



# ICSH guidelines for the laboratory diagnosis of nonimmune hereditary red cell membrane disorders

M.-J. KING\*, L. GARÇON†, J. D. HOYER‡, A. IOLASCON§, V. PICARD¶, G. STEWART\*\*\*, P. BIANCHI††, S.-H. LEE‡‡, A. ZANELLA††, FOR THE INTERNATIONAL COUNCIL FOR STANDARDIZATION IN HAEMATOLOGY

\*Membrane Biochemistry, NHS Blood and Transplant, Bristol, UK

†Laboratoire d'Hématologie, Centre de Biologie Humaine, CHU d'Amiens, Amiens, France

‡Department of Laboratory Medicine and Pathology, Mayo Clinic Rochester, Rochester, MN, USA

§Department of Molecular Medicine & Medical Biotechnologies, University Federico II of Naples, Naples, Italy

¶Hématologie Biologique, Bicêtre et Faculté de Pharmacie, AP-HP Hôpital, Université Paris-Sud, Le Kremlin Bicêtre, France

\*\*Division of Medicine, University College London, London, UK

††Hematology Unit, Physiopathology of Anemias Unit, Foundation IRCCS Cà Granada Ospedale Maggiore Policlinico, Milan, Italy

‡‡Haematology Department, St. George Hospital, SEALS Central, Sydney, NSW, Australia

## Correspondence:

May-Jean King, Membrane Biochemistry, NHS Blood and Transplant, 500 North Bristol Park, Northway, Filton, Bristol BS34 7QH, UK.

Tel.: +44 (0) 117 921 7601;

Fax: +44 (0) 117 912 5782;

E-mail: may-jean.king@nhsbt.nhs.uk

## SUMMARY

**Introduction:** Hereditary spherocytosis (HS), hereditary elliptocytosis (HE), and hereditary stomatocytosis (HSt) are inherited red cell disorders caused by defects in various membrane proteins. The heterogeneous clinical presentation, biochemical and genetic abnormalities in HS and HE have been well documented. The need to raise the awareness of HSt, albeit its much lower prevalence than HS, is due to the undesirable outcome of splenectomy in these patients.

**Methods:** The scope of this guideline is to identify the characteristic clinical features, the red cell parameters (including red cell morphology) for these red cell disorders associated, respectively, with defective cytoskeleton (HS and HE) and abnormal cation permeability in the lipid bilayer (HSt) of the red cell. The current screening tests for HS are described, and their limitations are highlighted.

**Results:** An appropriate diagnosis can often be made when the screening test result(s) is reviewed together with the patient's clinical/family history, blood count results, reticulocyte count, red cell morphology, and chemistry results. SDS-polyacrylamide gel electrophoresis of erythrocyte membrane proteins, monovalent cation flux measurement, and molecular analysis of membrane protein genes are specialist tests for further investigation.

**Conclusion:** Specialist tests provide additional evidence in supporting the diagnosis and that will facilitate the management of the patient. In the case of a patient's clinical phenotype being more severe than the affected members within the immediate family, molecular testing of all family members is useful for confirming the diagnosis and allows an insight into the molecular basis of the abnormality such as a recessive mode of inheritance or a *de novo* mutation.

---

<sup>1</sup>Icsh Representative.

doi:10.1111/ijlh.12335

Received 4 November 2014;  
accepted for publication 22  
January 2015

---

*Keywords*

Hereditary spherocytosis, hereditary elliptocytosis, hereditary stomatocytosis, red cell membrane defects, inherited hemolytic anemia

---



---

Table of Contents

---

1. Introduction
    - 1.1. Red blood cell membrane structure
  2. Hereditary spherocytosis
  3. Hereditary elliptocytosis and hereditary pyropoikilocytosis
  4. Hereditary stomatocytosis
  5. Coexistence of membranopathy and other red cell disorders
  6. Diagnostic tests for red cell membrane defects
    - 6.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of red cell membrane proteins
      - 6.1.1. Membrane protein defects in HS
      - 6.1.2. Membrane protein defects in HE and HPP
    - 6.2. Screening tests for hereditary spherocytosis
      - 6.2.1. The Osmotic Fragility Test
      - 6.2.2. The Acid Glycerol Lysis Time Test
      - 6.2.3. Osmotic gradient ektacytometry
      - 6.2.4. The Eosin-5'-Maleimide (EMA) Binding test
  7. Molecular genetic analysis
  8. Recommendations for laboratory diagnosis of red cell membrane Disorders
  9. Acknowledgements
  10. Conflict of Interest
  11. References
  12. Supporting information (Appendices) online version
    - Appendix A. Blood smears of different red cell disorders
    - Appendix B. Screening tests for hereditary spherocytosis
    - Appendix C. Identification of specific mutations in membrane protein genes
- 

## 1. INTRODUCTION

The designation for each of the hereditary red cell membrane disorders denotes the characteristic red cell morphology expected to be found on the peripheral blood smear of the patient. Abnormal red cell morphology is often an indicator but not necessarily specific for the named red cell disorder (Figure S1 in Appendix A). In light of new blood cell parameters being measured by the blood cell analyzers [1], it is possible to use some of the red cell parameters as indicators for an initial consideration of membranopathy.

The adoption of SDS-polyacrylamide gel electrophoresis and molecular genetic techniques since the 1970s has made significant inroads into understanding the pathophysiology of hereditary spherocytosis (HS), hereditary elliptocytosis (HE), and hereditary pyropoikilocytosis (HPP). Knowing the underlying causes of these red cell membrane disorders has also

enriched our basic knowledge in the structural organization of human red cell membrane. In the last 20 years, further progress has been made in understanding the pathophysiology of a group of rare membrane disorders caused by protein defects in the lipid bilayer, the hereditary stomatocytoses (HSt). The volumes of red cells in certain types of HSt can be markedly altered due to abnormal monovalent cation fluxes [2, 3]. Recently, a genome-wide association study of hemoglobin concentration and related parameters (Hb, MCH, MCHC, MCV, PCV, and RBC) has revealed possible effects of 75 independent genetic loci on the genetic mechanisms and biological pathways that control red blood cell formation and function [4].

