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New insights on hereditary erythrocyte membrane defects

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ABSTRACT

After the first proposed model of the red blood cell membrane skeleton 36 years ago, several additional proteins have been discovered during the intervening years, and their relationship with the pathogenesis of the related disorders have been somewhat defined. The knowledge of erythrocyte membrane structure is important because it represents the model for spectrin-based membrane skeletons in all cells and because defects in its structure underlie multiple hemolytic anemias. This review summarizes the main features of erythrocyte membrane disorders, dividing them into structural and altered permeability defects, focusing particularly on the most recent advances. New proteins involved in alterations of the red blood cell membrane permeability were recently described. The mechanoreceptor PIEZO1 is the largest ion channel identified to date, the fundamental regulator of erythrocyte volume homeostasis. Missense, gain-of-function mutations in the *PIEZO1* gene have been identified in several families as causative of dehydrated hereditary stomatocytosis or xerocytosis. Similarly, the *KCNN4* gene, codifying the so called Gardos channel, has been recently identified as a second causative gene of hereditary xerocytosis. Finally, *ABCB6* missense mutations were identified in different pedigrees of familial pseudohyperkalemia. New genomic technologies have improved the quality and reduced the time of diagnosis of these diseases. Moreover, they are essential for the identification of the new causative genes. However, many questions remain to solve, and are currently objects of intensive studies.

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Introduction

Red blood cell (RBC) membrane disorders are inherited conditions due to mutations in genes encoding for membrane or cytoskeletal proteins as well as for transmembrane transporters or channels, resulting in decreased red cell deformability and permeability, a reduced half-life and premature removal of the erythrocytes from the bloodstream. Extensive studies on the RBC membrane have allowed the comprehension of both structure and function of this subcellular compartment. Thus, the molecular bases of the overwhelming majority of cases of hemolytic anemia due to RBC membrane defects have been currently defined. They are counted as a subtype of hereditary hemolytic anemias that embrace a highly heterogeneous group of chronic disorders with a highly variable clinical picture.

In this review we summarize the biological, clinical and molecular aspects of red cell membrane defects, allowing for a better basis for diagnosis and treatment.

Red blood cell membrane: genesis, structure and function

During its long life span of 120 days, the RBC is forced to cross the pores of splenic sinusoids thousands of times. This cell has an ongoing relationship with the spleen that contributes to remodeling during the first week of its life, participating in the passage from reticulocyte to erythrocyte. Moreover the spleen plays a pri-

primary role in the removal of aged RBCs. In order to perform these journeys RBCs must possess and maintain a significant deformability. The main author of this property is certainly the membrane, that ensures both mechanical stability and deformability. After the first proposed model of the RBC membrane skeleton 36 years ago,¹ containing the core elements of the modern model, many additional proteins have been discovered during the intervening decades, and their structures and interactions have been defined. RBC membrane structure has been extensively covered by excellent reviews.^{2,3} Herein we summarize the main concepts. RBC membrane is composed by a fluid double layer of lipids in which approximately 20 major proteins and at least 850 minor ones are embedded.⁴ The membrane is attached to an intracellular cytoskeleton by protein-protein and lipid-protein interactions that confer the erythrocyte shape, stability and deformability. The transmembrane proteins have mainly a transporter function. However, several of these also have a structural function, usually performed by an intracytoplasmic domain interacting with cytoskeletal proteins.

The lipid bilayer acts as a barrier for the retention of cations and anions within the red cells, while it allows water molecules to pass through freely. Human erythrocytes have high intracellular K^+ and low intracellular Na^+ contents when compared with the corresponding ion concentrations in the plasma. The maintenance of this cation gradient between the cell and its environment involves a passive outward movement of K^+ , which is pumped back by the action of an ATP-dependent Na^+/K^+ pump in exchange for Na^+ ions. This protein belongs to a class of transmembrane proteins with a transport function (Figure 1).

The third and more important component of the RBC membrane is the cytoskeleton, a protein network that

laminates the inner surface of the membrane. Spectrin α - and β -chains, proteins 4.1, or 4.1R, and actin are the main components of this skeleton, maintaining the biconcave shape of the RBC. These components are connected to each other in two protein complexes; ankyrin and protein 4.1 complex. The former is composed by band 3 tetramers, Rh, RhAG, CD47, glycoprotein A and protein 4.2. Whereas the protein 4.1 complex is composed by band 3 dimers binding adducins α - and β -, glycoprotein C, GLUT1 and stomatin (Figure 1). The ends of spectrin tetramers converge toward a protein 4.1 complex (junctional complex). Electron microscopy (EM) shows that this latter links the tail of six spectrin tetramers, forming a pseudo-hexagonal arrangement.⁵ Spectrin tetramers include anion transporters (band 3 or chloride/bicarbonate exchange). The capability of these transporters to form aggregates could define the half-life of RBCs, causing antibody binding and removal by the spleen. Defects that interrupt this vertical structure (spectrin-actin interaction) underlie the biochemical and molecular basis of hereditary spherocytosis (HS), whereas defects in horizontal interactions (skeletal attachment to membrane proteins) cause hereditary elliptocytosis (HE).

Membrane protein synthesis is an important part of the differentiation process of erythroid cells in bone marrow and it starts very early. Cell culture studies established that this production is asynchronous (spectrin production starts before the synthesis of other cytoskeletal components) and is quantitatively exuberant (the production of α -spectrin exceeds that of β -spectrin three or four times).⁶ This pattern of production seems to play an important role in the genetics of both HS and HE: as a matter of fact only homozygous or double heterozygous defects of α -spectrin could cause HS; whereas the presence of hypomorphic alleles (such as α -LELY, Low expression Lyon) is complete-

