p.V282M mutant channel under conditions of maximal stimulation.[55] A 28-base-pair deletion, predicted to affect the KCNN4-calmodulin binding domain, has also been described.[56]

HX is often described as a rare condition, but there are several lines of evidence that indicate that it is rather underdiagnosed.[65] Estimates based on reported cases suggest ~1 case of HX in 50 000 births.[66] However, a recent study performed using a large US commercial laboratory database to search for complete blood count results consistent with HX estimated an incidence of ~1 case in 8000 adults.[67] In addition, a recent study described a *PIEZO1* gain-of-function polymorphism (E756del) predicted to be present in ≥1 allele in approximately one-third of the African population and demonstrated that individuals heterozygous for this variant have more dense RBCs that are less susceptible to infection by the malaria parasite *Plasmodium falciparum*.[68] It was recently shown that this variant may also be a disease-modifying polymorphism for individuals with sickle cell disease.[69]

Clinical picture and management The high degree of variability in the HX phenotype probably has many causes, including a large number of causative variants, with variable mechanisms of dysfunction; differences in transporters or other proteins that function in concert with the mutated protein; and coinheritance of other gene variants affecting RBC phenotype. HX patients typically present with mild to moderate hemolytic anemia or fully compensated hemolysis; a few cases with polycythemia (hemoglobin >16 g/dL) despite hemolysis have been reported.[56,70] Many patients may not come to medical attention until adulthood. Patients with HX due to *PIEZO1* variants typically have high reticulocyte count, high or high-normal MCHC, high MCH, and high MCV.[71] Peripheral blood smears reveal occasional stomatocytes, target cells, and possibly dense cells (Figure 5B). Splenomegaly is common. Some patients have history of transient perinatal edema or nonimmune hydrops fetalis. Total bilirubin is frequently increased, and many patients develop gallstones. Ferritin levels are typically elevated, and iron overload is disproportionate to transfusion history. Some individuals present with a primary clinical sign of hepatic and/or myocardial iron overload.[63] Osmotic fragility is frequently, but not always, decreased, limiting its value as a diagnostic test in these cases. Osmotic gradient ektacytometry typically shows a leftward shift of the curve, indicating dehydration (Figure 5D). RBC intracellular cation determinations reveal reduced K^+ content without corresponding increase in Na^+ content.[70]

In the largest study of HX to date, individuals with KCNN4-HX had more significant anemia, with lower reticulocyte count, and

Figure 5 (continued) results in a temporary decrease in cell volume and facilitates passage.[57-59] (D) Blood smear from a patient heterozygous for p.R2456H with macrocytosis (MCV 96 fL) showing occasional stomatocytes (arrows), target cells (arrowheads), and dense cells (thin arrows). (E) Osmotic gradient ektacytometry showing the typical HX curve with left shift due to decreased O_{min} and $O_{hyp,}$ indicating RBC dehydration. Ekatacytometry profiles are shown for 2 patients with HX due to *PIEZO1* p.R2456H and p.L2023V.

higher ferritin values consistent with worse iron overload in comparison with PIEZO1-HX.[56] Ektacytometry curve was dramatically left-shifted with reduced EI_{max} and O_{hyp} values consistent with severe cell dehydration in HX due to *KCNN4* p.V282M[55] but was near normal, with no clear evidence of cellular dehydration in HX due to *KCNN4* p.R352H, likely because of compensatory effects including K^+ influx through KCC, NKCC and Na/K ATPase channels.[54,56,72]

Gene sequencing with appropriately designed genetic panels is essential for an accurate diagnosis. Several patients with HX have received the diagnosis of HS or congenital dyserythropoietic anemia prior to genetic evaluation. Dysplastic and binucleated erythroblasts can be seen with stress erythropoiesis associated with brisk hemolytic anemia, while there may also be direct effects of abnormal PIEZO1 activation in erythropoiesis, as it was recently shown to delay erythroid maturation[73] and impair reticulocyte maturation[74] in vitro.