Two published Guidelines have already dealt with the diagnosis and management of hereditary spherocytosis [5, 6]. Although hereditary stomatocytosis (HSt) is about 30- to 40-fold less common than HS, its

inclusion in this guideline is to raise the awareness for the identification and differentiation between HS, overhydrated and dehydrated HSt. This is due to an increased risk of thromboembolic events in those patients with these two types of HSt postsplenectomy [7, 8]. Variable degrees of amelioration of anemia postsplenectomy have also been reported [9–11].

The writing of this guideline has made use of evidence-based materials in peer-reviewed publications from online literature search using key words relevant to the subject matter. The unpublished data presented herein are merely for illustration purpose. It is to the best knowledge of the contributors that the information presented is accurate. Omissions of other citations were unavoidable due to massive amounts of publications on these topics. Presented in the Appendices of this Guideline are supplementary materials, such as blood smears (Appendix A), background information on selected screening tests (Appendix B), and a selection of mutations associated with the protein genes (Appendix C).

### 1.1. Red blood cell membrane structure

The human erythrocyte membrane is a laminated structure comprising of two distinct layers with no direct contact with each other (Figure 1). Their compositions and physiological functions are very different. The outer layer is composed of two asymmetric

phospholipid leaflets. Embedded within this lipid bilayer are numerous transmembrane proteins and discrete lipid raft domains where GPI-anchored proteins reside. This 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 red cell has high intracellular  $K^+$  and low intracellular  $Na^+$  contents when compared with the corresponding ion concentrations in plasma. The maintenance of this cation gradient between the red 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. An imbalance of intracellular cation content can result in alteration of red cell volume (e.g. dehydration or swelling), which is the underlying cause of hereditary stomatocytosis, in which the leak rate of cation exceeds the pump rate [12].

Covering about 65% of the cytoplasmic cell surface of the lipid bilayer is a network of spectrin and actin, which form a stable ternary complex with protein 4.1 [13]. The contact points between the lipid bilayer and the spectrin-based network are through the band 3-ankyrin-protein 4.2 complex and the glycophorin C-P55-protein 4.1R complex (Figure 1). The entire framework maintains the shape, integrity, and deformability of the red cell. When there is either a qualitative or a quantitative defect in any one of the structural proteins [14], a shortened life span of the

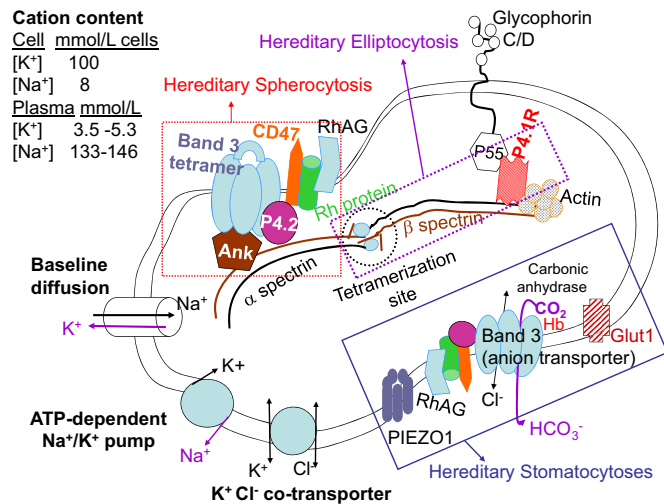


Figure 1. Structural organization and functions of various red cell membrane proteins in health and disease.

circulating red cells due to red cell fragility is manifested as hemolytic anemia. Abnormal red cell morphology is often the first clue to this type of red cells disorders, including hereditary spherocytosis (HS), hereditary elliptocytosis (HE), and hereditary pyropoikilocytosis (HPP) [15].

## 2. HEREDITARY SPHEROCYTOSIS

Hereditary spherocytosis has been reported worldwide, but the highest prevalence is found among the northern European populations (estimated 1: 2000 births). The uncoupling of an unstable cytoskeleton from the lipid bilayer in localized areas on the red cell membrane can hasten red cell vesiculation. Thus, the resulting spherocytes have a decreased surface/volume ratio, dehydrated with reduced deformability. The shortened life span of HS red cells reflects the intrasplenic destruction of red cells due to their sequestration within the splenic cords [16].

Typical HS patients present with anemia, jaundice, splenomegaly, and reticulocytosis (Table 1). The disease severity of HS is classified on the basis of the degree of anemia, namely asymptomatic state, mild, moderate, and severe [24]. Most of the patients have mild HS, and up to 20–30% have a purely compensated hemolysis due to a balance between reticulocyte production and red cell destruction. In this category, HS could be evoked when finding gallstone, persistent jaundice, clinical splenomegaly, or a transient anemia in a hemolytic crisis during infection or pregnancy (Table 1). The clinical picture for a family with HS is often fairly homogeneous. Variable clinical severity in isolated HS families (or kindred) has been linked to co-inheritance of low expression polymorphism in *trans* to a HS (Sp) allele, namely recessive HS and the  $\alpha$ -spectrin polymorphism Sp $\alpha^{\text{LEPRA}}$  allele (Low Expression Prague) [25, 26], which has a frequency of 3.6% in the normal population [25, 27], and a low expression band 3 allele (Allele Genas) in one family [28].

Diagnosis of HS can be difficult in neonates as spherocytic cells are often present after birth. Most of the HS newborns have a normal hemoglobin level at birth but develop a transient and sometimes severe anemia during the first few weeks of life, due to the inability of the infants to mount an appropriate reticulocyte response to compensate for the filtering function of the spleen [31, 32].