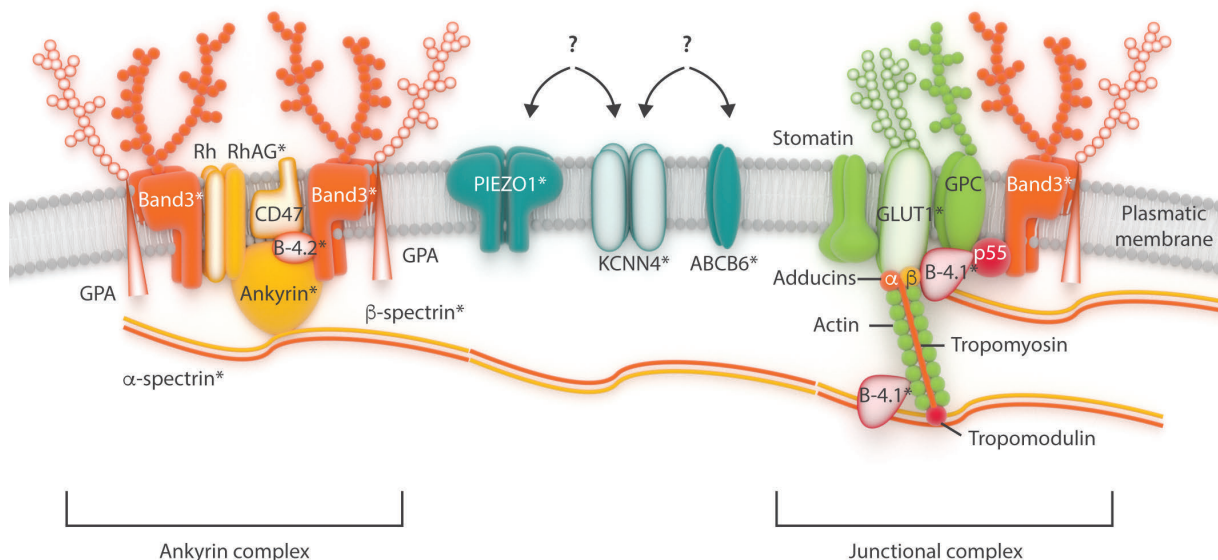


Figure 1. Simplified cross-section of the erythrocyte membrane. The red blood cell membrane is composed of integral membrane proteins incorporated into a phospholipid bilayer. The network of cytoskeletal proteins is anchored to the membrane via several transmembrane proteins with a transport function: band 3, anion transporter; GLUT1, glucose and L-dehydroascorbic acid transporter; RhAG, gas transporter, in particular CO_2 ; various cation pumps and transporters including, Na^+-K^+ -ATPase, Ca^{2+} -ATPase, $Na^+-K^+-2Cl^-$ and Na^+-Cl^- , Na^+-K^+ , K^+-Cl^- co-transporters and Gardos channel. The most recently described proteins PIEZO1, KCNN4 and ABCB6, involved in the modulation of RBC membrane permeability, and their putative interactions are also shown. The relative positions of the proteins to each other within the various complexes are mostly unknown. The shapes of the major proteins are mostly imaginary. GPA, glycoprotein A; Rh, Rhesus polypeptide; B-4.1, protein band 4.1; B-4.2, protein band 4.2; GPC, glycoprotein C; RhAG, Rh-associated glycoprotein; RBC: red blood cells. *Proteins that are known to be affected by pathogenic mutations so far.

ly asymptomatic. However, due to its limiting amount (with respect to α -spectrin), the deficiency of β -spectrin causes HS in the heterozygous state as well. Band 3 and ankyrin synthesis are the latest to occur and they seem to play a critical role in assembly. Protein 4.1 and ankyrin are the last cytoskeletal protein components to continue to be synthesized and assembled. This is at least partly due to the fact that ankyrin and protein 4.1 mRNA persist late into erythropoiesis when the levels of the majority of cytoplasmic RNAs, including those for band 3 and spectrins, have declined precipitously.⁷

Classification, diagnostic criteria and epidemiology of erythrocyte membrane defect-related anemias

From the genetic standpoint, 15 different types of anemias due to RBC membrane defects are currently included in the Online Mendelian Inheritance in Man (OMIM) compendium of human genes and genetic phenotypes (Table 1). Of note, the gene mutations identified so far refer only to a restricted number of patients; indeed, the molecular defect is still unknown for several patients. We can divide RBC membrane disorders into two main sub-

groups: (i) structural defects, and (ii) altered permeability of the RBC membrane. The first subgroup comprises: HS, HE, hereditary pyropoikilocytosis (HPP), and Southeast Asian ovalocytosis (SAO); the second subgroup contains: dehydrated hereditary stomatocytosis (DHS), overhydrated hereditary stomatocytosis (OHS), familial pseudohyperkalemia (FP), and cryohydrocytosis (CHC).

Hereditary anemias due to RBC structural defects

Hereditary spherocytosis

HS is the most common non-immune hemolytic anemia with a prevalence of 1:2000-5000 in the Caucasian population.⁸ This value is probably higher due to under-diagnosed mild/moderate forms. HS refers to a group of heterogeneous inherited anemias showing a broad spectrum of clinical severity, ranging from asymptomatic to severe transfusion-dependent forms, even within the same family. The intra-familial heterogeneity can be ascribed to the co-inheritance of genetic variants involved in erythrocyte defects themselves or in other disorders, such as enzymopathies, thalassemias and Gilbert syndrome.⁹

However, HS clinical findings are summarized by hemolytic anemia, jaundice and splenomegaly. Reticulocytosis (6-10% to 35% in severe cases), increased

Table 1. Classification of erythrocyte membrane disorders by OMIM database.

Disease symbol	Phenotype	Phenotype MIM number	Gene location	Protein name ^s	Inheritance
HS1	Hereditary spherocytosis type 1	182900	ANK1 <i>8p11.21</i>	Ankyrin-1	AD
HS2	Hereditary spherocytosis type 2	616649	SPTB <i>14q23.3</i>	Spectrin β chain, erythrocytic	AD
HS3	Hereditary spherocytosis type 3	270970	SPTA1 <i>1q23.1</i>	Spectrin α chain, erythrocytic 1	AR
HS4	Hereditary spherocytosis type 4	612653	SLC4A1 <i>17q21.31</i>	Band 3 anion transport protein	AD
HS5	Hereditary spherocytosis type 5	612690	EPB42 <i>15q15.2</i>	Erythrocyte membrane protein band 4.2	AR
HE1	Hereditary elliptocytosis 1	611804	EPB41 <i>1p35.3</i>	Protein band 4.1	AD
HE2	Hereditary elliptocytosis 2	130600	SPTA1 <i>1q23.1</i>	Spectrin α chain, erythrocytic 1	AD
HE3	Hereditary elliptocytosis 3	-	SPTB <i>14q23.3</i>	Spectrin β chain, erythrocytic	AD
HPP	Hereditary Pyropoikilocytosis	266140	SPTA1 <i>1q23.1</i>	Spectrin α chain, erythrocytic 1	AR
SAO	Ovalocytosis Southeast Asian type	166900	SLC4A1 <i>17q21.31</i>	Band 3 anion transport protein	AD
OHS	Overhydrated hereditary stomatocytosis	185000	RHAG <i>6p12.3</i>	Ammonium transporter Rh type A	AD
DHS1	Dehydrated hereditary stomatocytosis with or without pseudohyperkalemia and/or perinatal edema	194380	PIEZO1 <i>16q24.3</i>	Piezo-type mechanosensitive ion channel component 1	AD
DHS2	Dehydrated hereditary stomatocytosis 2	616689	KCNN4 <i>19q13.31</i>	Intermediate conductance calcium-activated potassium channel protein 4	AD
FP	Familial pseudohyperkalemia	609153	ABCB6 <i>2q35-q36</i>	ATP-binding cassette sub-family B member 6	AD
CHC	Cryohydrocytosis	185020	SLC4A1 <i>17q21.31</i>	Band 3 anion transport protein	AD