Management is supportive for anemia and complications of hemolysis with vigilant monitoring for iron overload. Ferritin and transferrin saturation should be monitored, and T2* magnetic resonance imaging of the liver and heart is indicated when iron overload is suspected. Poor correlation between ferritin level and mean liver iron content was shown for individuals with a ferritin level <1000 ng/mL, suggesting the value of evaluating iron content by imaging in patients with even moderate ferritin increases at diagnosis.[56] Phlebotomy or iron chelation are being used to address HX-associated iron overload, which can lead to cirrhosis and/or heart failure if left untreated.[56] A comparative efficacy study would be valuable, since the driver for hemochromatosis in HX may be the excessive erythropoiesis causing increased erythroferrone that suppresses hepcidin.[75] Therefore, the efficacy of phlebotomy to treat iron overload in HX, while it may further stimulate erythropoiesis, needs evaluation.[76] Splenectomy is strictly contraindicated in *PIEZO1*-associated HX. It is not of therapeutic benefit in reducing hemolysis, which is largely intravascular in HX, and results in a greatly increased risk of life-threatening venous and arterial thromboembolic complications,[77,78] which have been shown to happen within 1 month up to 28 years after splenectomy.[56] Interestingly, thrombotic complications were not observed in a total of 12 *KCNN4*-HX splenectomized patients after a follow-up time of 2 to 44 years.[54,56] These patients had well-compensated cellular dehydration based on MCHC and/or ektacytometry studies and also maintained active volume regulation with an overactivated Gardos channel; nevertheless, they continued to have significant hemolysis, even after splenectomy.[54,56] Because perinatal edema and nonimmune hydrops fetalis are common in HX, pregnancies should be closely monitored when either parent has HX.[56]

Specific drugs for the treatment of HX are not currently available. However, because KCNN4 has been demonstrated to be the major mediator of RBC dehydration in HX due to mutated PIEZO1,[79] a specific KCNN4 inhibitor, senicapoc, has been suggested as a candidate for treatment of HX due to either PIEZO1 or KCNN4 mutation. In a phase 3 clinical trial for the use of senicapoc for sickle cell disease, it reduced RBC dehydration and hemolysis, increased hemoglobin levels, and was well tolerated.[80] It has also been shown to inhibit both wild-type and mutated KCNN4 channels and reduce RBC dehydration in vitro.[81] Although small-molecule agonists have been useful in the study of PIEZO1 function, specific small-molecule inhibitors for PIEZO1 have not yet been identified, and it will likely be challenging to achieve the desired therapeutic improvement in RBC hydration by inhibiting PIEZO1 without affecting the wide range of its functions in various other cell types.[82]

Overhydration syndromes

Pathophysiology and genetics RBC overhydration syndromes are characterized by a large net increase in RBC intracellular Na^+ and water not adequately compensated by a decrease in intracellular K^+, leading to an increase in RBC volume without corresponding increase in membrane surface area.[83,84]

Overhydrated hereditary stomatocytosis (OHSt), caused by heterozygous missense mutations in *RHAG*, is a rare AD HHA. *RHAG* codes for Rhesus-associated glycoprotein (RhAG), which functions as an ammonium transporter,[85] and is a component of the band 3 macromolecular complex (Figure 1B). OHSt-associated mutant RhAG proteins expressed in *Xenopus* oocytes induce monovalent cation leak several-fold greater than the wild-type RhAG, probably by altering a cytoplasmic constriction in the pore of the protein.[86]

Cryohydrocytosis (CHC) is caused by certain heterozygous missense mutations in *SLC4A1* causing minimally increased RBC cation leak at physiologic temperature but a major leak as temperature approaches 5°C.[84] The CHC mutations are located at the band 3 transmembrane segment involved in anion exchange, altering it to mediate cation leak.[87] This leak can be inhibited by specific band 3 inhibitors in vitro.[88] In some cases, part of the observed cation leak may be mediated through band 3 regulation of other transporters.[89] Individuals with *SLC4A1* mutations generally have a milder form of OHSt than individuals with RhAG mutations.[65]

Another rare form of CHC, referred to as stomatin-deficient CHC, is caused by mutations in *SLC2A1* coding for GLUT1, the glucose transporter that also binds stomatin.[90-92] In stomatin-deficient CHC, there is a deficiency in GLUT1 and stomatin and a large RBC cation leak that increases in the cold. In addition to moderate hemolytic anemia, individuals with these mutations have cataracts due to the altered cation permeability and neurological disorder (seizures, mental retardation, dyskinesias) related to inadequate glucose transport into neurons.