A preliminary laboratory diagnosis includes examination of red cell morphology and full blood count results. Freshly collected blood samples should be used for preparation of blood smear to minimize artifacts. Spherocytes and hyperdense cells (% Hyper with Hb > 410 g/L) are a positive predictor for HS when the patient has both a family history and red cells indices consistent with HS [33], especially when  $\geq 4\%$  Hyper are shown in the CBC. In general, increased % Hyper indicates red cell dehydration [31]. Therefore, they are also present in other hemolytic conditions, such as infantile pyknocytosis, acquired immune spherocytosis, DHSt, and congenital dyserythropoietic anemia (CDA) type II. Among the red cell indices, MCV is not discriminative for HS. MCHC has been found useful as indicator for HS with concurrent neonatal jaundice [17]. In the case of HS with compensated hemolytic anemia, MCHC is often in the upper limit of the normal range or increased [33]. Both the MCV and the mean spheroid cell volume (MSCV) values for red cells are displayed by the Beckman Coulter cell analyzer. The MSCV value is expected to be greater than that of MCV as a result of cell volume increase after spherization in a hypo-osmolar solution during sample analysis. Under the same condition, the MSCV value obtained for HS red cells remains lower than that for the MCV value because HS red cells are unable to increase their volume further in a hypo-osmolar medium. Thus, a delta (MCV-MSCV) value > 9.6 fL is a good indicator for HS (90.6% specificity and 100% sensitivity) [34] when there is a negative DAT result.

Automated reticulocyte indices could be useful to differentiate hereditary from acquired spherocytosis [35, 36]. As membrane re-modeling begins at the reticulocyte stage in HS, a reduced reticulocyte volume under 100 fL is classically observed in HS, except in the neonates [37]. A two-step algorithm is established for identifying spherocytosis using the reticulocyte indices and the detection of hypochromic cells by the Sysmex analyzer [35]. The high predictive value for HS (100% sensitivity) relies on the use of reticulocyte indices to identify a population with a low percentage of immature reticulocyte fraction, i.e., with a high RET/IRF ratio (Rule #1). This ratio was particularly high (> 19) in mild or subclinical HS. Rule #2 for moderate and severe HS is based on a high percentage of microcytic cells (MicroR) and on a low proportion of

**Table 1.** Criteria for diagnosis of hereditary spherocytosis and general observations

Mode of inheritance: dominant in 75% cases, and 25% recessive or nondominant HS

Presentation and observation

A. Neonatal period/Infancy

- Neonatal jaundice: common in a few days of life. Risk of kernicterus for those with spherocytosis and requiring transfusion [17, 18]
- Hb level: normal at birth. Rapid fall in Hb levels within the 1st month after birth
- Anemia: mostly improves during the 1st year of life as high HbF level causes neonatal spherocytosis

B. Childhood/Adulthood

- Persistent jaundice, anemia, splenomegaly, gallstones
- Hemolysis: can be compensated in adults
- Sudden onset of acute anemia: aplastic crisis (Parvovirus B19); acute increase of hemolysis due to viral infections (e.g. CMV, Herpes 6, EBV)
- Pregnancy in mild HS: aggravated anemia and return to baseline after delivery [19]

C. Elderly: diagnosed in the 7–9th decade in life, often postinfection [20]

D. Chromosome 8p11 translocation or deletion (close to ankyrin gene): spherocytosis and dysmorphic or neurologic features [21–23]

E. Fatal hydrops fetalis: rare, more likely to happen with a family history of HS [29, 30]

Red cell indices, morphology, and other parameters

- Hemolysis indicators: low or no haptoglobin; raised LDH; hyperbilirubinemia (unconjugated)
- Red cell morphology: Predominantly spherocytes. Mild HS shows occasional spherocytes or only during hemolytic crisis. Mushroom-shaped cells (association with band 3 defects) (Appendix A). Anisocytosis in patients with severe hemolytic anemia

Red cell indices:

- Hb: variable, often lower than the normal range. May be normal
- Increased % hyperdense cells on Advia analyzer [32]
- MCHC in the high range or increased, reflecting RBC dehydration [17]
- Increased delta (MCV-MSCV) value on Beckman Coulter analyzers [34, 36]
- Reticulocyte count: increased. Low percentage of immature reticulocyte fraction (increased RET/IRF% ratio) on Sysmex analyzers, especially in mild HS [35]. Reduced reticulocyte volume under 100 fL, except in neonates [37]

Exclusions:

- Acquired spherocytosis: autoimmune hemolytic anemia or ABO incompatibility (DAT positive)
- Patients with CDAlI often presenting with a nondominant HS-like phenotype
- Gilbert's syndrome: elevated hyperbilirubinemia: no hemolytic feature except if when co-inherited with HS (increased risk of gallstones)
- Infantile pyknocytosis: may require top-up transfusion in 6–9 months after birth. Resolved with no further intervention [43]
- Occasional spherocytes in conditions unrelated to membranopathy: Pyrimidine-5'-nucleotidase deficiency (also shows basophilic stippling) [44]; G6PD deficiency in crisis; PK deficiency or unstable hemoglobin

hypochromic erythrocytes (Hypo-He) among the microcytes (i.e. an increased MicroR/Hypo-He ratio) [35].

Some patients with CDAlI are known to present with mild hemolytic anemia, splenomegaly, and microspherocytes that give increased osmotic fragility [38]. These cases could have been initially diagnosed to have HS and re-assigned as CDAlI [39, 40].

Although their etiology is different, in several cases, such patients were splenectomized based on this HS diagnosis without complete clinical remission post-splenectomy. The differentiation between HS and CDAlI depends on a correct assessment of red cell morphology on peripheral blood smear (poikilocytosis) and reticulocyte count which can also be compared against

the Hb level [41]. A reticulocyte count of  $< 150 \times 10^9/L$  is within the 90 percentile of the reticulocyte distribution in a majority of patients with CDAII [41, 42]. This value falls within the range of  $120 \times 10^9$ – $180 \times 10^9/L$  for suspected HS cases that would require consideration for differentiation from CDAII.

### 3. HEREDITARY ELLIPTOCYTOSIS AND HEREDITARY PYROPOIKILOCYTOSIS

Hereditary elliptocytosis (HE) is heterogeneous in respect to the mode of inheritance (dominant to recessive), clinical severity (compensated anemia to transfusion dependent) (Table 2), biochemical (Membrane protein defects in HE and HPP), and molecular defects (Table 5 and Table S1 in Appendix C). Finding a small percentage of elliptocytes does not necessarily rule out HE [51] as this varies between 10% and 100%. The diagnosis of HE is incidental (e.g. elliptocytes flagged up by blood counter or postinfection)

because most patients either have a compensated anemia or are asymptomatic. Red cell morphology (Figure S2 in Appendix A), Hb, and MCV are key indicators when assessing a patient's clinical severity.