^sProtein name reported in Uniprot database. AD: Autosomal dominant; AR: Autosomal recessive; ATP: adenosine triphosphate; Rh: Rhesus; OMIM: Online Mendelian Inheritance in Man; MIM: Mendelian Inheritance in Man.

mean corpuscular Hb concentration (MCHC > 34.5g/dL), increased RBC distribution width (RDW >14), and normal or slightly decreased MCV are the main laboratory findings. Anemia in most patients is mild (Hb >11 g/dL) or moderate (Hb 8-11 g/dL), due to poorly compensated hemolysis.¹⁰ Symptomless or mildly anemic patients are often diagnosed after hemolytic or aplastic crises, while in mildly affected women the condition often becomes evident during pregnancy, but transfusions are required only rarely. A small percentage of patients present a severe form (Hb 6-8 g/dL), which needs regular blood transfusions. One of the most common complications of chronic hemolytic anemia is cholelithiasis, which is more frequent in patients who co-inherited Gilbert Syndrome.¹¹ Of note, the co-inheritance of HS and Gilbert disease can be misdiagnosed as Crigler-Najjar syndrome type II. The third component of the HS triad is splenomegaly, observed in almost all adult patients. The spleen enlargement is mild or moderate, rarely massive: only one patient with spontaneous rupture has been described,¹² while in few patients splenic infarction has been observed.^{13,14} Extramedullary erythropoiesis and iron overload can also

be observed. Hemosiderosis is more relevant in transfusion-dependent patients or in those who have co-inherited mutations in the causative genes of hereditary hemochromatosis.¹⁵

The diagnosis of HS is based on clinical features, positive familial history and the observation of a peripheral blood (PB) smear, in which a variable percentage of spherocytes, related to the degree of anemia, mushroom red cells, poikilocytosis, acanthocytes and ovalostomatocytes can be found (Figure 2).¹⁶ The diagnostic guidelines of HS from the British Committee for Standards in Haematology do not recommend any additional tests for patients with classical clinical features and laboratory data.¹⁷ Whenever necessary, indirect tests can also be performed. Among these, the eosin-5'-maleimide (EMA) binding test shows high sensitivity (92-93%) and specificity (nearly 99%), although a positive test can also be obtained in patients affected by related conditions, such as congenital dyserythropoietic anemia type II (CDA II).¹⁶⁻¹⁸ Additional tests, such as the osmotic fragility (OF) test, acidified glycerol lysis test (AGLT) and the pink test, exhibit lower sensitivity compared to the EMA test (68%, 61% and 91%,

