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Review

Hereditary spherocytosis, elliptocytosis, and other red cell membrane disorders ☆

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ABSTRACT

Hereditary spherocytosis and elliptocytosis are the two most common inherited red cell membrane disorders resulting from mutations in genes encoding various red cell membrane and skeletal proteins. Red cell membrane, a composite structure composed of lipid bilayer linked to spectrin-based membrane skeleton is responsible for the unique features of flexibility and mechanical stability of the cell. Defects in various proteins involved in linking the lipid bilayer to membrane skeleton result in loss in membrane cohesion leading to surface area loss and hereditary spherocytosis while defects in proteins involved in lateral interactions of the spectrin-based skeleton lead to decreased mechanical stability, membrane fragmentation and hereditary elliptocytosis. The disease severity is primarily dependent on the extent of membrane surface area loss. Both these diseases can be readily diagnosed by various laboratory approaches that include red blood cell cytology, flow cytometry, ektacytometry, electrophoresis of the red cell membrane proteins, and mutational analysis of gene encoding red cell membrane proteins.

1. Introduction

A number of inherited red cell disorders due to altered membrane function have been identified. These include hereditary spherocytosis (HS), hereditary elliptocytosis (HE), hereditary ovalocytosis (SAO), and hereditary stomatocytosis (HSt).

HS and HE[1–6] are the most common red cell membrane disorders in the world with a prevalence of 1 out of 2000 affected cases in North America and Northern European countries and are likely to be even higher due to underdiagnosis of asymptomatic forms. Both diseases result from defects in genes encoding various membrane and skeletal proteins that play a role in regulating membrane cohesion and membrane mechanical stability.[1–6] In both HS and HE, red cell life span is shortened as result of splenic sequestration of red cells. The abnormal red cells with decreased membrane surface area and increased sphericity are trapped in the billroth canals in the spleen and phagocytozed by the splenic reticulo-endothelial system[7,8] resulting in regenerative hemolytic anemia, splenomegaly, and ictera with increased free bilirubin level. The severity of the disease depends on the extent of surface area loss and ranges from asymptomatic forms to severe neonatal or prenatal forms responsible for rare hydrops fetalis cases requiring transfusion *in utero*. In this review, we will summarize the substantial progress that has been made in our understanding of i) structural organization of the red cell membrane including comprehensive characterization of a large number of membrane proteins, ii) structural basis for the interactions between various membrane and skeletal proteins and how defects in these interactions due to mutations in genes encoding the various proteins lead to defective membrane function, and iii) appropriate methodologies including genetic analysis that enable the diagnosis of various red cell membrane disorders.

2. Red cell membrane structure

The red cell membrane is a composite structure consisting of a lipid bilayer anchored to the spectrin-based membrane skeleton through linking proteins interacting with cytoplasmic domains of membrane proteins embedded in the lipid bilayer. In addition, anionic phospholipids in the inner lipid monolayer interact directly with skeletal proteins spectrin and protein 4.1 and these interactions modulate membrane function.[9–13] In addition to phospholipids, the red cell membrane contains a large panel of proteins (more than 50 trans-membrane proteins and 10 skeletal proteins) that interact with each other, and which are responsible for the antigenic

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proprieties, the transport function and the mechanical proprieties of the red cell membrane[5,14–16] (Fig. 1). The red cell membrane is not a static structure but is highly dynamic enabling it to undergo extensive deformations necessary for traversing the vascular bed performing its function of oxygen delivery. The ordered and specific structural organization of various membrane components is responsible for the unique features of extensive deformability and mechanical stability of the membrane that are needed for the red cell to perform its physiologic function during its long life span of 120 days. Altered structural membrane organization due to various protein defects is responsible for a large panel of human disorders either constitutional or acquired.[17–25] In addition to their function as structural proteins, various membrane proteins play other important functional roles. For example, i) transport function (band 3: anion transporter, aquaporin 1: water transporter, GLUT1: glucose and L-dehydroascorbic acid transporter,[26] Kidd: urea transporter, RhAG: gas transporter in particular CO_2, various cation pumps and transporters including, Na^+–K^+-ATPase, Ca^{++}-ATPase, Na^+–K^+–$2Cl^-$ and Na^+–Cl^-, Na^+–K^+, K^+–Cl^- cotransporters and Gardos channel), ii) adhesion molecules (ICAM-4, Lu/BCAM, CD36, α4 and β1 integrins) and iii) the antigenic functions (Blood group antigens) and cell signaling such as β2 adrenergic receptor.[16,27–29]

Two protein complexes serve to anchor the spectrin/actin membrane skeleton to the phospholipid bilayer: the ankyrin complex and the 4.1R complex[5,30] (Fig. 1). The ankyrin complex is composed of the anion exchanger band 3 tetramers,[31] blood group antigens (Rh, RhAG),[16,27,32] CD47 (thrombospondin receptor), glycophorin A (GPA), and Lansteiner Wiener (LW) antigen.[33] RhAG and band 3 link the phospholipid bilayer to red cell membrane skeleton by interacting with peripheral protein 4.2 and ankyrin. The 4.1R complex[34] is composed of band 3 dimers, blood group antigens (Rh, Duffy, Kell, XK),[27] and glycophorin C (GPC).[14] The GPC, Rh, Duffy, and XK interact directly with 4.1R while band 3 binds to adducin.[30] The loss of linkages between the spectrin-based skeleton and the lipid bilayer leads to loss of membrane cohesion resulting in lipid vesicle formation responsible for the loss of surface area, a mechanism leading to the spherocyte generation.

Spectrin-based membrane skeleton is composed of spectrin tetramers formed by head to head association of αβ spectrin heterodimers and the junctional complex composed of αβ spectrin tails interacting with actin, protein 4.1R, adducin, dematin, tropomyosin and tropomodulin.[18] The spectrin dimer–dimer interaction and the spectrin–actin–protein 4.1R junctional complex play a key role in regulating membrane deformability and membrane mechanical stability. Weakening of either of these lateral interactions results in elliptocytosis and decreased membrane mechanical stability leading to membrane fragmentation.

3. Hereditary spherocytosis

Hereditary spherocytosis (HS) (known as well as the Minkowski Chauffard disease) is the most common inherited red cell membrane disorder with one case out of 2000–3000 individuals, and probably even higher prevalence due to underdiagnosis of minor or moderate forms of HS (Table 1). Although more often diagnosed in Europe and North America, HS is reported in other continents and countries, without a founder effect. The inheritance is dominant in 75% of cases.