Other band 3 disorders that result in some degree of RBC overhydration include SAO and band 3 Ceinge. SAO RBCs were described above with the cytoskeleton disorders but have also been shown to have a cation leak in the cold similar to that observed in CHC mutations.[46] Band 3 Ceinge (*SLC4A1* p.Gly796Arg) is an unusual, dominantly inherited variant associated with mild hemolytic anemia with dyserythropoiesis.[93] The mutation is located in the same membrane-spanning domain as many CHC mutants and has a similar cation leak at low temperature. Since membrane cation transport pathways are regulated by phosphorylation-dephosphorylation events, the RBC membrane tyrosine phosphorylation profile was evaluated, revealing membrane association of the Syk and Lyn tyrosine kinases and tyrosine phosphorylation of band 3 and stomatin.[93]

Clinical picture and management Patients with overhydration syndromes may have a mild to severe hemolytic anemia. Stomatocytes are usually abundant on peripheral blood smears along with occasional spherocytes. MCV is typically increased and MCHC decreased. Osmotic fragility is increased, and the osmotic gradient ektacytometry curve is shifted to the right. Intracellular cation determinations reveal greatly increased RBC Na^+ content and reduced K^+ content.

Management is supportive for anemia and complications of hemolysis. Splenectomy is partially effective in reducing hemolysis, but it carries a high risk of thromboembolic complications and pulmonary hypertension.[77,78]

Conclusions

The broader availability of next-generation sequencing over the last decade has vastly facilitated accurate diagnosis in patients with HHA and has allowed genotype-phenotype correlations and natural history studies in patient cohorts with rare but well-defined pathogenesis. These studies have revealed complications such as iron overload in HX, amenable to monitoring, treatment, and prevention, and made clear that the one-size-fits-all approach of splenectomy for any patient with jaundice is not always effective or safe. Careful phenotypic assessment in clinical or research laboratories is necessary to assist in the interpretation of variants of unknown clinical significance revealed by genetic evaluation. It is exciting to see the emerging possibility of targeted treatments to manipulate transporter activity[94] or structural organization of cytoskeletal proteins via phosphorylation[95] that have the potential to offer safer therapeutic options to improve morbidity and mortality.

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ORCID profiles: M.R., 0000-0002-5917-2267; T.A.K., 0000-0002-0426-9686.

Correspondence: Theodosia A. Kalfa, Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center, 3333 Burnet Ave, MLC 7015, Cincinnati, OH 45229-3039; e-mail: theodosia.kalfa@cchmc.org.

Footnote

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REFERENCES

1. Lux SE, Palek J. Disorders of the red cell membrane. In: Handin RI, Stossel TP, eds. Blood: Principles and Practice of Hematology. Lippincott: Philadelphia, PA; 1995.1701-1818.
2. Mohandas N, Gallagher PG. Red cell membrane: past, present, and future. *Blood*. 2008;112(10):3939-3948.
3. Salomao M, Zhang X, Yang Y, et al. Protein 4.1R-dependent multiprotein complex: new insights into the structural organization of the red blood cell membrane. *Proc Natl Acad Sci USA*. 2008;105(23):8026-8031.
4. Mohandas N, Evans E. Mechanical properties of the red cell membrane in relation to molecular structure and genetic defects. *Annu Rev Biophys Biomol Struct*. 1994;23(1):787-818.
5. Sung LA, Vera C. Protofilament and hexagon: a three-dimensional mechanical model for the junctional complex in the erythrocyte membrane skeleton. *Ann Biomed Eng*. 2003;31(11):1314-1326.
6. Kalfa TA, Pushkaran S, Mohandas N, et al. Rac GTPases regulate the morphology and deformability of the erythrocyte cytoskeleton. *Blood*. 2006;108(12):3637-3645.
7. Gokhin DS, Fowler VM. Feisty filaments: actin dynamics in the red blood cell membrane skeleton. *Curr Opin Hematol*. 2016;23(3):206-214.
8. Burton NM, Bruce LJ. Modelling the structure of the red cell membrane. *Biochem Cell Biol*. 2011;89(2):200-215.
9. Bryk AH, Wiśniewski JR. Quantitative analysis of human red blood cell proteome. *J Proteome Res*. 2017;16(8):2752-2761.
10. Gautier EF, Leduc M, Cochet S, et al. Absolute proteome quantification of highly purified populations of circulating reticulocytes and mature erythrocytes. *Blood Adv*. 2018;2(20):2646-2657.
11. Lux SE IV. Anatomy of the red cell membrane skeleton: unanswered questions. *Blood*. 2016;127(2):187-199.
12. Mohandas N. Inherited hemolytic anemia: a possessive beginner's guide. *Hematology Am Soc Hematol Educ Program*. 2018;2018:377-381.
13. Eber S, Lux SE. Hereditary spherocytosis—defects in proteins that connect the membrane skeleton to the lipid bilayer. *Semin Hematol*. 2004;41(2):118-141.
14. Gallagher PG. Abnormalities of the erythrocyte membrane. *Pediatr Clin North Am*. 2013;60(6):1349-1362.
15. Eber SW, Gonzalez JM, Lux ML, et al. Ankyrin-1 mutations are a major cause of dominant and recessive hereditary spherocytosis. *Nat Genet*. 1996;13(2):214-218.
16. Gallagher PG. Red cell membrane disorders. *Hematology Am Soc Hematol Educ Program*. 2005;2005:13-18.
17. Iolascon A, Miraglia del Giudice E, Perrotta S, Alloisio N, Morlé L, Delaunay J. Hereditary spherocytosis: from clinical to molecular defects. *Haematologica*. 1998;83(3):240-257.
18. Hammill AM, Risinger MA, Joiner CH, Keddache M, Kalfa TA. Compound heterozygosity for two novel mutations in the erythrocyte protein 4.2 gene causing spherocytosis in a Caucasian patient. *Br J Haematol*. 2011;152(6):780-783.
19. Kalfa TA, Connor JA, Begtrup AH. EPB42-related hereditary spherocytosis. In: Adam MP, Ardinger HH, Pagon RA, eds., et al. GeneReviews, Seattle, WA: University of Washington; 2016:
20. Wichterle H, Hanspal M, Palek J, Jarolim P. Combination of two mutant alpha spectrin alleles underlies a severe spherocytic hemolytic anemia. *J Clin Invest*. 1996;98(10):2300-2307.
21. Gallagher PG, Maksimova Y, Lezon-Geyda K, et al. Aberrant splicing contributes to severe α-spectrin-linked congenital hemolytic anemia. *J Clin Invest*. 2019;129(7):2878-2887.