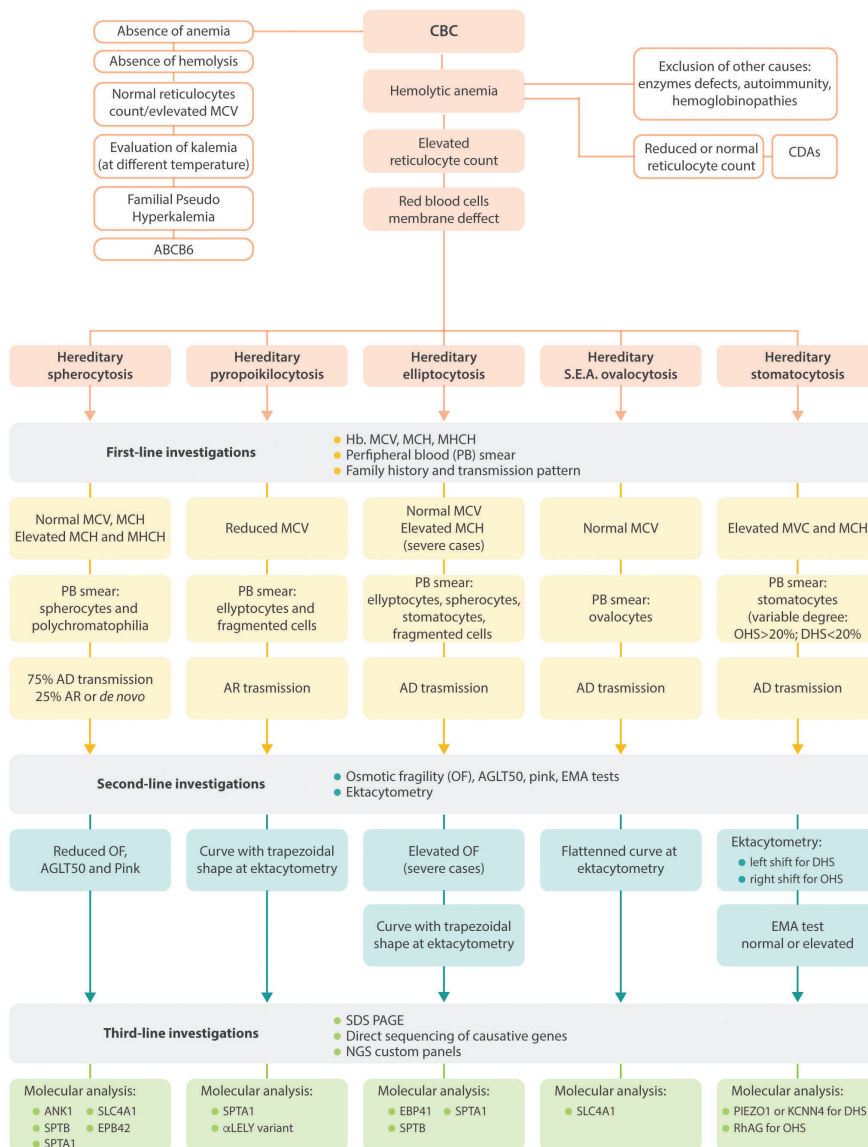


Figure 2. Flow diagram for the differential diagnosis of hemolytic anemias due to RBC membrane defects. The flow diagram shows the main steps for guiding the clinical suspicion toward the diagnosis of different subtypes of hereditary erythrocyte membrane disorders. First-, second-, and third-line investigations are also shown. The cut-off for the EMA binding test is still debated: currently, a test with a reduction of EMA binding > 21%, in comparison with controls, is defined positive, whereas a test with a reduction of EMA binding < 16% is considered negative. Values between 16-21% are not conclusive, although a cut-off of 11% has been proposed. Hb: hemoglobin; MCH: mean corpuscular hemoglobin; MCV: mean cellular volume; MCHC: mean corpuscular hemoglobin concentration; CBC: complete blood count; RBC: red blood cells; OHS: overhydrated hereditary stomatocytosis; DHS: dehydrated hereditary stomatocytosis; AD: autosomal dominant; AR: autosomal recessive; EMA: eosin-5'-maleimide; SDS: Sodium dodecyl sulfate; NGS: next generation sequencing; RHAG: rhesus blood group-associated glycoprotein. PB: peripheral blood.

respectively). Nevertheless, the combination of the EMA and pink tests or those of the EMA and AGLT tests improves the sensitivity to 99% and 100%, respectively.¹⁹ Ektacytometry is a highly sensitive test of membrane deformability.¹⁰ In HS the characteristic features of ektacytometry are: decreased DI max, in conjunction with a shift of the Omin point to the right (reduced surface to volume ratio), and a shift of the O' or hyper point to the left (increased dehydration of the red cells) (Figure 3).¹⁶

As a third-line of investigation, the analysis of major erythrocyte membrane proteins *via* SDS-PAGE still represents invaluable support for the identification of different subsets of HS patients; however, several subjects remain unclassified by this technique. As discussed later in this review, the current availability of advanced genomic surveys, such as next generation sequencing (NGS) technologies, allows one to overcome the limitations of previous analytic methods. However, the biochemical analyses may be of great use in the interpretation of NGS data in order to assess the pathogenicity of identified genetic variants.

In HS, the phenotype variability is linked to different molecular defects. The increased membrane fragility is caused by heterogeneous molecular defects due to deficiency and/or dysfunction in erythrocyte membrane proteins,

ankyrin (ANK1), α - and β -spectrin (SPTA and STPB), band 3 (SLC4A1), and protein 4.2 (EPB42). Approximately 75% of HS cases exhibit an autosomal dominant (AD) pattern of inheritance, associated with mutations in *ANK1*, *SPTB*, *EPB42* and *SLC4A1* genes. In the remaining 25% of patients autosomal recessive (AR) and *de novo* mutations were observed (Table 1). In rare cases, HS can be associated to psychomotor developmental delay and autism in contiguous gene syndromes due to large genomic deletions, including *ANK1*^{20,21} or *SPTB* genes.^{22,23} Finally, prenatal hydrops fetalis has been rarely observed in patients with mutations in *SLC4A1*²⁴ and *SPTA-SPTB* genes.^{25,26}

Hereditary elliptocytosis and pyropoikilocytosis

HE is characterized by the presence of elliptical-shaped erythrocytes (elliptocytes) on the PB smear associated to variable clinical manifestations. The worldwide incidence of HE is 1:2000-4000 individuals, but it results higher in some African regions (1:100). The majority of patients present no anemia or hemolysis, and diagnosis is made incidentally, after worsening of anemia due to infections or after diagnosis in symptomatic relatives. Severe anemia was observed only in rare cases. A good indicator for the severity of the disease is the percentage of spectrin dimers. A subtype of HE is HPP, a rare severe hemolytic anemia characterized by poikilocytosis and fragmented erythro-