3.1. Clinical and biological feature

HS may be revealed early in infancy, even in the neonatal period or during pregnancy in the severe forms. HS is responsible for hemolytic anemia. The clinical features are pallor due to anemia, jaundice due to the hyperbilirubinemia and splenomegaly, the spleen being the site of sequestration and phagocytosis of the undeformable HS red blood cells. The jaundice may be the most important sign in the neonates (splenomegaly is often absent) and may require an exsanguinotransfusion in order to avoid the nuclear ictera, but most often phototherapy is sufficient to eliminate excess bilirubin. Hydrops fetalis of HS is rare and is likely due defects in either band 3[35] or spectrin.[36,37] Late in infancy and in adult, the classical triad (pallor/regenerative anemia, jaundice and splenomegaly) of the hemolytic anemia, in association with gallstones is noted. Careful examination of the blood smear, with the presence of spherocytic red cells usually confirms the diagnosis. A family history of HS, splenectomy and/or cholecystectomy can also be very helpful for the diagnosis since spherocytic red cells are also a feature of certain form of auto-immune hemolytic anemias (AIHA). The clinical manifestations of HS are highly variable from very mild to severe (Table 1). As a consequence, the anemia may vary from absent to mild or severe, with a reticulocyte count $>150 \times 10^9$/l. Of note, only 35% of the HS neonates exhibit an increased reticulocyte count of more than 10%.[38] Spherocytic red cells on the blood smear result from a reduced surface to volume ratio primarily related to loss of membrane surface area,[39] the main characteristic of the HS. The loss of surface area in HS is due to the disruption of the vertical linkages between the phospholipid bilayer and the membrane skeleton. The decreased surface area is a feature of both the reticulocytes and mature red cells in HS.[39] Mutations in genes encoding for various red cell

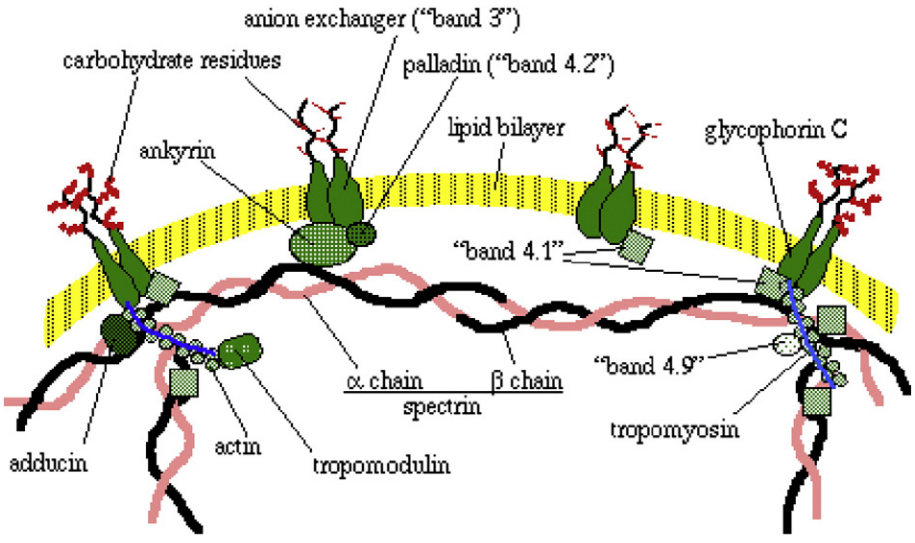

Fig. 1. Red cell membrane network with the two complexes, ankyrin and 4.1R embedded in the phospholipid bilayer.

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Table 1
HS classification.

	Minor HS	Moderate HS	Moderate to severe HS	Severe HS
Hb (g/l)	Normal	>80	60–80	<60
Reticulocytes (%)	<6%	6–10%	>10%	>10%
Bilirubin (μmol/l)	17.1–34.2	>34.2	>34.2–51.3	>51.3
Red blood smear	Few spherocytes	Spherocytes	Spherocytes	Microspherocytes and poikilocytosis
Osmotic fragility (fresh blood)	Normal or slight increased	Increased	Increased	Increased
Osmotic fragility (incubation at 37 °C)	Increased	Increased	Increased	Increased
Splenectomy	Rarely	If the capacity level is decreased and depending on certain cases	Necessary >5 y-old	Necessary >2–3 y-old
Transfusions	0–1	0–2	>2	Regularly
SDS-PAGE (protein defect)	Normal	Sp, Ank + Sp, band 3, protein 4.2	Sp, Ank + Sp, band 3	Sp, Ank + Sp, band 3
Inheritance	AD	AD, *de novo*,	AD, *de novo*	AR

membrane protein including ankyrin, band 3, protein 4.2, α or β-spectrin and RhAg[40–48] (Table 2) result in the assembly of an intrinsically unstable membrane leading to vesiculation of the lipid bilayer resulting in increased cell sphericity and reduced cellular deformability. The sequestration of the non-deformable spherocytes in the spleen and their subsequent phagocytosis by the splenic macrophages is responsible for the anemia and splenomegaly. The number of spherocytes is highly variable from patient to patient: very few in 25 to 35% of mild cases of HS and in 33% of HS neonates to very large numbers in the more severe forms of HS. The severity of the HS is directly related to extent of membrane surface area loss and consequently the severity of spherocytosis.[38] Spherocytic red cells are associated with polychromatophilia and various red cell shape abnormalities depending on the associated membrane defect (Table 2)[49] (Fig. 2A, B,C). For example, "mushroom" shaped red cells are generally a feature of band 3 defects (Fig. 2A, green arrow). The Mean Cell Volume (MCV) is decreased variably in HS with largest decreases noted in severe forms of HS due to significant decreases in the spectrin content of the red cell membrane.[38] Importantly, the reticulocyte MCV (MCVr) is also decreased to variable extent depending on the severity of anemia. This feature is useful for distinguishing HS from autoimmune hemolytic anemia (AIHA) or ABO incompatibility, which is also associated with spherocytic red cells[38,39,50] but does not exhibit decreases in MCVr. Another important feature of HS red cells is cell dehydration as revealed by an increased percentage of hyperdense cells (cells with a hemoglobin concentration of more than 41 g/dl)[38,39,51,52] in association with increased Mean Corpuscular Hemoglobin Concentration (CHCM) values of >36 g/dl of mature red cells as well as of reticulocytes (CHCMr). In contrast, CHCMr values are normal in AIHA. It is important to note that the red cell and reticulocyte indices derived depend on the technologies used by various hematological analyzers and as such appropriate interpretation of CBC needs a thorough understanding of the technologies used to derive the various cellular parameters.

3.2. Confirmation of HS diagnosis

Patients with a family history of HS and typical HS biological manifestations (hemolytic anemia with high CHCM >36 g/dl, high percentage of hyperdense cells >4%, spherocytic cells on the blood smears) do not require any additional tests (grade 1 recommendation, grade A evidence on the latest 2011 guidelines for the diagnosis and management of HS[53]).

More specific biological tests may be required in cases where the HS diagnosis is not readily evident (lack of HS family history, lack of expression of typical biological manifestation including normal osmotic fragility when the test is performed and iron deficiency, which may mask the regeneration and the increased reticulocyte count).

3.2.1. EMA-binding and other tests

The osmotic fragility,[54] glycerol lysis [55] or Pink tests[56] may be used as first line of clinical laboratory tests. However, the sensitivity of these tests for diagnosis is low (68% for osmotic fragility test performed on fresh blood, 61% for the glycerol lysis test and 91% for the Pink test).[38,57,58] As a result, flow cytometry measurement of the mean red cell fluorescence, associated red cells following labeling with the dye eosin-5′ maleimide (EMA) to document surface area loss is being used an alternate test for diagnosis of HS.[53,58–71] The EMA binds covalently to the Lys430 on the first extracellular loop of the band 3 protein predominantly but it also interacts with sulfhydryl groups expressed by Rh, RhAg and CD47. This test is able to detect HS with a sensitivity of 92.7% and a specificity of 99.1%, with a positive predictive value of 97.8% (meaning that if the test is positive, the

Table 2
Molecular defects in HS.