22. Chonat S, Risinger M, Sakthivel H, et al. The spectrum of *SPTA1*-associated hereditary spherocytosis [published correction appears in *Front Physiol*. 2019;10:1331]. *Front Physiol*. 2019;10:815.

23. Becker PS, Tse WT, Lux SE, Forget BG. Beta spectrin kissimmee: a spectrin variant associated with autosomal dominant hereditary spherocytosis and defective binding to protein 4.1. *J Clin Invest*. 1993;92(2):612-616.

24. Miraglia del Giudice E, Nobili B, Francese M, et al. Clinical and molecular evaluation of non-dominant hereditary spherocytosis. *Br J Haematol*. 2001;112(1):42-47.

25. Ribeiro ML, Alloisio N, Almeida H, et al. Severe hereditary spherocytosis and distal renal tubular acidosis associated with the total absence of band 3. *Blood*. 2000;96(4):1602-1604.

26. Kager L, Bruce LJ, Zeitlhofer P, et al. Band 3 null^{VIENNA}, a novel homozygous SLC4A1 p.Ser477X variant causing severe hemolytic anemia, dyserythropoiesis and complete distal renal tubular acidosis. *Pediatr Blood Cancer*. 2017;64(3):e26227.

27. Christensen RD, Yaish HM, Gallagher PG. A pediatrician's practical guide to diagnosing and treating hereditary spherocytosis in neonates. *Pediatrics*. 2015;135(6):1107-1114.

28. Delhommeau F, Cynober T, Schischmanoff PO, et al. Natural history of hereditary spherocytosis during the first year of life. *Blood*. 2000;95(2):393-397.

29. Tchernia G, Delhommeau F, Perrotta S, et al; ESPHI working group on hemolytic anemias. Recombinant erythropoietin therapy as an alternative to blood transfusions in infants with hereditary spherocytosis. *Hematol J*. 2000;1(3):146-152.

30. del Giudice EM, Perrotta S, Nobili B, Specchia C, d'Urzo G, Iolascon A. Coinheritance of Gilbert syndrome increases the risk for developing gallstones in patients with hereditary spherocytosis. *Blood*. 1999;94(7):2259-2262.

31. Palek J, Sahr KE. Mutations of the red blood cell membrane proteins: from clinical evaluation to detection of the underlying genetic defect. *Blood*. 1992;80(2):308-330.