The variable clinical severity observed in patients with HE is the consequence of either a membrane protein deficiency or a qualitative defect, respectively, in protein 4.1R and the spectrin variants. Both can effect an impairment of protein–protein interaction within the cytoskeleton. The mild HE clinical picture presented by a partial 4.1 deficiency can arise from a variety of mutational events [52, 53], which are different to those mechanisms producing the 4.1 null phenotype [54, 55].

The black populations have a higher incidence of spectrin defects, whereas protein 4.1 abnormalities are prevalent among Caucasians. Patients with HE with spectrin defects tend to present a more severe clinical phenotype [56] than those having a partial 4.1 deficiency. This can be related to the severity of the spec-

**Table 2.** Characteristic features of hereditary elliptocytosis

1. Ethnicity: higher prevalence in countries in the malaria-endemic regions: probable protection against malarial infection [45]. 1 in 100 births in some African countries [46] and 1: 5000 births among Caucasians. Also found in the populations in the Mediterranean countries, the Middle East, Japan [47], the Indian subcontinent, and the Far East.
2. Classification based on clinical severity
  - Asymptomatic carrier state, nonhemolytic HE: normal Hb level and normal reticulocyte count
  - HE with varying degrees of hemolysis: normal Hb but raised reticulocyte count in mild hemolysis; reduced Hb and raised reticulocyte counts in severe hemolysis
  - HE with transient infantile poikilocytosis: transfusion dependent up to the first year, and evolving quickly into the milder phenotype of the HE parent
  - Hereditary pyropoikilocytosis (HPP): severe transfusion-dependent hemolytic anemia, starting in the neonatal period with Hb of 60–100 g/L and poikilocytosis
  - Fatal hydrops fetalis: only one case due to homozygosity in  $\beta$ -spectrin mutation [48] note: Asymptomatic or mild elliptocytosis can be exacerbated by an infection (e.g. CMV)
3. Red cell morphology
  - Elliptocytes present in asymptomatic and nonhemolytic HE
  - Hemolytic HE: increasing severity in hemolysis results in red cell fragmentation, showing poikilocytosis due to a decreased membrane mechanical stability
  - HPP: severe microspherocytosis, micropoikilocytosis. MCV between 50 and
4. Exclusions:
  - Iron deficiency anemia, thalassemia, and other causes of acquired elliptocytosis, such as burns patients, myelodysplasia [49], and blood specimens inadvertently kept at high temperature
  - Blood group Leach phenotype (Ge-2,-3,-4) in the blood group Gerbich (Ge) system: Individuals with Gerbich null phenotype are hematologically well and healthy. Their elliptocytic RBCs lack both GPC/D and 20% reduction of protein 4.1 [50]

trin self-association impairment (see Membrane protein defects in HE and HPP). Hereditary pyropoikilocytosis (HPP) is one form of severe HE which exhibits *in vivo* red cell fragmentation, especially prominent during the first year of life. The percentage of microcytes (mainly dehydrated mature red cells) is the best indicator of the severity of the hemolytic anemia [57]. The marked microspherocytosis in HPP is caused by a decrease of  $\alpha$ -spectrin content in these red cells but normal spectrin in HE red cells. Furthermore, HPP is a biallelic disorder. One of the modifying factor is a low expression allele  $Sp\alpha^{LELY}$  (Low Expression LYon) [58, 59], which has a frequency of 20% in all ethnic groups studied. Unlike HS that asymptomatic parents can produce offspring with typical HS, in a majority of HE/HPP, at least one parent is expected to have a HE phenotype.

#### 4. HEREDITARY STOMATOCYTOSIS

The hereditary stomatocytoses (HSt) are a group of hemolytic conditions in which the 'primary' lesion is a 'leak' to the monovalent cations sodium ( $Na^+$ ) and

potassium ( $K^+$ ) [8, 60], resulting in an altered hydration status shown by a significant change in mean cell volume (MCV) [3]. Four subtypes have been identified: overhydrated HSt, dehydrated HSt, cryohydrocytosis (CHC), and familial pseudohyperkalemia (FP) (Table 3). Only FP is an asymptomatic trait, whereas the other three conditions vary in both clinical severity and in qualitative features. All subtypes are inherited as dominant, but new mutations are not uncommon.

Overhydrated HSt (OMIM 185000, also known as hydrocytosis) is the prototypical example, characterized by a moderate-to-severe hemolytic anemia with a mild macrocytosis and often numerous typical stomatocytes (Figure S3 in Appendix A). The intracellular  $Na^+$  and  $K^+$  levels are very abnormal, probably due to an increased pore size of the mutated Rh-associated glycoprotein (RhAG) that facilitates a more rapid rate of cation leak through the red cell membrane [65].

The incidence of dehydrated HSt (OMIM 194380, also known as xerocytosis) is the highest in the stomatocytosis group (Table 3). There are variable

**Table 3.** Summary features for subtypes of hereditary stomatocytosis

	Overhydrated HSt (OHSt)	Dehydrated HSt (DHSt)	Cryohydrocytosis (CHC)	Familial Pseudo-hyperkalemia (FP)
Prevalence	1 in million births	1 in 10 000 births	Rare	Rare
Morphology	Macrocytosis, stomatocytosis	Stomatocytes Target cells	Stomatocytes	Normal
Hb	80–100 g/L	120–150 g/L	100–120 g/L	Normal range
MCV	120–140 fL	98–120 fL	Normal	Normal or high
MCHC	240–280 g/L	350–370 g/L	Normal	Normal
Reticulocytosis	10–15%	About 10%	About 8%	Normal
Haptoglobin	Undetectable	Undetectable	Undetectable	Present
Intracellular cations	About 40× normal $Na^+/K^+$ transport rate	Abnormal, more subtle than n OHSt	$[K^+]$ leak at low temperature and at 4 °C	High plasma $[K^+]$ when blood specimen left at RT for several hours
Glycolytic metabolites	Reduced 2,3 DPG oxidized glutathione, creatine and ergothioneine [61]	Reduced 2,3 DPG	Not known	Normal

#### Exclusions:

- Altered RBC cation transport: (a) subtype of HS with a partial band 3 deficiency [62], (b) Band 3 Campinas [63]: a splicing mutation.
- SAO and CHC have indistinguishable cation permeability defects [64].

presentations of DHSt: with and without pseudohyperkalemia, and perinatal edema [66]. Recently, DHSt is linked to several mutations of PIEZO1 protein gene (*FAM38A*) [67, 68]. This mechanosensitive channel protein probably regulates the response of ion fluxes accompanying mechanical stress when circulating through narrow passages in capillaries [69]. The mutated PIEZO1 proteins can cause a gain-of-function (or increased permeability) [70], and that seems to explain why a healthy athlete with a firm diagnosis of DHSt presented with hemolysis after every training session [71].