Molecular defect	Prevalence in HS population	Inheritance	Prevalent mutation	Protein low expressed	Disease severity	Cytologic feature (MGG coloration of the blood smears)
Ankyrin-1 (*ANK1*)	40–65% in Europe and USA	AD, AR, *de novo*	AD or *de novo*: null mutation AR: missense or promoter mutation	Spectrin and ankyrin: 11% à 50%	Minor to moderate form	Spherocytes
Band 3 (*SLC4A1*)	20–35%	AD	Functionally null mutation	Band 3: 31%–35%	Minor to moderate form	Spherocytes, rare mushroom red cells
α spectrin (*SPTA1*)	<5%	AR	α-LEPRA and null mutation	α spectrin: 50–75%	Severe	Spherocytes, poikilocytosis, contracted red cells
β spectrin (*SPTB*)	15–30%	AD, *de novo*	Null mutation	β spectrin: 15–40%	Minor to moderate form	Spherocytes, 5–10% acanthocytes
4.2 Protein (*EPB42*)	<5% in Europe and USA (45–50% in Japan)	AR	Missense mutation	4.2 Protein: 95–100%	Minor to moderate form	Spherocytes, ovalostomatocytes

AD: autosomal dominant, AR: autosomal recessive, LEPRA: Low expression allele Prague.

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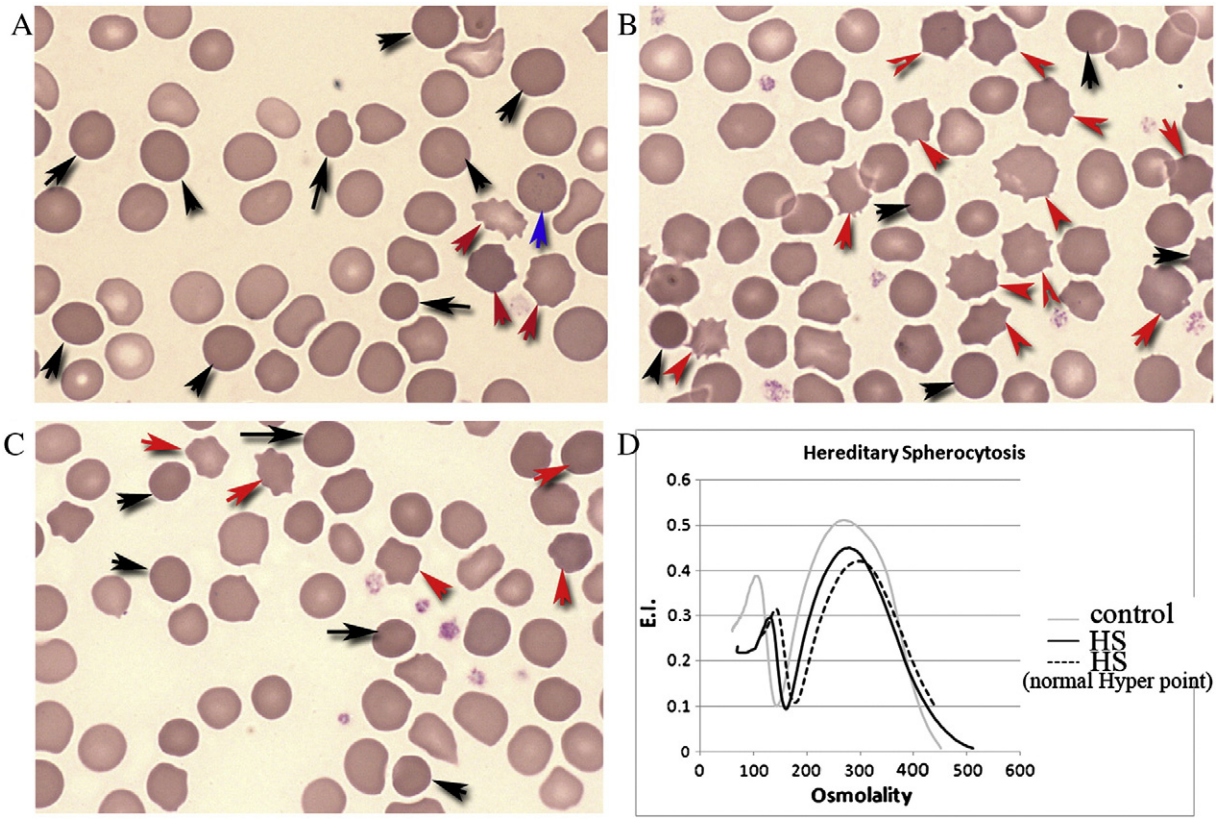

Fig. 2. Red cells under microscopic examination in HS. A) Band 3 defect with the classical mushroom feature (green arrow) in association with typical spherocytic red cells, which appear dense and hyperchromic (black arrows), few acanthocytes (red arrows) and basophilic red cell due to the hematopoietic stress (blue arrow). B) and C) β spectrin (*SPTB*) defect with a lot of acanthocytes (red arrows) in association with spherocytic red cells (black arrows). D) Ektacytometry curve of two different HS patients, dark line the typical feature with a decreased in the DImax as a sign of a decreased red cell deformability due to the surface area loss, a shift to the right of the Omin, which corresponds to the reduced surface to volume ratio and a shift to the left of the O'/Hyper point, which corresponds to the dehydration of the red cells.

diagnosis of HS is confirmatory in 97.8% of the cases) and a negative predictive value of 96.9% (meaning that if the test is negative, HS can be ruled out in 96.9% of the cases). This test is able to detect HS due to defects in band 3, spectrin and 4.2 but less effective for HS due to ankyrin defects.[70] The test can also be positive in pyropoikilocytosis (HPP) due to significant extent of cell fragmentation leading to generation of red cells with decreased surface area. However, if fragmented red cells are gated out by their distinct forward scatter signal, the non-fragmented red cells do not express decreased membrane associated fluorescence, which is in contrast to HS where the entire population of red cells exhibit decreased membrane associated fluorescence. The EMA test may be problematic in the case of South East Asian ovalocytosis (SAO) due to a nine amino acid deletion in the band 3 protein, with mutant protein failing to bind EMA. King et al. described decreased membrane associated fluorescence in patients with congenital dyserythropoietic anemia type II (CDAII) with abnormal glycosylation of band 3 and in 2 patients with cryohydrocytosis (positive test). However, it is to be noted that these two latter patients carried the band 3 memphis polymorphism.[70] The EMA-binding is not dependent on the phenotype and is positive also in compensated HS. However, the sensitivity is increased in splenectomized HS compared to non-splenectomized HS.[58] It is also independent of the molecular defect in the red cell membrane protein but it may be less sensitive for diagnosis if HS is with undefined molecular defects and with ankyrin defects.[58,70] The cut-off above which the test is considered positive is much debated. Classically, the mean fluorescence of patient red cells is compared to the mean fluorescence of red cells obtained from 6 age-matched controls on the same day. Ratio of mean fluorescence of the patient red cells to mean fluorescence derived for the 6 controls is derived and when the ratio is decreased by >21%, the test is considered positive while a value of <16% is considered negative. Values between 16 and 21% are considered indeterminate and additional studies are needed to confirm or rule out the diagnosis of HS.[72] The cut-off point in the extent of decreased fluorescence to validate diagnosis of HS is still a matter of debate. For Crisp et al.,[71] it is at 17% of mean channel fluorescence decrease and recently, Bianchi et al.,[58] described a lower cut-off at 11% to separate 150 HS patients from normal controls, which may lead to over-diagnose of HS if the laboratories perform only flow cytometry. Additional large-scale studies are needed in order to define the best cut-off and the best strategy to adopt for the diagnosis of HS. However, this test should replace the much lower sensitive and specific tests since: 1) it is easy to perform particularly in neonates, since only 5 μl of peripheral blood is needed and since a flow cytometer is available in most hematology laboratories; 2) the test results are available in 2 to 3 h; 3) the samples may be analyzed up to 7 days after the blood sampling[72]; and 4) the gating on the abnormal red cells allows the avoidance of the bias due to presence of transfused red cell enabling the diagnosis of HS in patients with a recent transfusion history.[67]