32. Englum BR, Rothman J, Leonard S, et al; Splenectomy in Congenital Hemolytic Anemia Consortium. Hematologic outcomes after total splenectomy and partial splenectomy for congenital hemolytic anemia. *J Pediatr Surg*. 2016;51(1):122-127.

33. Rice HE, Englum BR, Rothman J, et al; Splenectomy in Congenital Hemolytic Anemia (SICHA) Consortium. Clinical outcomes of splenectomy in children: report of the splenectomy in congenital hemolytic anemia registry. *Am J Hematol*. 2015;90(3):187-192.

34. Pincez T, Guitton C, Gauthier F, et al. Long-term follow-up of subtotal splenectomy for hereditary spherocytosis: a single-center study. *Blood*. 2016;127(12):1616-1618.

35. Harper SL, Sriswasdi S, Tang HY, Gaetani M, Gallagher PG, Speicher DW. The common hereditary elliptocytosis-associated α-spectrin L260P mutation perturbs erythrocyte membranes by stabilizing spectrin in the closed dimer conformation. *Blood*. 2013;122(17):3045-3053.

36. Gallagher PG. Hereditary elliptocytosis: spectrin and protein 4.1R. *Semin Hematol*. 2004;41(2):142-164.

37. Reid ME, Mohandas N. Red blood cell blood group antigens: structure and function. *Semin Hematol*. 2004;41(2):93-117.

38. Niss O, Chonat S, Dagaonkar N, et al. Genotype-phenotype correlations in hereditary elliptocytosis and hereditary pyropoikilocytosis. *Blood Cells Mol Dis*. 2016;61:4-9.

39. Maillet P, Alloisio N, Morlé L, Delaunay J. Spectrin mutations in hereditary elliptocytosis and hereditary spherocytosis. *Hum Mutat*. 1996;8(2):97-107.

40. Lacy JN, Ulirsch JC, Grace RF, et al. Exome sequencing results in successful diagnosis and treatment of a severe congenital anemia. *Cold Spring Harb Mol Case Stud*. 2016;2(4):a000885.

41. Fournier CM, Nicolas G, Gallagher PG, Dhermy D, Grandchamp B, Lecomte MC. Spectrin St Claude, a splicing mutation of the human alpha-spectrin gene associated with severe poikilocytic anemia. *Blood*. 1997;89(12):4584-4590.

42. Kuma H, Abe Y, Askin D, et al. Molecular basis and functional consequences of the dominant effects of the mutant band 3 on the structure of normal band 3 in Southeast Asian ovalocytosis. *Biochemistry*. 2002;41(10):3311-3320.

43. Tanner MJ, Bruce L, Martin PG, Rearden DM, Jones GL. Melanesian hereditary ovalocytes have a deletion in red cell band 3. *Blood*. 1991;78(10):2785-2786.

44. Genton B, al-Yaman F, Mgone CS, et al. Ovalocytosis and cerebral malaria. *Nature*. 1995;378(6557):564-565.

45. Laosombat V, Viprakasit V, Dissaneevate S, et al. Natural history of Southeast Asian Ovalocytosis during the first 3 years of life. *Blood Cells Mol Dis*. 2010;45(1):29-32.

46. Bruce LJ, Ring SM, Ridgwell K, et al. Southeast Asian ovalocytic (SAO) erythrocytes have a cold sensitive cation leak: implications for in vitro studies on stored SAO red cells. *Biochim Biophys Acta*. 1999;1416(1-2):258-270.

47. Picard V, Proust A, Eveillard M, et al. Homozygous Southeast Asian ovalocytosis is a severe dyserythropoietic anemia associated with distal renal tubular acidosis. *Blood*. 2014;123(12):1963-1965.

48. Zarychanski R, Schulz VP, Houston BL, et al. Mutations in the mechanotransduction protein PIEZO1 are associated with hereditary xerocytosis. *Blood*. 2012;120(9):1908-1915.

49. Andolfo I, Alper SL, De Franceschi L, et al. Multiple clinical forms of dehydrated hereditary stomatocytosis arise from mutations in PIEZO1. *Blood*. 2013;121(19):3925-3935.

50. Albuisson J, Murthy SE, Bandell M, et al. Dehydrated hereditary stomatocytosis linked to gain-of-function mutations in mechanically activated PIEZO1 ion channels. *Nat Commun*. 2013;4(1):1884.

51. Glogowska E, Lezon-Geyda K, Maksimova Y, Schulz VP, Gallagher PG. Mutations in the Gardos channel (KCNN4) are associated with hereditary xerocytosis. *Blood*. 2015;126(11):1281-1284.