'Cryohydrocytosis,' a name coined to describe red cells which become wet at refrigerator temperatures, comes in three forms. The key abnormality is that the cells leak  $\text{Na}^+$  and  $\text{K}^+$  profusely at refrigerator temperatures (4–8 °C) [72]. Hemolysis is variable. The low-temperature leaks cause 'pseudohyperkalemia,' artifactually high plasma  $\text{K}^+$  readings due to loss of this cation from the abnormal red cells when they are cooled down from body to room temperatures after venesection. The mildest form of cryohydrocytosis presents with pseudohyperkalemia only; the next most severe presents with a mild hemolytic state, normocytic red cells, some stomatocytes, and normal MCHC. The most severe form caused by mutations in GLUT1 [73] presents with marked hemolysis, marked pseudohyperkalemia, and a pediatric neurological syndrome which is due to deficiency in glucose transport across the blood–brain barrier ('GLUT1 deficiency').

'Familial pseudohyperkalemia' denotes mild hemolytic conditions which present with artifactually high plasma  $\text{K}^+$  due to a red cell leak of potassium. Mild cryohydrocytosis is an example but there are others. The hematology shows minimal abnormalities. The pseudohyperkalemia can be quite misleading. Fludrocortisone can be given for presumed adrenal insufficiency. In a French family in which hypertension was present [74], a diagnosis of familial hyperkalemic hypertension (Gordon's syndrome) [75] was made before the artifactual nature of the hyperkalemia was recognized.

Stomatocytosis and hemolysis are also encountered in phytosterolemia (also known as sitosterolemia) [76], a rare recessively inherited metabolic condition in which those steroid alcohols made by oily plants (sitosterol, iso-fucasterol, others) are absorbed in unlimited quantities from the gut. In normal individu-

als, these sterols are rejected at the gut. The presence of these sterols in the plasma causes a mild hemolytic state with very marked stomatocytosis with no detectable cation leak. The key factor which differentiates these cases from the cation-leaky stomatocytoses is the macrothrombocytopenia (MPV about 10–15 fL), which is presumably caused by the presence in the plasma of these abnormal sterols. The sterols can be measured by mass spectrometry of the patient's plasma.

## 5. COEXISTENCE OF MEMBRANOPATHY AND OTHER RED CELL DISORDERS

A persistently unexplained hemolysis with or without bizarre red cell morphology (anisocytosis and poikilocytosis together with a MCV below 70 fL) is often the cause of attention for further investigation. Taking detailed family history reveals heterogeneous clinical presentations among the affected parent(s) and siblings. When faced with the proband's confounding clinical presentation and blood smears (Figure S2 in Appendix A), the best approach is to undertake a family study to determine the segregation pattern of two or more concurrent red cell disorders.

The incidence of HE is about 2% in different regions of Africa [46] while thalassemia [77] and South East Asian ovalocytosis (SAO) (incidence of 1–1.5%) are prevalent in South East Asia and the Melanesia. SAO (caused by a deletion of nine amino acids in Band 3) can be a hemolytic condition during early childhood [78] but hematologically normal when reaching adulthood. The co-presence of heterozygous  $\alpha$ -thalassemia and SAO does not seem to produce more severe hemolysis and clinical symptoms. Co-inheritance of SAO with distal renal tubular acidosis (dRTA) is well documented for different ethnic groups in South East Asia. Other reported compound heterozygotes of dRTA (with 25–60% ovalocytes on blood smears) are associated with HbE,  $\alpha^+$  thalassemia, and Hb H disease, respectively [79]. Autosomal dominant and autosomal recessive dRTA due to band 3 gene (*SLC4A1*) mutations are found in distinct geographic locations [80]. The former is localized mainly in the temperate zone while the latter is exclusively in the tropical regions encompassing Oman in the west to the Papua New Guinea in the east [80]. Three cases of severe HS with dRTA (without SAO) have been reported [30, 81, 82].



## 6. DIAGNOSTIC TESTS FOR RED CELL MEMBRANE DEFECTS

### 6.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of red cell membrane proteins

SDS-polyacrylamide gel electrophoresis (PAGE) is the standard technique used not only for the quantitation of relative membrane protein contents [e.g. decreased proteins in both HS (Figure 2a) and HE, and increased spectrin dimer in HE/HPP (Figure 3a)], but it can also reveal the abnormal proteins present in the red cells, such as CDAII band 3 (Figure 2a) and spectrin variants in HE/HPP (Figure 3b). Except for the visualization of band 3 (25% of total red cell protein) and stomatin (band 7.2b in Figure 2a), this technique can not detect GLUT1, RhAG, and Piezo1 for hereditary stomatocytosis.