3.2.2. Ektacytometry

Bianchi et al.[58] point out the fact that the combination of different diagnosis tests can increase test sensitivity for diagnosis of HS when the EMA-binding test is combined with the acidified glycerol lysis test (AGLT)[73] (100% sensitivity) or with the Pink test (99% sensitivity). In our experience and since we have access in our laboratory to an ektacytometer,[23,74,75] we prefer to combine the EMA-binding test to the ektacytometry, generally acknowledged to be the reference technique for diagnosis of red cell membrane disorders.[21,38,39,57] In the 2011 guidelines, the recommended screening tests if the HS diagnosis is equivocal, are the cryohemolysis test[71] and EMA-binding test (grade 1 recommendation, grade A evidence)[53] and the osmotic fragility test is not recommended for routine use. However, the technique of osmotic gradient ektacytometry has not been evaluated in

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formulating the guidelines because of lack of its general availability, in spite of its recognition as the reference technique for diagnosis of red cell membrane disorders. The ektacytometer is a viscometer, in which the deformation of red cells suspended in a viscous PVP solution at defined values of applied shear stress is monitored as a continuous function of suspending medium osmolality. Three distinct features of the osmotic gradient ektacytometry profiles are: the Omin point, 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) value, which represents the maximal cellular deformability of the red cell population, and the O' or hyper point, which corresponds to the osmolarity at DImax/2, which reflects the hydration status of the red cells. In the case of HS,[38,39] the characteristic features are a decrease in the DImax, 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 left (increased dehydration of the red cells) (Fig. 2D). However, either Omin or O' may be in the normal range without ruling out the HS diagnosis (Fig. 2D, dashed curve). The amount of blood required to perform the test is small (100 μl) and as with EMA dye test ektacytometry can be performed on blood samples from neonates. The limitations of ektacytometry are the limited availability of the instrumentation and the need to perform the analysis within 48 h of blood sampling. Importantly, the ektacytometer generates distinct osmotic deformability profiles enabling diagnosis of not only HS but also the other red cell membrane disorders such as elliptocytosis, HPP, stomatocytosis and SAO.

3.2.3. Identification of the molecular defects (Tables 1 and 2)

The molecular defects responsible for HS are in large part detected by SDS-PAGE electrophoresis performed on red cell ghosts prepared from the fresh blood using a 4% to 12% gradient acrylamide gels according to Fairbanks[76] and/or discontinuous buffer system of Laemmli with linear gradient of acrylamide from 6% to 14%.[77] The use of SDS-PAGE is recommended[53] 1) when the clinical phenotype is more severe than predicted from the red cell morphology; 2) when the red cell morphology is more severe than predicted from parental blood films where one parent is known to have HS; 3) if the diagnosis is not clear; and 4) in any case prior to splenectomy, in order to avoid misdiagnosis in particular with stomatocytosis. In this latter case, the splenectomy will lead to lethal thrombosis, and is strictly contraindicated.[78] The molecular defect may be difficult to detect in case of ankyrin defects due to in part to large numbers of reticulocytes, which contain excess amounts of ankyrin and mask the reduction in ankyrin content of mature red cells, and in 20% of cases the molecular defect cannot be detected especially in asymptomatic carriers or individuals with very mild HS.[41]

- Mutations in *ANK1* gene are most frequently associated with HS and account for 50% of the cases. Sporadic or *de novo* mutations are often described in recessive HS associated with *ANK1* gene mutation while transmission is dominant in the familial cases. No homozygous mutation has been identified to date. All forms of mutations have been reported: frameshift (17), nonsense (8), abnormal splicing (4), missense (4), and even in the promoter region (mutations *in trans* to mutations in the coding sequence) (2).[53] Ankyrin Florianopolis, which results in one nucleotide insertion in exon 14 is a recurrent mutation, which has been found in three unrelated severe HS patients.[79] On SDS-PAGE electrophoresis, ankyrin defect when present is sometimes associated with reduced spectrin content and consistently with decreased levels of band 4.2.
- Mutation in *SPTB* gene, encoding for the β-spectrin protein is mutated in 20% of the HS cases. As with *ANK1* gene, transmission is dominant but some sporadic mutations have also been identified. No homozygous mutation has been identified to date. On SDS-PAGE gels, only the spectrin bands are reduced. More than 20 mutations (10 null mutations, 10 nonsense or in non-coding sequence, 5 missense) are reported[53] amongst them the spectrin Kissimmee, which alters the β-spectrin binding to 4.1 (p.Trp202Arg) or the spectrin Primassao, which modifies the translational initiation start site.
- Mutations in *SLC4A1* gene, encoding for the anion exchanger protein band 3, are identified in ~15 to 20% of the HS cases that include missense (23), nonsense (18), and larger mutant proteins (3).[53] Band 3 and protein 4.2 are decreased on the SDS-PAGE gels to different extents depending on the mutation. A case of homozygous null mutation (Coimbra mutation)[35] results in complete deficiency of band 3 and very severe anemia. Inheritance of two heterozygous *SLC4A1* mutations with different levels of expression has been shown to worsen the clinical phenotype in one affected HS patient.[80,81] Transmission is dominant and no sporadic mutations have been reported.
- Mutations in *EPB42* gene, encoding for protein 4.2 are rare and the SDS-PAGE exhibits the absence of protein 4.2 as a result of homozygous or compound heterozygous mutations. Only 10 mutations have been described, which include missense (4), nonsense or deletion (3) and splicing (2) mutations.[53,82] All the known mutations are reported in the NH_2-terminal region of protein 4.2, which binds to band 3. Mutations in *EPB42* gene are often described in the Japanese population.
- Mutations in *SPTA1* gene, encoding for the α-spectrin chain are extremely rarely associated with HS. When present, only homozygous mutation or compound heterozygous mutations are responsible for HS. Indeed, due to synthesis of a large excess of α-spectrin in erythroid cells, the defect of α-spectrin needs to be severe to lead to a HS phenotype. Of particular interest, is the spectrin Prague mutation leading to the exon 37 skipping and frameshift.[83,84] This mutation is in *trans* of allele α^{LEPRA} (LEPRA: Low Expression PRAgue), a low expression recurrent allele of the *SPTA1* gene (3.3%). This allele is silent if carried by a normal individual. Allele α^{LEPRA} carries the C>T transition at position −99 of intron 30. It activates an alternative acceptor site at position −70 of intron 30 causing mis-splicing and frameshift in about 80% of the pre-mRNA. Allele α^{LEPRA} is associated *in cis* with a functionally neutral polymorphism, Bug Hill (GCT>GAT codon 970; Ala>Asp). Spα^{LEPRA} allele is prevalent in non-dominant HS[83,85] and can result in a marked deficiency of both α and β spectrin bands in the affected propositus but spectrin content is normal in both parents.

At present, routine screening for mutations in various genes accounting for analysis of the affected genes in HS is not well established although progress is made in this avenue. Furthermore, knowledge of the molecular defect doesn't influence the clinical management of HS.

3.2.4. Other biological indicators

It is important to emphasize the fact that cytology with a meticulous microscopic examination of the blood smear examination be the first important step for the diagnosis of HS and all the red cell disorders. Otherwise it is likely that misdiagnosis or over-diagnosis of these red cell disorders will occur.

Other biological tests that are important in the diagnosis to confirm the hemolytic anemia include: increased indirect bilirubinemia >17 μmol/l, increased LDH >300 IU/l, a decrease in haptoglobin <10 mg/dl (signature of the hemolytic anemia) and a DAT test to rule out an immune hemolytic anemia (AIHA or ABO incompatibility).

We propose the flow chart for laboratory diagnosis of HS in Fig. 3A and B.