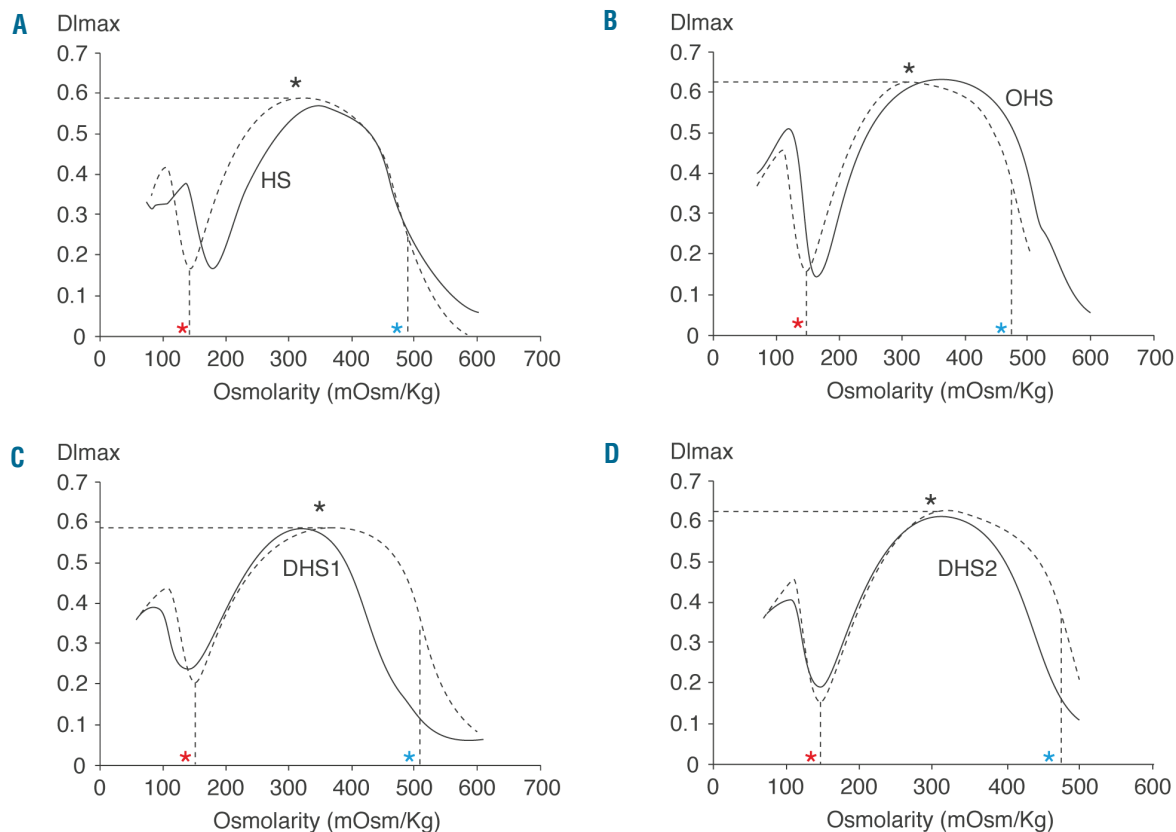


Figure 3. Examples of ektacytometric curves of different hereditary erythrocyte membrane disorders. The ektacytometer is a laser diffraction viscometer that measures RBCs (red blood cells) deformability at constant shear stress as a continuous function of suspending Osmolarity. Three principal features of the osmotic gradient ektacytometry profiles are: the Omin point (red asterisk), which corresponds to the osmolarity at which 50% of the red cells are lysed in the classical osmotic fragility test and represents the surface area to volume ratio; the maximal deformability index (DImax, black asterisk) value, which represents the maximal cellular deformability of the red cell population; and the O' or hyper point (blue asterisk), which corresponds to the osmolarity at DImax/2, which reflects the hydration status of the red cells. Ektacytometric analysis of (A) HS, (B) OHS, (C) DHS1 and (D) DHS2 patients are shown. The dotted line is those relative to the control subjects. HS: hereditary spherocytosis; OHS: overhydrated hereditary stomatocytosis; DHS1: dehydrated hereditary stomatocytosis 1; DHS2: dehydrated hereditary stomatocytosis 2.

cytes, resulting in low MCV (50-60 fL) and microspherocytes.¹⁶ HPP patients show marked splenomegaly, and splenectomy is therefore usually recommended. There is a strong association between HE and HPP. The main defect in HE erythrocytes is mechanical weakness or fragility of the erythrocyte membrane skeleton due to defective horizontal connections of cytoskeletal proteins, such as spectrin dimer-dimer interactions and spectrin-actin-protein 4.1 at the junctional complex. For the main part, HE is inherited as AD disease, with rare cases of *de novo* mutations, whereas HPP patients exhibit an AR inheritance. HE can be due to mutations in *EPB41*, *SPTA1* and *SPTB* genes that lead to serious damage in the association of spectrin dimers/tetramers.²⁷ Also, HE shows high inter- and intra-familial phenotypic variability, due to the modifier alleles. One example is the α -LELY in the *SPTA1* gene, a hypomorphic haplotype composed of two variants, the missense Leu1857Val and the splicing variant in intron 45. This hypomorphic haplotype alone causes minimum damage in both heterozygous and homozygous states since the spectrin α chains are produced in excess (3- to 4-fold compared to β -chains); otherwise, when it is associated with a HE mutation in *SPTA1*, the resulting phenotype is severe, i.e., HPP (Table 1).²⁷

Southeast Asian ovalocytosis

SAO is a very common condition in the aboriginal peoples from Papua New Guinea, Indonesia, Malaysia, the Philippines and southern Thailand, in areas where malaria is endemic, with prevalence varying between 5% and 25%. Indeed, this condition offers protection against all forms of malaria.²⁷ Despite the reduced *in vitro* deformability of SAO erythrocytes, patients are asymptomatic and the diagnosis is made accidentally as a result of a PB smear examination, showing the characteristic rounded elliptocytes (ovalocytes). However, in newborns it may manifest as hemolytic anemia and require phototherapy. SAO is an AD condition caused by the deletion of 27 nucleotides in the *SLC4A1* gene, leading to the loss of the amino acids 400-408 of protein band 3.² The deletion is in linkage disequilibrium with the Memphis polymorphism (p.Lys56Glu) in *SLC4A1* (Table 1). SAO erythrocytes show a slight loss of monovalent cations when exposed to low temperatures, with a reduction of anions flux. Thus, the condition may be classified as a genetic disease affecting the permeability of the RBC membrane. Despite the frequency of heterozygotes, only one case homozygous for the 27 nucleotide deletion has been described so far. This patient showed severe phenotype with intrauterine transfusions, transfusion dependent anemia and distal renal tubular acidosis due to the loss of band 3, which is also expressed in the kidneys.²⁸

Other conditions

Erythrocyte abnormalities can also be observed in other hereditary and acquired conditions. For example, the autoimmune hemolytic anemias are characterized by shortened RBC survival due to the presence of auto-antibodies directed toward red cells, with a positive Coombs test. RBCs are typically coated with auto-antibodies and trapped by macrophages in the cords of the spleen. The interaction of trapped RBCs with splenic macrophages may result in phagocytosis of the entire cell or partial phagocytosis with the formation of spherocytes, present in the blood film.²⁹ Micro- and macrospherocytes, associ-

ated with increased osmotic fragility, were also seen in patients affected by chronic hepatitis C virus treated with protease inhibitors (telaprevir and boceprevir). In these patients oxidative stress, induced by drugs, damages membrane-cytoskeletal stability, reducing α - and β -spectrins.³⁰ Alterations in the RBCs membrane are also present in neuroacanthocytosis, a heterogeneous group of diseases that include chorea-acanthocytosis, McLeod and Huntington's disease-like syndromes. These conditions are characterized by alterations of post-translational modifications, mostly phosphorylation, of erythrocyte membrane proteins and significant neurological symptoms.³¹

Anemias due to altered permeability of RBC membrane

Dehydrated hereditary stomatocytosis or xerocytosis

HST includes both DHS and OHS, which show alteration of the RBC membrane permeability to monovalent cations Na⁺ and K⁺, with a consequent alteration of the intracellular cationic content and alterations of cell volume.³² DHS is the most highly represented among HST, with an incidence of approximately 1:50000 births. It is 10-20 times less frequent than HS, with which it may be, however, confused. Of note, based on our experience, the incidence of this anemia could be underestimated because it is often undiagnosed.

The phenotype ranges from asymptomatic to severe forms, with massive hemolysis. Generally, DHS patients show hemolytic well-compensated anemia, with a high reticulocyte count, a tendency to macrocytosis and mild jaundice. The main characteristic of RBCs is cell dehydration caused by the loss of the cation content, with a subsequent increase of MCHC (>36 g/dL). At blood smear the stomatocytes, erythrocytes with a characteristic central mouth-shaped spot, are quite rare, which often makes diagnosis difficult. In addition, it may be difficult when the clinical picture is associated with pseudohyperkalemia and/or perinatal edema, in the so-called pleiotropic syndrome form.^{33,34} For these reasons, the condition may be overlooked for years or decades before reaching a conclusive diagnosis. Osmotic gradient ektacytometry is a useful tool to diagnose this condition; it shows a leftward shift of the minimum in the deformability index (Omin) at low osmolarities, as well as a decrease in DImax (Figure 3).