#### 6.1.1. Membrane protein defects in HS

Confirmation of HS is often based on detecting a reduction of spectrin ( $\alpha$  and  $\beta$  spectrin), ankyrin, band 3, or protein 4.2 (Figure 2). A specific membrane protein deficiency can represent either a direct quantitative effect due to a mutation in that protein gene or an outcome secondary to the primary mutational event in a different membrane protein gene. For instance, a primary ankyrin gene (*ANK1*) mutation can cause a secondary partial  $\alpha$ -spectrin reduction or a combined deficiency of ankyrin/spectrin or spectrin/protein 4.2. An isolated partial protein 4.2 deficiency can result from either an *EPB42* (protein 4.2 gene) mutation or a *SLC4A1* (band 3 gene) mutation by which the protein 4.2 binding site on the mutated Band 3 is abrogated. The highest number of mutations linking to HS resides in the primary genes *SPTB*, *ANK1*, and *SLC4A1* [15],

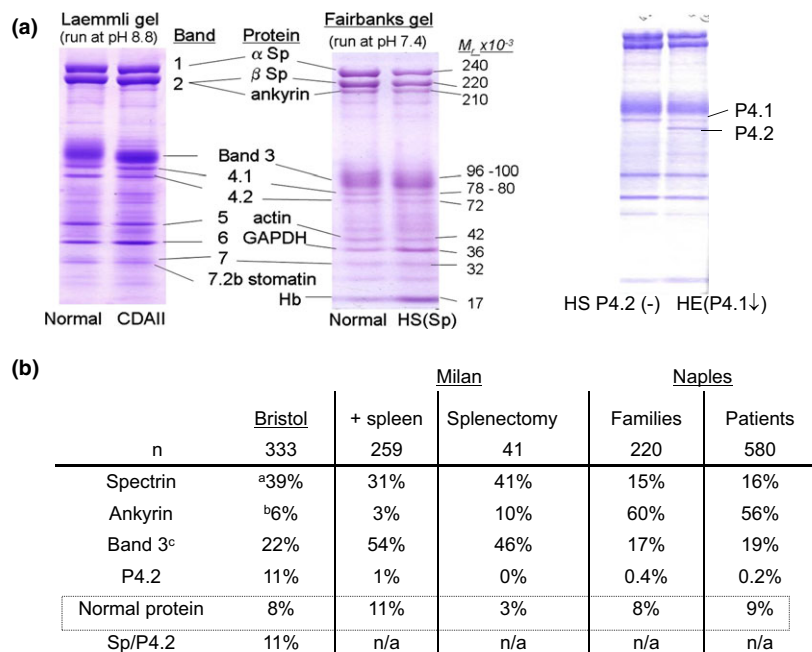
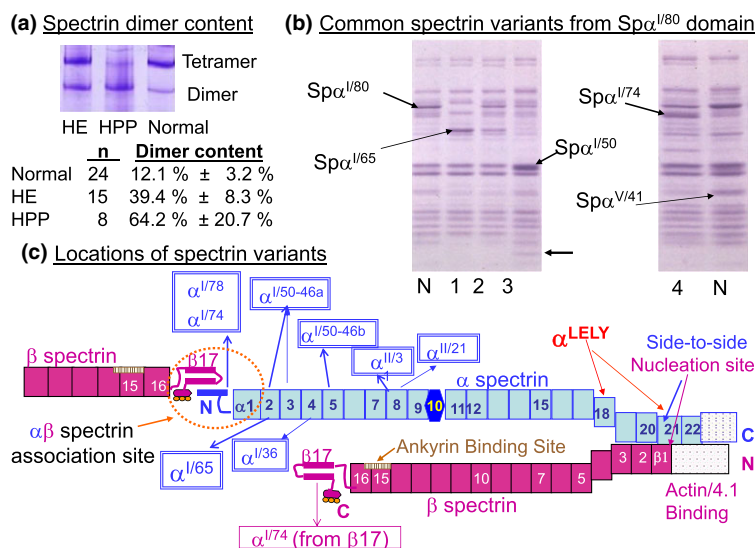


Figure 2. Membrane protein deficiencies in HS (a) Laemmli and Fairbanks buffer systems are used for gradient gels. Band 2 in Laemmli gel (4–16% linear gradient) is separated out into  $\beta$  spectrin and ankyrin in the Fairbanks gel (3.5–17% exponential gradient). (b) Membrane protein deficiencies identified in three laboratories: Bristol (unpublished data), Milan [83], and Naples [84]. <sup>a</sup>: the spectrin deficient group comprised of 31%  $\alpha$ -spectrin deficiency only and 8% combined  $\alpha\beta$ -spectrin deficiency. <sup>b</sup>: Ankyrin deficiency included Ankyrin only ( $n = 7$ ), combined Ank/P4.2 ( $n = 10$ ), Ank/Sp ( $n = 7$ ), and Ank/Sp/P4.2 ( $n = 7$ ). <sup>c</sup>: Band 3 deficiency included both isolated band 3 and a combined B3/P4.2 reduction (for the three laboratories).



**Figure 3.** Spectrin analysis for confirmation of HE/HPP and the locations of spectrin variants (a) Dimer content for HE/HPP; (b) Spectrin variants for HE/HPP from the domain I of  $\alpha$ -spectrin ( $M_r$  80 kDa) after trypsin digestion on ice for 20 h [86],  $\alpha$ -spectrin is cleaved into five domains ( $\alpha$ I to  $\alpha$ V) and  $\beta$  spectrin into four domains (I to IV). The numbers for the spectrin variants indicate the  $M_r$  of each tryptic peptide;  $\alpha^{I/36}$ ,  $\alpha^{I/46-50}$  (lane 3, including a 25-kDa peptide),  $\alpha^{I/65}$  (lane 1: homozygote; lane 2: heterozygote),  $\alpha^{I/74}$  (lane 4), and  $\alpha^{I/78}$ ; N: normal control. (c) Locations of spectrin variants on the  $\alpha$ -spectrin repeats ( $\alpha$ 1 to  $\alpha$ 22 repeats) and the location of Sp $\alpha^{LELY}$  allele. *Note:* Most of  $\alpha$  and  $\beta$  chains are composed of linear repeats (i.e. homologous triple helical segments). Each repeat is about 106 amino acids in length and is organized into a triple helix. The arrangement is that helix 2 and 3 in one repeat lock into helix 1 of the next repeat. The self-association site (circle with dotted line) comprises the N-terminus of  $\alpha$  chain (mainly the  $\alpha$ I domain,  $M_r$  80 kDa) and the C-terminus of the  $\beta$  chain. Mutations in Sp $\alpha^{LELY}$  allele affect repeats  $\alpha$ 18 and  $\alpha$ 21. Mutations in the  $\alpha$ -spectrin and  $\beta$ -spectrin (*SPTB*) genes can result in producing the same  $\alpha^{I/74}$  variant. The Sp $\alpha^{I/74}$  variant from mutations in exon 30 of *SPTB* has been found mainly among Caucasian [90] and reported in a Filipino family [91]. (cf. Table S1 in Appendix C)

and their corresponding membrane protein deficiencies are shown in Figure 2b.