3.3. Clinical management of affected HS individuals

In the familial HS, with the exception of some severe cases of HS, in which there is a need for *in utero* transfusion program during the pregnancy,[86] the management of the mild to moderate HS begins at

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birth, the hemoglobin being often normal at birth but decreasing rapidly after birth. The educational program of both parents is mandatory. It must emphasize the signs of anemia at this age of life (pallor, difficulties to thrive, dyspnea). The French orphanet website (http://www.orpha.net/) has posted very helpful information for the families.[57] The weekly follow-up on hemoglobin and the other red cell parameters including the reticulocyte count and blood smear exam is necessary until the hemoglobin levels stabilize with a reticulocyte count corresponding to the degree of anemia and hemoglobin levels. Care of the infant by a hematologist in concert with the pediatrician or the general practitioner is important for good clinical management. After the stabilization of hemoglobin values, the duration of which may be different from one patient to another, a once a year visit that includes growth measurement and medical exam is sufficient. In the absence of symptoms, the most recent recommendations do not suggest an annual blood test.[53] The parents should be warned about the risk of parvovirus B19 infection (enhancement of the pallor, asthenia, dyspnea, fever and the classical skin eruption) and the need for their infants and children to seek immediate medical attention. Confirmation of diagnosis leads to transfusion of red cells to correct the sudden aregenerative anemia, due to the erythroid tropism of the parvovirus leading to the erythroblastopenia. The HS children with severe anemia need to be carefully monitored during breakout of any viral infections.

The vaccination against hepatitis B is recommended since the HS patients may need regular transfusions, in addition to the usually recommended vaccinations. During the neonate period, treatment is

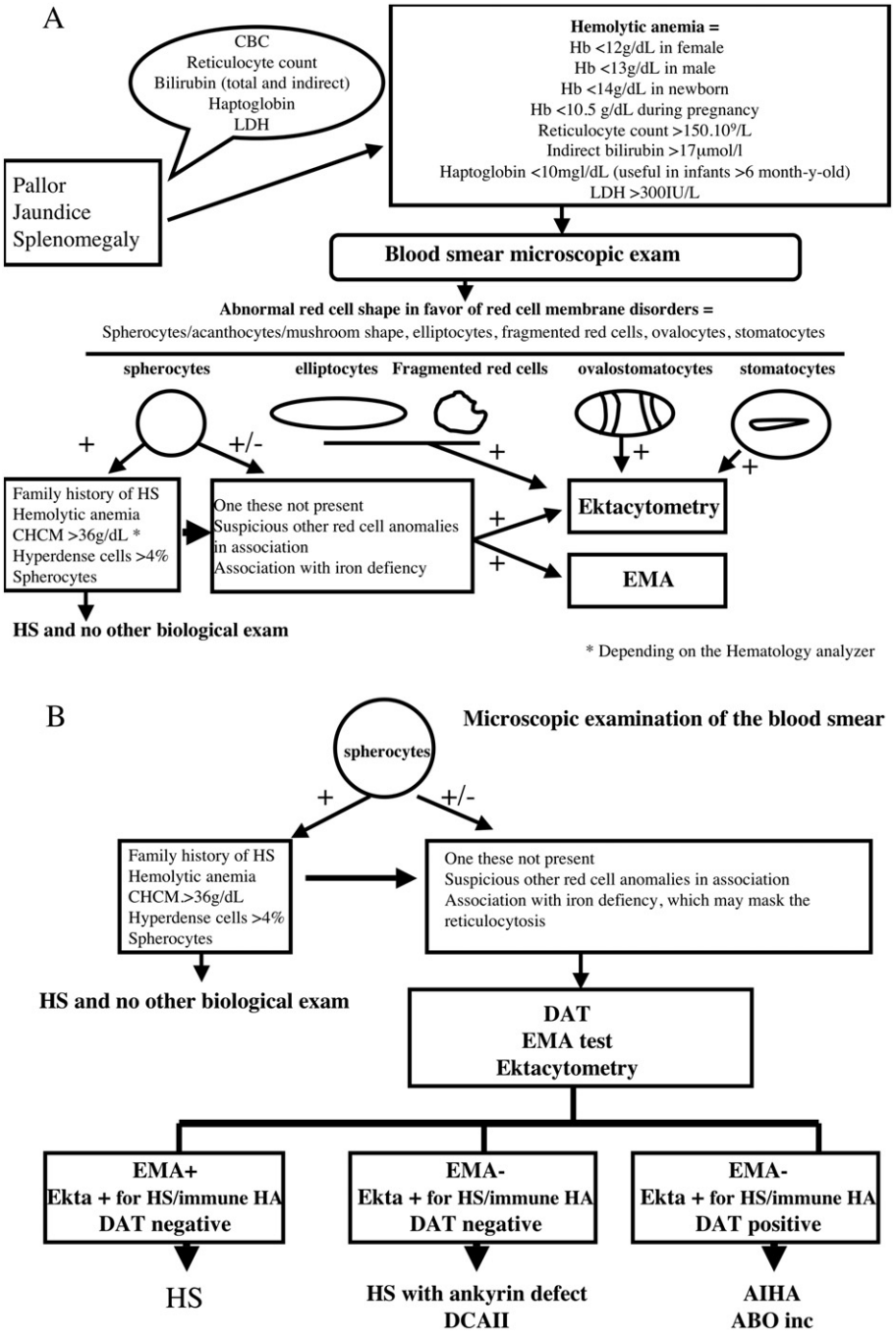

Fig. 3. Flow chart proposal for laboratory diagnosis of HS and other red cell membrane disorders (A) and in particular HS (B).

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necessary for moderate to severe forms of HS with folate supplementation at the dose of 2.5 mg/D to manage nutritional requirements of stress erythropoiesis. Recombinant erythropoietin (EPO) treatment at the dose of 1000 IU/kg/sem in 3 subcutaneous injections has been evaluated in 16 transfusion-dependent neonates and infants and resulted in avoidance of transfusions in 10 of 16 and the need for only one transfusion in 3 of 6 HS infants.[87] However, use recombinant EPO is still debated and large and multicenter clinical studies are needed to validate its effectiveness.

Coinheritance of a hemochromatosis gene can aggravate the iron overload in a chronic hemolytic anemia, in which the iron absorption is already increased. Red cells matched for antigens in the Rhesus and Kell systems should be used for transfusion when the hemoglobin falls below a non-tolerable level, but there is no set threshold of hemoglobin value below, which transfusion is needed. Only the tolerance of anemia should be an important determinant in the decision to transfuse or not.

Splenectomy should not be performed simply based on HS diagnosis but only on the basis of severity of anemia. Thus, splenectomy is indicated in severe HS, with significant anemia and gallstone complications, while splenectomy should be considered in moderate HS if anemia has an important effect on quality of life. Indeed, splenectomy abolishes anemia and gallstone complication in such cases. But, it should not be performed in cases of mild HS except in specific cases with large reductions in exercise tolerance. The laparoscopic surgical approach is recommended but is dependent on the availability of appropriately trained surgeons and suitable equipment.[88] In any case, splenectomy should be considered only after 6 years of age[53] with the usual precautions (vaccinations according to the national guidelines, penicillin antibiotics and parent education in case of fever >38 °C) due to the infectious risks with some encapsulated germs such as *haemophilus B*, *meningococcus*, and *Streptococcus pneumonia* bacteria. It is important to ascertain that the splenectomized patients adhere to the latest vaccination guidelines (Price et al., for US[89]). The patients and parents should be aware of life-long risk of overwhelming sepsis following splenectomy. However, there is no consensus regarding the need for reimmunization and its frequency, the type and the duration of the prophylactic post-splenectomy antibiotics.[53] The risk of thrombosis in HS patients following splenectomy is the same as in the general population and the prophylactic anticoagulation to prevent thrombosis should adhere to standard protocols. However, the HS diagnosis needs to be fully validated prior to splenectomy since in certain red cell disorders, such as stomatocytosis splenectomy is accompanied by lethal thrombosis or because splenectomy is less effective in disorders such as CDAII.