52. Rapetti-Mauss R, Lacoste C, Picard V, et al. A mutation in the Gardos channel is associated with hereditary xerocytosis. *Blood*. 2015;126(11):1273-1280.

53. Andolfo I, Russo R, Manna F, et al. Novel Gardos channel mutations linked to dehydrated hereditary stomatocytosis (xerocytosis). *Am J Hematol*. 2015;90(10):921-926.

54. Fermo E, Bogdanova A, Petkova-Kirova P, et al. "Gardos channelopathy": a variant of hereditary stomatocytosis with complex molecular regulation. *Sci Rep*. 2017;7(1):1744.

55. Rivera A, Vandorpe DH, Shmukler BE, et al. Erythrocyte ion content and dehydration modulate maximal Gardos channel activity in KCNN4 V282M/+ hereditary xerocytosis red cells. *Am J Physiol Cell Physiol*. 2019;317(2):C287-C302.

56. Picard V, Guitton C, Thuret I, et al. Clinical and biological features in *PIEZO1*-hereditary xerocytosis and Gardos channelopathy: a retrospective series of 126 patients. *Haematologica*. 2019;104(8):1554-1564.

57. Danielczok JG, Terriac E, Hertz L, et al. Red blood cell passage of small capillaries is associated with transient Ca^{2+}-mediated adaptations. *Front Physiol*. 2017;8:979.

58. Dyrda A, Cytlak U, Ciuraszkiewicz A, et al. Local membrane deformations activate Ca2+-dependent K+ and anionic currents in intact human red blood cells. *PLoS One*. 2010;5(2):e9447.

59. Cahalan SM, Lukacs V, Ranade SS, Chien S, Bandell M, Patapoutian A. Piezo1 links mechanical forces to red blood cell volume. *eLife*. 2015;4:e07370.

60. Bagriantsev SN, Gracheva EO, Gallagher PG. Piezo proteins: regulators of mechanosensation and other cellular processes. *J Biol Chem*. 2014;289(46):31673-31681.

61. Bae C, Gnanasambandam R, Nicolai C, Sachs F, Gottlieb PA. Xerocytosis is caused by mutations that alter the kinetics of the mechanosensitive channel PIEZO1. *Proc Natl Acad Sci USA*. 2013;110(12):E1162-E1168.

62. Glogowska E, Schneider ER, Maksimova Y, et al. Novel mechanisms of PIEZO1 dysfunction in hereditary xerocytosis. *Blood*. 2017;130(16):1845-1856.

63. Andolfo I, Russo R, Rosato BE, et al. Genotype-phenotype correlation and risk stratification in a cohort of 123 hereditary stomatocytosis patients. *Am J Hematol*. 2018;93(12):1509-1517.

64. Maher AD, Kuchel PW. The Gárdos channel: a review of the Ca2+-activated K+ channel in human erythrocytes. *Int J Biochem Cell Biol*. 2003;35(8):1182-1197.

65. Andolfo I, Russo R, Gambale A, Iolascon A. Hereditary stomatocytosis: an underdiagnosed condition. *Am J Hematol*. 2018;93(1):107-121.

66. Andolfo I, Russo R, Gambale A, Iolascon A. New insights on hereditary erythrocyte membrane defects. *Haematologica*. 2016; 101(11):1284-1294.

67. Kaufman HW, Niles JK, Gallagher DR, et al. Revised prevalence estimate of possible Hereditary Xerocytosis as derived from a large U.S. Laboratory database. *Am J Hematol*. 2018;93(1):E9-E12.

68. Ma S, Cahalan S, LaMonte G, et al. Common PIEZO1 allele in African populations causes RBC dehydration and attenuates plasmodium infection. *Cell*. 2018;173(2): 443-455.e12.

69. Ilboudo Y, Bartolucci P, Garrett ME, et al. A common functional PIEZO1 deletion allele associates with red blood cell density in sickle cell disease patients. *Am J Hematol*. 2018;93(11):E362-E365.

70. Risinger M, Glogowska E, Chonat S, et al. Hereditary xerocytosis: diagnostic considerations. *Am J Hematol*. 2018;93(3):E67-E69.

71. Risinger M, Black V, Hsieh L, et al. Evaluation of phenotype-genotype correlation in two common PIEZO1 mutations p.R2456H and p.L2495_E2495dup [abstract]. *Blood*. 2018; 132(suppl 1). Abstract 1040.