DHS patients also exhibit a tendency toward having iron overload, regardless of the transfusion regimen or splenectomy.³⁵ The study of the iron metabolism in this condition is an open and interesting field of investigation, enabling the discovery of new drugs to treat the iron overload.

DHS is inherited as an AD trait. The candidate gene locus was first localized at 16q23-24.^{36,37} Several years later, *PIEZO1* was identified as the causative gene of both isolated and syndromic forms of DHS1 by exome sequencing (Table 1).^{38,39} *PIEZO1* encodes a mechanoreceptor, an ion channel activated by pressure. This protein has been identified in the RBC membrane, and in mice it has been shown to form a tetramer of about 1.2 million daltons; it is therefore the largest ion channel identified to date, and moreover it regulates mechanotransductive release of ATP from human RBCs.⁴⁰⁻⁴³ The identified mutations are missense and mainly located in the highly conserved C-terminus of the protein, recently described to form the pore of the channel.⁴⁴ Several electrophysiology studies demonstrated that the mutations cause a gain-of-function phenotype with delayed inactivation of the channel,^{38,39,45} sug-

gesting increased cation permeability leads to DHS erythrocyte dehydration. PIEZO1 is currently the subject of intense research and has been shown to be involved in several physiological and pathophysiological processes. The study of this mechanoreceptor will shed light on the hydration pathways in healthy and diseased RBCs.

Recently, a novel gene, *KCNN4*, has been identified as causative of a second form of DHS, named DHS2, in six different families (Table 1).⁴⁶⁻⁴⁸ The *KCNN4* gene encodes the Gardos channel, a widely expressed Ca²⁺-dependent K⁺ channel of intermediate conductance that mediates the major K⁺ conductance of erythrocytes.⁴⁶⁻⁴⁸ Mutated *KCNN4* channels showed a higher current compared to WT resulting from changes in the open probability, in the trafficking, and in the unitary conductance of the channel. This is suggestive of the pathogenic mechanism associated with several mutations affecting PIEZO1. In addition, this observation could suggest that PIEZO1 and the Gardos channel might act in the same stretch-induced cation pathway involved in cell volume homeostasis.

Unlike DHS1, patients affected by DHS2 show a normal pattern of ektacytometry analysis (Figure 3),^{46,47} whereas they exhibit iron overload similar to that in DHS1 patients.

HST can also be associated with band 3 mutations, characterized by the conversion of band 3 from an anion exchanger to a cation transporter.⁴⁹

Overhydrated hereditary stomatocytosis

OHS is a very rare subtype among HST, with 20 cases reported overall worldwide. Contrary to DHS, RBCs are hydrated due to an increase, from 20 to 40 times, in the loss of cations.³² OHS is associated with more severe phenotypes compared to DHS. In addition to reticulocytosis, it is characterized by a sharp increase in MCV (>110 fL) and decreased MCHC (24-30 g/dL). The number of stomatocytes is usually much higher than that observed in DHS. The causative gene of this condition is *RHAG*, encoding the Rh-associated glycoprotein (RhAG) which acts as an ammonia channel (Table 1).⁵⁰ Stomatatin has been found at low or absent levels in OHS patients, but no mutations have been found in the encoding gene so far.³⁴ Moreover, a complex syndrome named stomatin-deficient cryohydrocytosis has been described. It is characterized by mental retardation, seizures, cataracts and massive hepatomegaly. RBCs showed dramatic resumption of the leak *in vitro* when stored at low temperatures and in the absence of stomatin.^{51,52} Recently, this syndrome was associated with mutations in *SLC2A1* that cause both loss of glucose transport and a cation leak.⁵³

Familial pseudohyperkalemia and cryohydrocytosis

FP and CHC are additional forms of stomatocytosis. FP is not associated with hemolytic anemia and stomatocytes are rarely observable on PB smear. Conversely, CHC patients show hemolytic anemia of variable degrees.⁵⁴ RBCs from FP patients exhibit a loss of K⁺ at low temperatures (<37°C, mostly 8-10°C), but not at 37°C. In CHC the main feature is the temperature dependence of the loss of cations: instead of being around 8-10°C, the minimum is approximately 23°C. The gene responsible for FP was mapped at 2q35-q36,⁵⁵ and later identified in the *ABCB6* gene,⁵⁶ encoding the homonymous protein, ABCB6. It belongs to the family of ABC transporters with the binding cassette for ATP, one of the most abundant families of

integral membrane proteins. ABCB6 was previously identified as a porphyrin transporter, thus we now find that it is currently highly debated because several other studies identified its expression in the plasma membrane of RBCs and in the endo-lysosomal compartment, excluding the mitochondrial uptake of porphyrins.⁵⁷ Moreover, in erythrocyte membranes it bears the Langereis (Lan) blood group antigen system.^{58,59} ABCB6 expression increases during erythroid differentiation of CD34+ cells.⁵⁶ The ABCB6 missense mutations in FP does not alter mRNA or protein levels, or subcellular localization in mature erythrocytes or erythroid precursor cells, but are predicted to have a pathogenic consequence on protein function. Recently, ionic flux assays on the mutations found in FP patients have demonstrated that the mutations are gain-of-function, causing an abnormal loss of potassium from cells at low temperatures.⁶⁰ These changes could lead either to a cation leak through the normal substrate translocation pathway of ABCB6, or to the generation of a novel constitutive or cyclic leak pathway through the protein. Recently, the ABCB6 variants R723Q, V454A, and R276W were found in FP patients who are regular blood donors.^{60,61} The blood of these patients exhibited increased potassium leakage upon storage at temperatures below 37°C. Of note, all these variants are annotated in public databases, suggesting that FP is common in the general population. Particularly, the variant R276W has been found in one of 327 random blood donors (0.3%). The storage of blood of these subjects leads to significantly increased K⁺ levels, with serious clinical implications for neonates and infants receiving large-volume transfusions of whole blood. This interesting finding encouraged further study on the implications for neonates and infants receiving transfusion of whole blood from undiagnosed FP subjects.⁶⁰

CHC is due to mutations in the *SLC4A1* gene; these are gain-of-function mutations, since they are able to transform the band 3 anion exchanger to a cation transporter (Table 1).