Partial splenectomy can be performed instead of total splenectomy in order to reduce the infectious risk, while eliminating most of the red cell destruction site, reducing anemia, reticulocytosis and hyperbilirubinemia.[90–100] The largest multicenter study so far on 62 HS infants from 1990 to 2008[96] has confirmed the benefit of the partial splenectomy with no post-splenectomy sepsis complication in 18 years of follow-up and with only 4.84% of the patients undergoing subsequent total splenectomy.

The question of splenectomy and cholecystectomy has finally been resolved and cholecystectomy should not be performed if there is no cholelithiasis at the time of splenectomy. Splenectomy reduces hyperbilirubinemia and thus no pigment stones are formed following splenectomy, reducing the risk of gallstones.[101] However, Gilbert disease in HS patients increases the risk of gallstone formation by 5 fold.[102] Ultrasound measurement is the reference procedure used to detect gallstones and recommended after the age of 5 years. It also allows an accurate measurement of the spleen size, which is important before laparoscopic splenectomy.[102] Cholecystectomy should be performed if the gallstones are symptomatic otherwise it has been shown that cholecystectomy alters the bile salt metabolism and increases the risk of colon carcinoma later in life.[103] However, if the gall bladder is left *in situ* even after gallstones extraction, a close follow-up using ultrasound is necessary. The other scenario of whether the spleen should be removed if cholecystectomy is needed in case of symptomatic gallstones in mild HS is still being debated. Recently, Alizai et al.[104] reported that only 3 mild HS individuals out of 16 who underwent cholecystectomy without splenectomy have been splenectomized subsequently within 2 to 5 years.

4. Hereditary elliptocytosis (HE), pyropoikilocytosis (HPP), South East Asian ovalocytosis (SAO)

Hereditary elliptocytosis is another red cell membrane disorder characterized by mutations in genes encoding membrane or skeletal proteins, which alters membrane function and reduces red cell deformability. HE is due to defects in the horizontal protein connections of the red cell membrane skeletal network including the spectrin dimer–dimer interaction or the spectrin–actin–protein 4.1R junctional complex. The genes mutated in HE are thus the α-spectrin (*SPTA1*), the β-spectrin (*SPTB*) or protein *4.1R* genes.[105–117] An acquired deficit in 4.1R is reported in myelodysplastic syndromes and HE diagnosis in adults, with no history of hemolytic anemia during infancy should consider this possibility in differential diagnosis.[118–120]

HE is a common red cell hemolytic anemia (3 to 5 affected individuals for 10,000), with a worldwide distribution but a higher prevalence like for other red cell defects in malaria endemic regions.[121] The red cell shape is classically elliptic, with different features from the short stick shape (4.1 deficiency) (Fig. 4A,B, red arrows) to the shape more oblong on the blood smears (Fig. 4A, B, blue arrows).[49] The phenotype and genotype are heterogeneous with autosomal dominant inheritance with the exception of the pyropoikilocytosis (HPP). The vast majority of the HE affected individuals are asymptomatic and the HE is discovered fortuitously on a blood smear, while some patients exhibit hemolytic anemia with anemia, jaundice and splenomegaly. Neonatal jaundice, hemolytic anemia and hydrops fetalis are also reported.[37,122] It may be difficult to distinguish HE with neonatal poikilocytosis from HPP (Fig. 4B, black arrows). In HE with neonatal poikilocytosis, fragmentation and hemolysis decline with time and the phenotype after 4 months to 2 year of age is a mild common HE. The worsening of the hemolysis in the first months of life has been attributed to the particularities of the fetal erythropoiesis and the large amount of the fetal hemoglobin in the first months in the red cells, which is responsible for the increase in 2,3-DPG concentration in the cell, which destabilizes the spectrin–actin–protein 4.1 complex, enhancing membrane instability.[123] Splenectomy with the usual precautions may be a good option in order to increase the red cell life-span and increase the hemoglobin levels but it should be considered only for severe forms of elliptocytosis and after 5 years of age. In HPP, splenectomy reduces but does not eliminate hemolysis completely.

The heterozygous mutated patients are classically asymptomatic while the clinically evident HE patients exhibit anemia that ranges from mild to severe including the severe HE variant, HPP due to a homozygous mutation or compound heterozygous mutations. In the severe form HPP, extensive red cell fragmentation is responsible for a large decrease in the MCV. Some of the fragmented red cells are counted as platelets by the hematology analyzer and as such overestimate the platelets counts. In these instances platelet count should be performed manually. Another feature of red cells in HPP is their increased sensitivity to thermal fragmentation at a lower temperature (45° to 46 °C) rather than normal (49 °C). The more specialized tests include ektacytometry, SDS-PAGE electrophoresis and analysis of spectrin tetramer–dimer ratios using non-denaturing gels. Molecular biological studies (screening of the low expression polymorphism α-^{LELY}) are not necessary for the diagnosis but may be useful in the severe and persistent forms, including HPP (Fig. 4B). In the typical HE forms, the ektacytometry curve exhibits a trapezoidal form with a decrease in the red cell deformability

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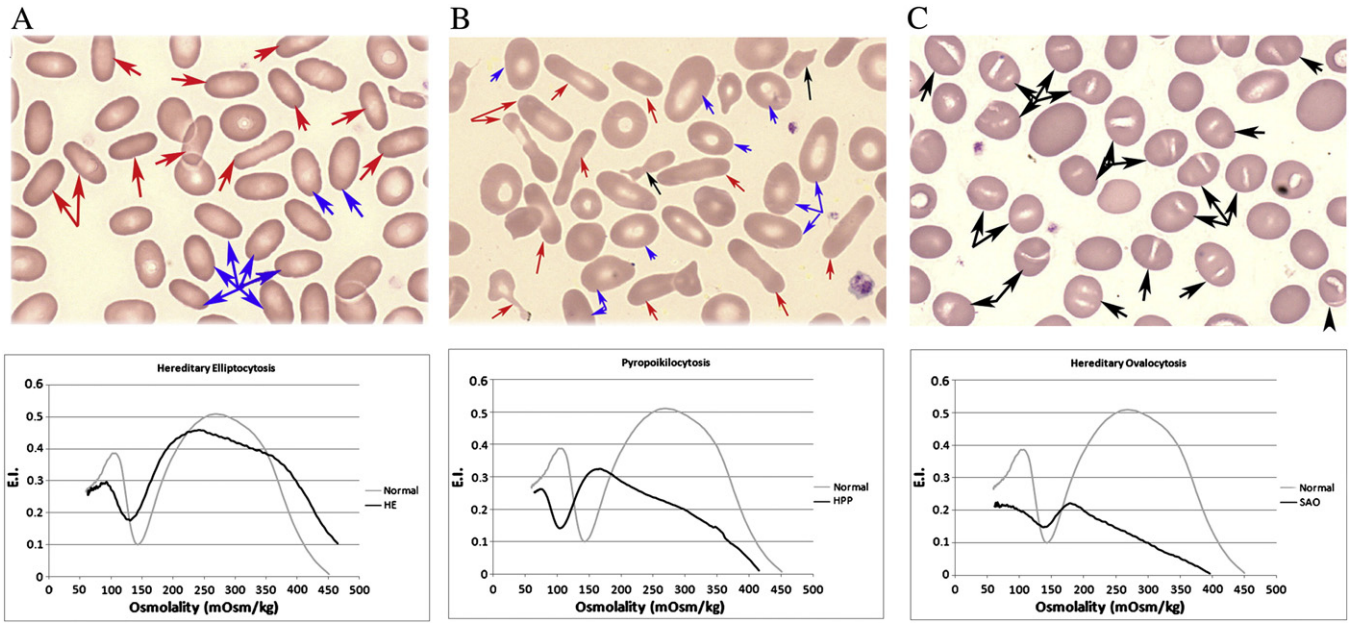