72. Zaninoni A, Fermo E, Vercellati C, et al. Use of laser assisted optical rotational cell analyzer (LoRRca MaxSis) in the diagnosis of RBC membrane disorders, enzyme defects, and congenital dyserythropoietic anemias: a monocentric study on 202 patients. *Front Physiol*. 2018;9:451.

73. Caulier A, Jankovsky N, Demont Y, et al. PIEZO1 activation delays erythroid differentiation of normal and hereditary xerocytosis-derived human progenitors. *Haematologica*. 2020;105(3):610-622.

74. Moura PL, Hawley BR, Dobbe JGG, et al. PIEZO1 gain-of-function mutations delay reticulocyte maturation in hereditary xerocytosis. *Haematologica*. 2020;105(3):e268-e271.

75. Archer NM, Shmukler BE, Andolfo I, et al. Hereditary xerocytosis revisited. *Am J Hematol*. 2014;89(12):1142-1146.

76. Kim A, Nemeth E. New insights into iron regulation and erythropoiesis. *Curr Opin Hematol*. 2015;22(3):199-205.

77. Stewart GW, Amess JA, Eber SW, et al. Thrombo-embolic disease after splenectomy for hereditary stomatocytosis. *Br J Haematol*. 1996;93(2):303-310.

78. Iolascon A, Andolfo I, Barcellini W, et al; Working Study Group on Red Cells and Iron of the EHA. Recommendations regarding splenectomy in hereditary hemolytic anemias. *Haematologica*. 2017;102(8): 1304-1313.

79. Rapetti-Mauss R, Picard V, Guitton C, et al. Red blood cell Gardos channel (KCNN4): the essential determinant of erythrocyte dehydration in hereditary xerocytosis. *Haematologica*. 2017;102(10):e415-e418.

80. Ataga KI, Reid M, Ballas SK, et al; ICA-17043-10 Study Investigators. Improvements in haemolysis and indicators of erythrocyte survival do not correlate with acute vaso-occlusive crises in patients with sickle cell disease: a phase III randomized, placebo-controlled, double-blind study of the Gardos channel blocker senicapoc (ICA-17043). *Br J Haematol*. 2011;153(1):92-104.

81. Rapetti-Mauss R, Soriani O, Vinti H, Badens C, Guizouarn H. Senicapoc: a potent candidate for the treatment of a subset of hereditary xerocytosis caused by mutations in the Gardos channel. *Haematologica*. 2016; 101(11):e431-e435.

82. Xiao B. Levering mechanically activated piezo channels for potential pharmacological intervention. *Annu Rev Pharmacol Toxicol*. 2020;60:195-218.

83. Bruce LJ. Hereditary stomatocytosis and cation-leaky red cells—recent developments. *Blood Cells Mol Dis*. 2009;42(3): 216-222.

84. Gallagher PG. Disorders of erythrocyte hydration. *Blood*. 2017;130(25):2699-2708.

85. Westhoff CM, Ferreri-Jacobia M, Mak DO, Foskett JK. Identification of the erythrocyte Rh blood group glycoprotein as a mammalian ammonium transporter. *J Biol Chem*. 2002;277(15):12499-12502.

86. Bruce LJ, Guizouarn H, Burton NM, et al. The monovalent cation leak in overhydrated stomatocytic red blood cells results from amino acid substitutions in the Rh-associated glycoprotein. *Blood*. 2009;113(6): 1350-1357.

87. Barneaud-Rocca D, Borgese F, Guizouarn H. Dual transport properties of anion exchanger 1: the same transmembrane segment is involved in anion exchange and in a cation leak. *J Biol Chem*. 2011;286(11):8909-8916.

88. Bruce LJ, Robinson HC, Guizouarn H, et al. Monovalent cation leaks in human red cells caused by single amino-acid substitutions in the transport domain of the band 3 chloride-bicarbonate exchanger, AE1. *Nat Genet*. 2005;37(11):1258-1263.

89. Barneaud-Rocca D, Pellissier B, Borgese F, Guizouarn H. Band 3 missense mutations and stomatocytosis: insight into the molecular mechanism responsible for monovalent cation leak. *Int J Cell Biol*. 2011;2011:136802.

90. Weber YG, Storch A, Wuttke TV, et al. GLUT1 mutations are a cause of paroxysmal exertion-induced dyskinesias and induce hemolytic anemia by a cation leak. *J Clin Invest*. 2008;118(6):2157-2168.