Differential diagnosis

Hemolytic anemias caused by RBC membrane defects can often be misdiagnosed with other hemolytic anemias. In particular, HS can be confused with autoimmune hemolytic anemia that shows spherocytes on the PB smear. Thus, it is crucial to perform additional diagnostic tests, such as a Coombs' test (the direct antiglobulin test) to distinguish between these conditions. Other conditions, including liver disease, thermal injury, micro- and macroangiopathic hemolytic anemias, clostridial sepsis, transfusion reaction with hemolysis, severe hypophosphatemia, ABO incompatibility, and poisoning with certain snake, spider, and hymenoptera venoms can be associated with the presence of spherocytes on the PB smear. It is critical to evaluate the disorder in the proper clinical context and to evaluate the family history and transmission pattern.¹⁰

Additionally, membrane defects can be misdiagnosed with enzymatic defects, particularly with glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase (PK) deficiencies. In cases where red cell enzyme deficiency is suspected, the diagnosis should be confirmed by performing the most common red cell enzyme assays and by analyzing the inheritance of the disease that is X-linked for

G6PD deficiency and autosomal recessive for PK defect.

CDA II also shares some characteristics with HS; in fact, CDA II patients are often erroneously diagnosed as HS.⁶² It is critical to evaluate the reticulocyte count, which is elevated in HS while it is inadequate for the degree of anemia in CDA II, because the marrow stress is higher in respect to HS for the same Hb level. This observation is confirmed by the increased sTfR levels observed in CDA II patients compared to those with HS. RDW is also characteristically increased in CDA II, while the Hb distribution width (HDW) is increased in HS, resulting in an RDW/HDW ratio that is significantly greater in CDA II than HS.⁶³ Additionally, a new clinical index, named BM responsiveness index (BMRI), has been developed to discriminate a well-compensated hemolytic anemia from an ineffective erythropoiesis one. BMRI is calculated as [(patient's absolute reticulocyte count) × (patient's Hb/normal Hb)] and showed high specificity and sensitivity.⁶⁴ This index represents the degree of production of new RBCs in a condition of anemia. Of note, it is crucial to distinguish between these anemias because HS patients take advantage of splenectomy; conversely, this intervention lacks substantial improvement in CDA II patients.

Furthermore, both OHS and DHS can be erroneously diagnosed with CDA type I (CDA I). Both CDA I and HST present macrocytosis associated with hemolytic signs. Likewise in this case, the accurate evaluation of the reticulocyte count can be enlightening, since it is inadequate for the degree of anemia in CDA I. Whenever applicable, a bone marrow aspirate can provide a clue, since it shows hypercellularity and erythroid hyperplasia with the pathognomonic morphological features of CDA I, i.e. the presence of thin chromatin bridges between the nuclei pairs of erythroblasts.⁶²

Therapy and management

A correct diagnosis of RBC membrane disorders should be obtained before starting treatment. The first-line treatment is often based only on supportive care. For example, HS neonatal patients often need phototherapy or exsanguinous-transfusions, in case of significant jaundice.¹⁶ In the children of affected parents, bilirubin values should be strictly monitored. Moreover, some newborns affected by erythrocyte membrane defects may necessitate transfusions: in these patients recombinant erythropoietin (rEPO) can reduce transfusion requirements.⁶⁵ Similarly, transfusions may be administered as needed in adulthood, mostly during aplastic or hemolytic crises.

In patients with increased folate demands, such as children, pregnant women or in patients with evidence of folate deficiency, supplements of this vitamin is suggested.

Splenectomy could be suggested in several cases of HS and HE/HPP, resulting in an increased life span of RBCs. Currently, total splenectomy is recommended in adult patients with severe forms of HS and HPP; otherwise, in patients with mild or moderate anemia, splenectomy is not recommended thus far. However, splenectomy increases the risk of both infections (mostly from encapsulated organisms) and thrombosis.⁶⁶ Vaccinations against encapsulated bacteria, such as *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*,¹⁶ should be administered before surgery, even if thus far there is no standard protocol regarding antibiotic prophylaxis after

splenectomy. Whenever possible, splenectomy in children should be delayed until they reach 6 years of age, otherwise partial splenectomy (residual splenic mass 20-30%) can be performed.¹⁷ Laparoscopic splenectomy is preferred for shorter postoperative discomfort and hospital stay, even if it involves a longer operative time and needs more surgical experience.⁶⁷ However, laparoscopy is not applicable for spleens which are too large. In the presence of cholelithiasis, contemporary cholecystectomy can be performed.

In patients with moderate HS, with poor resistance to fatigue or growth retardation, the role of splenectomy is still debated. Recently Pincez and colleagues have proposed subtotal splenectomy (residual splenic mass 15-25%) for severe and moderate HS.⁶⁸ They propose this approach in order to reduce long-term complications: after a long follow-up, subtotal splenectomy led to a good hematologic response in many patients, but 5 out of 79 patients remained transfusion dependent, 3 were considered as asplenic and 1 showed no significant increase of Hb. In our opinion, in severe HS cases total splenectomy would be preferred, while the role of subtotal splenectomy in symptomatic non-transfusion dependent patients needs to be evaluated case by case.

In a few HPP patients, subtotal splenectomy (resection of 80-90% of spleen volume) has been performed, with no advantage as compared to total splenectomy.⁶⁹ Conversely, splenectomy is contraindicated in DHS due to the increased risk of thromboembolic complications,^{70,71} probably related to the augmented number of undestroyed stomatocytes in the bloodstream. Thrombotic complications (arterial and venous events, including portal vein thrombosis and pulmonary hypertension) have been described at a high rate following splenectomy.^{70,71}

In literature, data on splenectomy of OHS patients are lacking. Splenectomy should be avoided in this condition because the percentage of stomatocytes is even higher compared to DHS.

Animal models

Several animal models have been developed for the study of RBC defects (Table 2). Concerning the mouse models, although the main stream of human hemolytic anemias caused by RBC membrane defects are inherited as AD, in mice these conditions are usually inherited as AR (Table 2). Recently, within hereditary stomatocytosis, mouse models of PIEZO1 were developed. Mice deficient in Piezo1 die *in utero* at mid-gestation due to defective vasculogenesis.⁷² Thus, another model of Piezo1 was developed by specific deletion in the hematopoietic system (Vav1-P1cKO mice).⁷² Hematological analysis of blood from Vav1-P1cKO mice revealed elevated MCV, MCH and reduced MCHC. RBCs exhibited increased osmotic fragility, demonstrating that Piezo1-deficient RBCs were overhydrated. At the moment a knock-in mouse model carrying missense mutations found in DHS1 patients doesn't exist; its creation will further elucidate the role of PIEZO1 in the hydration pathways of RBCs.