Fig. 4. Red cell in hereditary elliptocytosis and other elliptic red cell membrane disorders. A) Top panel: hereditary elliptocytosis with the classical form (red arrows) and more ovalocytic red cells (blue arrows). Bottom panel: Ektacytometry curve in HE with the classical trapezoidal feature and characterized with a decreased DImax and normal Omin and Hyper points. B) Top panel: elliptic (red arrows) and ovalocytic red cells (red arrows) with fragmented cells (black arrows), which characterized the severe form of HE, pyropoikilocytosis (HPP). Bottom panel: the ektacytometry curve with a large decrease in DImax and a shift to the left of both Omin and Hyper points. C) Top panel: classical feature of ovalocytic red cells in South-east Asian ovalocytosis (SAO) (black arrows). Bottom panel: the ektacytometry curve, which exhibits the undeformable feature of the red cells with close to null DImax.

(decreased DImax) (Fig. 4A, bottom panel). On the SDS-PAGE, in the severe homozygous HE due to mutation in *4.1R* gene, an absence of 4.1 and p55, and 30% of the normal content of the glycophorins C and D are observed. In addition the two classical forms of 4.1R defect can be seen with a truncated 4.1R (4.1 65/68) or an elongated high molecular weight of 4.1R (4.1 95).[4] In addition, HE due to 4.1R defect exhibits a large number of thin and elongated elliptocytes, such as bacillus on the blood smear (Fig. 4A). In HE due to mutation in either α- or β-spectrin there is an increase in spectrin dimer to tetramer ratio as documented by non-denaturing gel electrophoresis of spectrin extracted at 4 °C. In HPP, additional deficiency in spectrin/band 3 ratio is observed on the SDS-PAGE electrophoresis and explains the presence of spherocytes on the blood smears amongst with fragmented cells. Diagnosis of HPP, due to low expression LELY allele can be easily performed by the molecular screening of the low expression polymorphism α^{LELY}. Spα^{LELY} is a very common polymorphism, with 42% heterozygosity and 9% homozygosity in European Caucasian population. Its identification in the proband and the molecular screening analysis of both parents with a meticulous blood smear exam with one parent exhibiting elliptocytes, the other no red cell morphology abnormality and but carrying the α-^{LELY} polymorphism are sufficient to confirm the HPP diagnosis in the proband. Spα^{LELY} is a combination of two linked mutations, one in exon 40 and one in intron 45 on the α-spectrin gene. Exon 40 mutation, the G>C transition leads to the p.Leu1857Val amino acid change. The p.Leu1857Val is linked to the other allele variation C>T transition in intron 45, minus 12 nucleotides from the 3′ splice site of exon 46. This latter allelic variation leads to exon 46 skipping in 50% of α spectrin mRNA, which fails to assemble into stable spectrin dimer and α^{LELY} △exon 46 alleles which are degraded. This allele variation is responsible for low allele expression. The α^{LELY} polymorphism is completely asymptomatic at the heterozygous but also at the homozygous state, due to the large excess by 3 to 4 fold of the α spectrin chains, but when the α^{LELY} is associated *in trans* with a mutation of the α spectrin gene, the mutated spectrin forms increase and are responsible for the HPP phenotype. The severity of HE is dependent on the extent of the membrane instability,[112] *per se* dependent on the extent of the loss of surface area. Indeed, marked red cell fragmentation is a key feature of HPP. In this case, either a homozygous mutation or heterozygous compound mutations in the spectrin genes are reported but also the association between the low expression V/41 polymorphism α^{LELY} *in trans* and a mutation on the α-spectrin *SPTA1* gene *in cis*.[124,125]

The hereditary ovalocytosis, also designated as South-east Asian ovalocytosis (SAO) has a geographical distribution mostly in the malaria endemic regions of Indonesia, Philippines, Melanesia and Southern Thailand as SAO phenotypes offer protection against both *Plasmodium falciparum* and *Plasmodium vivax* malaria.[126,127] Deformability of SAO red cells quantitated by either ektacytometry (Fig. 4C, bottom panel) or by the micropipette aspiration technique is dramatically decreased.[20,128] Paradoxically, this extent of decreased deformability *in vitro* has little effect on red cell survival *in vivo* and the adult affected individuals are completely asymptomatic and diagnosis is usually made coincidentally on examination of blood smears with the characteristic feature of oval shaped red cells with 1 to 2 transverse ridges (Fig. 4C, top panel). SAO may however express as a hemolytic anemia in the neonates and requires phototherapy. The inheritance is autosomal dominant and to date only heterozygous individuals have been reported.[129] SAO results a mutation in the gene encoding band 3 and 3 (*SLC4A1*), characterized by a deletion of the 27 nucleotides encoding the amino acids 400 to 408 of band 3. While various hypotheses have been proposed to explain the discrepancy between the mild phenotype and the strong effect of the band 3 mutation on the red cell membrane rigidity, no clear explanations have emerged.

5. Stomatocytosis

This rare red cell disorder is divided into two different entities: the xerocytosis or dehydrated hereditary stomatocytosis (DHSt) and the overhydrated hereditary stomatocytosis (OHS).[1,2,4,41,130] Both exhibit a cation leak to the univalent cations Na^+ and K^+ resulting in altered intracellular cation content and cell volume alterations. The inheritance of stomatocytosis is autosomal dominant. The phenotype can vary from asymptomatic to severe hemolytic form.

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In the DHSt, the most frequent form, the main characteristic is red cell dehydration, due to the loss of the cation content, in particular K^+ and cell water. As a consequence, the MCHC is increased (>36 g/dl) and the ektacytometric osmotic deformability profile is shifted to the left. Strikingly, the red cell survival is not significantly compromised. The phenotype varies from mild to moderate anemia with a normal or slightly increased MCV in spite of cell dehydration, with the presence of stomatocytes with the classical mouth feature on blood smears (Fig. 5A, B,C). The diagnosis may be difficult when the number of stomatocytes is low. DHSt diagnosis may be difficult to diagnose when associated to pseudohyperkalemia or perinatal edema.[131] DHSt may be responsible for hydrops fetalis.[132,133] The locus of the candidate gene has been located in 16q23-24[134] and redefined more recently in 16q24.2-16qter from two large families from Rochester, USA and Manitoba, Canada.[135] Recently, Zarychanski et al.[136] by copy number analyses, linkage analysis, and exome sequencing, have identified that mutations in PIEZO1 protein encoded by *FAM38A* gene are associated with DHSt in all affected DHS patients from both North American families. PIEZO proteins are the pore-forming subunits of channels that control the mechanotransduction and stretch-activated cation channel activation in mammalian cells. PIEZO protein has been identified in the red cell membrane. PIEZO proteins, which have been only recently identified[137] may play an important role in the red cell cation and volume homeostasis.