A higher incidence of partial spectrin deficiency is associated with moderate and severe HS. Since only a handful of pathogenic *SPTA* mutations have been reported, this observation can be attributed to the effect of the *cis* inheritance of the Sp $\alpha^{LEPRA}$  allele either homozygous or heterozygous for this low expression polymorphism [25–27]. There is no definitive correlation between clinical phenotype and specific membrane proteins although a spectrin deficiency was found more frequently in children than in adults at presentation [83].

Between 8 and 11% of patients with HS gave with no detectable reductions in membrane proteins (Figure 2b). Splenectomy seemed to have improved the sensitivity of SDS-PAGE in detecting a partial ankyrin

deficiency (the Milan data) [83]. This finding is consistent with the masking of ankyrin deficiency in HS by reticulocytosis [85] due to higher ankyrin content in young red cells than in aged red cells.

### 6.1.2. Membrane protein defects in HE and HPP

One-dimensional SDS-PAGE can detect both protein 4.1R deficiency and spectrin variants. The latter are the same for both patients with HE and patients with HPP (Figure 3). The severe hemolysis in HPP is attributed to higher spectrin dimer content [56] with a concomitant reduction in  $\alpha$ -spectrin (Figure 3a). By contrast, it is normal spectrin content in asymptomatic HE. Limited trypsin digestion of spectrin reveals the defective region in the spectrin protein as a trypsin-resistant peptide, which is often localized to the

domain I of  $\alpha$  spectrin (designated as  $\alpha^{I/80}$ ; M<sub>r</sub> 80 000) [86] (Figure 3b). The defects identified within this domain correlate with the extent of impairment in the spectrin self-association site, and the severity of hemolytic anemia. This conclusion is based on two findings: (i) The proximity of the  $\alpha$ -spectrin defect (or variant) to the self-association site of  $\alpha$  and  $\beta$  chains (Figure 3c) and (ii) The proportion of the mutant HE spectrin  $\alpha$  chain recruited into the cytoskeleton, which, in turn, depends on the presence in *trans* (or in *cis*) of the low expression Sp $\alpha^{\text{LELY}}$  allele [58, 59].

Identification of spectrin variants from other domains requires two-dimensional gel electrophoresis of tryptic spectrin digest. The detection of Sp $\alpha^{\text{II/21}}$  [87], Sp $\alpha^{\text{II/31}}$  [88], or Spectrin St. Claude [89] in the homozygous probands is easier than in the heterozygous family members due to the elevated spectrin dimer content found only in the homozygotes. Since the locations of these variants are further away from the spectrin self-association site (Figure 3c), the increased dimer content solely in homozygotes indicates a long-range effect of a spectrin mutation on self-association site. It could be a conformational change in the helix harboring the mutation within the triple helical repeat unit [56].

## 6.2. Screening tests for hereditary spherocytosis

The screening tests presented in Table 4, except ektacytometry, are in regular use in general hematology laboratories.

### 6.2.1. The osmotic fragility test

The traditional OF test uses 14 concentrations of NaCl, ranging from 0.1 to 0.8 g/dL NaCl [92]. However, modifications are available: a 4-tube method using four concentrations of NaCl solution (0.5 g/dL for unincubated cells, 0.60, 0.65, and 0.75 g/dL NaCl for incubated RBCs) [93], and a 17-tube method with the last 3 tubes at 0.85, 0.9, and 1.0 g/dL of NaCl (Milan). Spherocytes have less resistance to lysis at each NaCl concentration when compared to normal RBCs. Pre-incubation of the whole blood sample for 24 h at 37 °C will enhance the degree of cell lysis.

The drawback of the OF test is a lack in specificity, as other congenital red cell defects or conditions can

also give a positive result (i.e. increased red cell lysis). These include immune hemolytic anemia, recent blood transfusion (i.e. lysis of recently transfused RBCs *ex vivo* due to depletion of ATP in these cells), RBC enzyme deficiencies (e.g. G6PD and pyruvate kinase deficiencies), and unstable hemoglobin variants. The OF test result has to be interpreted together with family history and examination of the peripheral blood smear.

A flow cytometric osmotic fragility test is available, which is based on a greater susceptibility of HS red cells to lyse in a medium spiked with deionized water. Although it shows high specificity and sensitivity for HS [94], it is not known whether this method can also detect red cells with abnormal membrane permeability to cation.

### 6.2.2. The acid glycerol lysis time test

The original glycerol lysis time (GLT) test [95] measures the time taken for 50% hemolysis of a blood sample in a buffered solution of hypotonic saline/glycerol. Glycerol retards the entry of water into the red cells, thus prolonging the lysis time. Addition of 0.0053M sodium phosphate lowered the pH of the buffered solutions to 6.85 with improvement of the sensitivity and specificity of this test for HS. This is the basis of the acidified glycerol lysis time (AGLT) test [96]. As a result, the lysis of normal red cells can be measured at a more manageable time of > 900s, instead of lysis at 23s–45s. The AGLT could also give positive results in acquired spherocytosis, such as AIHA, in about one-third of the pregnant women, and in some patients with chronic renal failure and with myelodysplastic syndrome [96, 97].

### 6.2.3. Osmotic gradient ektacytometry

Osmotic gradient ektacytometry monitors the extent of red cell shape change within a range of osmotic gradient (i.e. modulation of cellular water content) under a known shear stress (Appendix B.1). An osmotic gradient deformability index (DI) profile provides the information about cell water content and heterogeneity of deformability. The advantage of this technique is the production of distinct deformability profiles for several types of red cell disorders (Figure 4).