Beyond mouse models, which in several cases don't recapitulate the main characteristics of human hematological disease, a powerful model for the study of erythropoiesis is the zebrafish, *Danio rerio*, because of its small size, its ability to generate a large number of embryos, and

Table 2. Animal models for the study of RBC membrane defects.

Organism	Mutant	Altered gene	Type of alteration	Phenotype	References
<i>Mus musculus</i>					
	ja/ja	Sptb	Deficiency of β -spectrin	Severe hemolytic anemia, reticulocytosis	84
	sph/sph	Spta	Deficiency of α -spectrin		85
	Nan	Klf1	Missense E339D	Neonatal anemia; in adult mice, hemolytic anemia with decreased RBCs, hematocrit, Hb, and elevated zinc protoporphyrin levels	86
	ENU-generated	Ank1	Nonsense E924X	Heterozygous mice: low MCV, elevated RBC counts, reticulocytosis, reduced EMA intensity, and increased osmotic fragility	87
	wan	Slc4a1	Premature stop codon	Homozygous mice: severe anemia with marked anisocytosis and spherocytosis on the PB smear	88
	sph ^{Dem} /sph ^{Dem}	Spta	Inframe deletion of 46 amino acids that alters spectrin dimer/tetramer stability	Increased MCV, decreased MCHC, marked reticulocytosis (50%). PB smears: elliptocytes, spherocytes and occasional poikilocytes, as seen in severe human HE	89
	Vav1-P1cKO	Piezo1	Gene deletion in the hematopoietic system	Elevated MCV, MCH and reduced MCHC. RBCs exhibit increased osmotic fragility	72
<i>Danio rerio</i>					
	merlot	Epb41	Mutation	Spiculated RBC membranes; hemolytic anemia, cardiomegaly, splenomegaly	90
	Morpholino	Piezo1	Knockdown of gene expression	Severe anemia with swollen, fragile and spherocytic RBCs	74
	ZFN knockout	Piezo1	Frameshift in exon 8	No anemia or dysmorphic erythrocyte morphology	75

RBC: red blood cells; Hb: hemoglobin; MCV: mean cellular volume; EMA: eosin-5-maleimide; PB: peripheral blood; MCHC: mean corpuscular hemoglobin concentration; HE: hereditary elliptocytosis; MCH: mean corpuscular hemoglobin.

its transparency that facilitates the visualization of erythroid cell migration.⁷³ Notably, the high conservation of hematopoietic genes among vertebrates and the ability to successfully transplant hematopoietic cells have enabled the establishment of models of human anemic diseases in zebrafish (Table 2). Recently, zebrafish models have also been created for PIEZO1. Morpholino-knockdown of Piezo1 expression in the *Danio rerio* was reported to result in severe anemia.⁷⁴ However, the phenotype observed in the morpholino-knockdown model was not present in an independent zebrafish model carrying a predicted truncated form of PIEZO1 (Table 2).⁷⁵ The debate on the phenotype observed in the two different models is still open.^{76,77} It is notable that patients with homozygous loss-of-function mutations in human PIEZO1 show lymphatic dysplasia and an asymptomatic, fully compensated, very mild hemolytic state of incomplete penetrance.^{78,79}

In conclusion, both mouse and zebrafish models appear not to better recapitulate the human pathogenesis, but they are useful to study the function of newly identified proteins such as PIEZO1.

Conclusions and perspectives

Hereditary anemias due to RBC membrane defects represent a heterogeneous group of hereditary defects with very overlapping phenotypes. Indeed, the clinical definition of patients is often difficult. For some conditions, the great phenotypic variability is partially explained by the high genetic heterogeneity; otherwise, it is sometimes complicated to distinguish one form from the others since the signs can be veiled in symptom-free carriers or in mildly affected patients. Moreover, some subtypes of RBC membrane disorders can be easily confused with

other clinically-related hereditary hemolytic conditions, as classically reported for differential diagnosis of HS and CDA II.⁶⁴ Thus, when there is a suspected hereditary erythrocyte membrane disorder, after the exclusion of other common diseases, it is essential to perform a depth analysis of PB smear and pedigree transmission of the disease. Biochemical tests can be useful, especially in HS, but they do not have high sensitivity. The combination of an EMA test with ektacytometry is of great help for the majority of these conditions, but ektacytometry analysis is of limited availability. Thus, the genetic analysis becomes crucial, mainly in cases with an ambiguous phenotype. The absence of clear genotype/phenotype correlations is often problematic for both genetic counseling and suitable treatments. It is proper in this context that new genomic technologies are utilized. In the last few years, remarkable progress has been made in discovering new disease genes involved in these disorders by means of unbiased genomic approaches, such as whole exome sequencing.^{39,46,47,56} However, the increasing genetic heterogeneity underlines the problem of a very complex differential diagnosis. In the next generation sequencing (NGS) era, the genetic testing is going to move from few candidate genes to wider panels of genes, namely targeted (t)-NGS. Recent studies have already demonstrated the usefulness of t-NGS as a comprehensive and invaluable diagnostic tool by means of achieving a correct diagnosis and proceeding with careful management of these patients.^{80,81} Indeed, t-NGS approaches will be increasingly useful to accelerate the analysis, reduce costs and provide a clear diagnosis. One of the most important aspects of the use of t-NGS gene panels in clinical practice is their ability to be easily upgradable in view of novel discoveries. Despite their wide use in clinical practice, the major drawback of current NGS applications is represented by the data pro-

cessing steps, mainly by the difficulty in determining the pathogenicity of the numerous identified variants. One of the ways to overcome this limitation is the simultaneous evaluation of all family members, allowing one to establish the inheritance pattern of the identified variants and thus to understand its pathogenetic role, although functional characterizations are often necessary.

Finally, a future scenario in this field will be the implementation of novel therapeutic molecules. This is the case of Gardos channel blockers, such as senicapoc (ICA-17043), and PIEZO1 channel inhibitors for the treatment of primary and secondary disorders of erythrocyte hydra-

tion. Indeed, a clinical trial with senicapoc has already been established for the treatment of disorders of secondary erythrocyte hydration, such as sickle cell disease, demonstrating increased Hb and reduced markers of hemolysis in treated patients.^{82,83}

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