In the overhydrated hereditary stomatocytosis (OHS), in contrast to DHSt, the red cells are hydrated due to a 20 to 40 fold increase in the cation leak. This form of stomatocytosis is rare (20 reported cases worldwide) but leads to the most severe phenotypes. In addition to reticulocytosis, hemolytic anemia in OHS is characterized by a large increase in MCV (>110 fl but up to 150 fl in some cases) and decreased MCHC (between 24 and 30 g/dl), with a shift to the right of the ektacytometric osmotic deformability profile. All these red cell features reflect the over-hydration of red cells. In this form, the number of the stomatocytes on the blood smear is usually much higher than that in the DHSt and the diagnosis is easy to establish. Recently, mutations p.Ile61Arg and p.Phe65Ser of the RhAg (Rh-associated glycoprotein) have been identified in the OHS. RhAg is a member of the band 3 complex and is considered as an ammonium transporter and/or gas channel.[138,139] Stomatin protein has not been found mutated in OHS but has been found to be expressed at low levels or absent in OHS.[140] As no defect in gene encoding stomatin could be identified and stomatin protein expression appears normal it is likely that observed stomatin defect is secondary. It has been suggested that the stomatin defect increases the glucose uptake through its interacting with Glut1 transporter.

Other forms of stomatocytosis have been described: the familial pseudohyperkalemia and the cryocytosis. Familial pseudohyperkalemia is particular in that it is not associated with hemolytic anemia and stomatocytes are uncommon but red cells exhibit a leak of K^+ at room temperature but not at physiologic temperature. The gene responsible for this such form is yet to be defined but two different loci have been described: one involved in DHSt and the other one maps to chromosome 2q35-q36.[141,142] In the cryocytosis (CHC) form, affected patients exhibit mild to moderate hemolytic anemia. Band 3 mutations (p.Ser731Pro, p.His734Gln, and others affecting the amino acids D705, R760,[143] and the p.Gly796Arg[144]), which transform band 3 anion exchanger into a cation transporter, are described. The band 3 mutation p.Leu687Pro is responsible for an intermediate form between the CHC and the pseudohyperkalemia (Blackburn stomatocytosis).[143] The complexity is even higher when some forms of stomatocytosis, with band 3 mutation p.Asp705Tyr and p.Arg760Gln have been reclassified as HS with a low temperature leak (HS-LTL)[143] or when the mutated p.Gly796Arg band 3 is associated not only with stomatocytosis but also to congenital dyserythropoietic type I (CDAI) feature.[144,145]

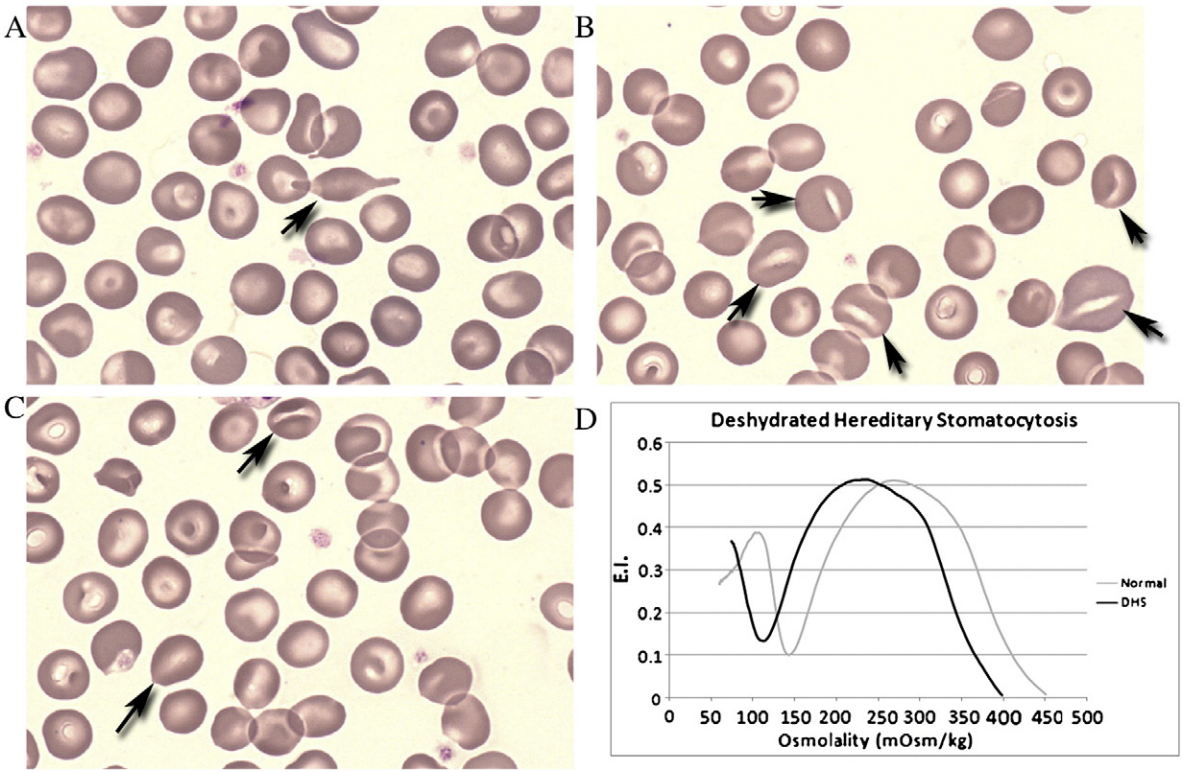

Fig. 5. A, B, and C: three cytological features of dehydrated stomatocytosis in A only the mouth red cell noticed; the number of stomatocytic red cell being sometimes very low. B and C: more stomatocytic red cells (black arrows). D) Classical ektacytometry curve of DHSt with a normal DImax and a shift to the left of Omin and Hyper point, corresponding to the dehydrated red cells.

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The stomatocytosis entities are still incompletely understood but it is anticipated that in the near future, the comprehensive understandings of the various red cell transporters will shed new exciting insights in deciphering mechanistic basis for these rare red cell disorders.

Practice points:

- Red cell membrane disorders are common and responsible for hemolytic anemia.
- The clinical severity is extremely variable ranging from asymptomatic to severe hemolytic anemia.
- Underdiagnosis or overdiagnosis or misdiagnosis of the red cell membrane defects is not uncommon.
- Osmotic fragility test is not sensitive and its usefulness is limited.
- The best diagnostic reference technique is osmotic gradient ektacytometry.
- Diagnosis of the hereditary spherocytosis is easy.
- Quantitation of membrane associated fluorescence following the EMA-labeling of red cells by flow cytometry can effectively diagnose HS with the use of controls and choice of appropriate cutoff values to avoid both overdiagnosis and underdiagnosis.
- False positive EMA test can be eliminated by a meticulous microscopic cytological exam. The microscopic analysis should always be the most important component of the diagnostic strategy of all cases of red cell membrane disorders.
- Molecular biological approaches while useful have to be further developed to be of use in routine diagnosis of membrane disorders except particular cases of spherocytosis and elliptocytosis ($sp\alpha^{LEPRA}$ and $sp\alpha^{LELY}$ respectively).
- Genes involved in hereditary stomatocytosis are yet to be fully defined.
- Splenectomy is contraindicated and should not be performed in cases of hereditary stomatocytosis.

Research agenda:

- Genes involved in stomatocytosis.
- Consensus cut-off of the positive EMA test, which currently depends on the experience of individual laboratories.
- Comprehensive understanding of the discordance between *in vitro* finding of marked red cell rigidity of SAO red cells and lack of *in vivo* consequences.
- Role of the molecular mutation analysis for diagnosis using Next generation sequence technologies.

Conflict of interest statement

None of the authors have any conflicts of interest to declare.

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