91. Flatt JF, Guizouarn H, Burton NM, et al. Stomatin-deficient cryohydrocytosis results from mutations in SLC2A1: a novel form of GLUT1 deficiency syndrome. *Blood*. 2011; 118(19):5267-5277.

92. Bawazir WM, Gevers EF, Flatt JF, et al. An infant with pseudohyperkalemia, hemolysis, and seizures: cation-leaky GLUT1-deficiency syndrome due to a SLC2A1 mutation. *J Clin Endocrinol Metab*. 2012;97(6):E987-E993.

93. Iolascon A, De Falco L, Borgese F, et al. A novel erythroid anion exchange variant (Gly796Arg) of hereditary stomatocytosis associated with dyserythropoiesis. *Haematologica*. 2009;94(8):1049-1059.

94. Rivera A, Vandorpe DH, Shmukler BE, et al. Erythrocytes from hereditary xerocytosis patients heterozygous for KCNN4 V282M exhibit increased spontaneous Gardos channel-like activity inhibited by senicapoc. *Am J Hematol*. 2017;92(6):E108-E110.

95. Kesely KR, Pantaleo A, Turrini FM, Olupot-Olupot P, Low PS. Inhibition of an erythrocyte tyrosine kinase with imatinib prevents plasmodium falciparum egress and terminates parasitemia. *PLoS One*. 2016;11(10):e0164895.

96. King MJ, Smythe JS, Mushens R. Eosin-5-maleimide binding to band 3 and Rh-related proteins forms the basis of a screening test for hereditary spherocytosis. *Br J Haematol*. 2004;124(1):106-113.

97. King MJ, Jepson MA, Guest A, Mushens R. Detection of hereditary pyropoikilocytosis by the eosin-5-maleimide (EMA)-binding test is attributable to a marked reduction in EMA-reactive transmembrane proteins. *Int J Lab Hematol*. 2011;33(2):205-211.

98. Bessis M, Mohandas N, Feo C. Automated ektacytometry: a new method of measuring red cell deformability and red cell indices. *Blood Cells*. 1980;6(3):315-327.

99. Mohandas N, Clark MR, Jacobs MS, Shohet SB. Analysis of factors regulating erythrocyte deformability. *J Clin Invest*. 1980;66(3): 563-573.

100. Da Costa L, Suner L, Galimand J, et al; French Society of Hematology (SFH). Diagnostic tool for red blood cell membrane disorders: assessment of a new generation ektacytometer. *Blood Cells Mol Dis*. 2016;56(1):9-22.

101. Rets A, Clayton AL, Christensen RD, Agarwal AM. Molecular diagnostic update in hereditary hemolytic anemia and neonatal hyperbilirubinemia. *Int J Lab Hematol*. 2019;41(S1 Suppl 1):95-101.

102. King MJ, Garçon L, Hoyer JD, et al; International Council for Standardization in Haematology. ICSH guidelines for the laboratory diagnosis of nonimmune hereditary red cell membrane disorders. *Int J Lab Hematol*. 2015;37(3):304-325.

103. Saotome K, Murthy SE, Kefauver JM, Whitwam T, Patapoutian A, Ward AB. Structure of the mechanically activated ion channel Piezo1. *Nature*. 2018;554(7693):481-486.

104. Zhao Q, Zhou H, Li X, Xiao B. The mechanosensitive Piezo1 channel: a three-bladed propeller-like structure and a lever-like mechanogating mechanism. *FEBS J*. 2019; 286(13):2461-2470.

105. Zhao Q, Zhou H, Chi S, et al. Structure and mechanogating mechanism of the Piezo1 channel. *Nature*. 2018;554(7693):487-492.

106. Guo YR, MacKinnon R. Structure-based membrane dome mechanism for Piezo mechanosensitivity. *eLife*. 2017;6:e33660.

107. Haselwandter CA, MacKinnon R. Piezo's membrane footprint and its contribution to mechanosensitivity. *eLife*. 2018;7:e41968.

108. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera–a visualization system for exploratory research and analysis. *J Comput Chem*. 2004;25(13):1605-1612.

109. Fanger CM, Ghanshani S, Logsdon NJ, et al. Calmodulin mediates calcium-dependent activation of the intermediate conductance KCa channel, IKCa1. *J Biol Chem*. 1999; 274(9):5746-5754.