**Table 4.** Laboratory tests for screening red cell disorders

	OF test (Mayo and Milan data)	AGLT test (Milan data)	Ektacytometry (Bicêtre data in Fig 4)	EMA Binding test (Data from Bicêtre, Bristol, Mayo, and Milan laboratories)
Result for normal controls	Traditional OF: (14 tubes) 17 tube assay (Milan) plus 0.85, 0.9 and 1.0 g/dL NaCl 4-tube OF: % Cell lysis NaCl (g/dL) Male Female 0.50 (fresh) 0.0 – 47.8 0.0-31.1 Incubated: 0.60 18.7 – 67.4 10.9 – 65.5 0.65 4.4 – 36.6 0.2 – 39.3 0.75 0.8 – 9.1 0.0 - 10.9	> 900 sec	normal values are defined in each assay. Ranges: $O_{min}$ $O_{hyper}$ $DI_{max}$ 135-155 335-360 > 0.4 (mosmol/kg)	Lab Instrument n <sup>1</sup> MCF(SD) <sup>2</sup> Ratio (SD) <sup>3</sup> %Reduction (SD) Bristol <sup>4</sup> FC500 120 53.9 (1.9) 1.00 (0.04) *0 % Canto II 120 12412 (638) 1.01 (0.05) *0 % Bicêtre <sup>5</sup> Navios 391 70.4 (5.2) 1.00 (0.04) 0% (4%) Milan <sup>5</sup> Canto II 1445 10830 (2290) 1.00 (0.05) 0% (5%) Mayo FACSCaliburs 213 629.2 (34.4) 1.00 (0.05) * 0%
HS	Increased fragility 4-tube assay: positive when lysis greater than the normal ranges for all 4 NaCl concentrations 17-tube assay: positive when lysis greater than the normal range or abnormal results for at least 2 consecutive NaCl concentrations	< 900 sec	↓ $DI_{max}$ (↓ deformability) ↑ $O_{min}$ (i.e., ↑ osmotic fragility), and/or ↓ $O_{hyper}$ Positive result when at least two abnormal parameters including decreased $DI_{max}$	Reduced fluorescence Lab Instrument n MCF(SD) Ratio (SD) %Reduction (SD) Bristol <sup>4</sup> FC500 43 42.9 (4.7) 0.78 (0.09) * -22% Canto II 43 9660 (1098) 0.77 (0.08) * -23% Bicêtre <sup>5</sup> Navios 228 53.5 (8.4) 0.75 (0.11) -25% (-11%) Milan <sup>5</sup> Canto II 381 7800 (1900) 0.75 (0.1) -27% (-10%) Mayo FACSCaliburs 213 444.3 (34.4) 0.71 (0.05) * -29%
AIHA	Normal to increased fragility	Normal to HS-like	Normal to HS-like DI	Normal fluorescence
HE (non-hemolytic)	Normal	Normal	↓ $DI_{max}$ (Trapezoidal profile)	Normal or borderline MCF
HPP	Increased fragility	↓ lysis time	↓↓ $DI_{max}$ (↓↓ deformability) distorted trapezoidal profile	↓↓ fluorescence (lower than the HS range)
SAO	Reduced fragility	No data	No deformability	↓ fluorescence (within the range for HS) <sup>¶</sup>
CDA type II	Increased fragility for some cases	Normal to HS-like	Isolated ↓ deformability to HS-like DI profile (↓ $DI_{max}$ , $O_{min}$ and $O_{hyper}$ are within the normal range)	↓ fluorescence within the HS range for some, otherwise normal result
Cryohydrocytosis	No data	No data	No data	↓ fluorescence (within range for HS) <sup>¶</sup>
Dehydrated HSt	Reduced fragility (exclusion of thalassemia)	> 900 sec	Normal $DI_{max}$ (deformability), ↓ $O_{hypo}$ and ↓ $O_{hyper}$ . DI profile left of normal (dehydration)	Normal or ↑ fluorescence
Overhydrated HSt	Increased fragility (HS-like)	No data	Normal deformability DI profile right of normal deformability (over-hydration)	Normal or ↑ fluorescence

\*MCF: mean channel fluorescence readings are shown.

<sup>†</sup>The ratio was obtained by (MCF of test sample/mean MCF of normal controls used).

<sup>‡</sup>The % reduction was obtained by (mean MCF of Normal controls used – MCF of Test)/mean MCF of normal controls used.

<sup>§</sup>These data were taken from a prospective study in which the same samples were analyzed contemporaneously using FC500 and Canto II [102]. The normal controls shown represent a separate group from the normal controls used for calculating ratios. Six normal controls were used per assay.

<sup>¶</sup>Both the Milan and Bicêtre laboratories use % reductions in reporting patient results [42, 108]. The MCF readings are not used at all.

\*\*The % Reduction shown for Bristol and Mayo was calculated using the following equation: (1- ratio) × 100%. Both laboratories use MCF in result reporting.

<sup>††</sup>Distinct red cell morphology for SAO (Figure S3-L in Appendix A).

<sup>‡‡</sup>The amount of Band 3 reduction detected in SDS-PAGE cannot explain the severity of the patient’s hemolytic anemia. An increase of plasma [K<sup>+</sup>] on storage of patient’s blood sample in the refrigerator or a delay of 5–6 h in sample arrival at the testing laboratory [72].

For producing consistent DI profiles, same day blood collection and testing are preferred. If necessary, overnight storage of blood specimens at 4 °C is still acceptable. Red cells stored over 24 h will give a profile of overhydration. Antibodies bound to red cells can affect each parameter ( $DI_{max}$ ,  $O_{min}$ ,  $O_{hyper}$ ) (Appendix B.1).

The DI profiles for HS show a decreased  $DI_{max}$  together with an increased  $O_{min}$  (reflecting osmotic fragility) and/or decreased  $O_{hyper}$  reflecting cell dehy-

dration (Figure 4). All blood samples are required to give a negative result in the direct antiglobulin test prior to performing ektacytometry. This is due to overlapping results obtained for HS and the spherocytes produced in AIHA [37], especially among jaundiced neonates [31].

HE is characterized by a trapezoidal curve with moderately decreased  $DI_{max}$  but normal  $O_{min}$  and  $O_{hyper}$  results. By contrast, the DI profile of HPP red cells presents a markedly reduced  $DI_{max}$  (Figure 4).

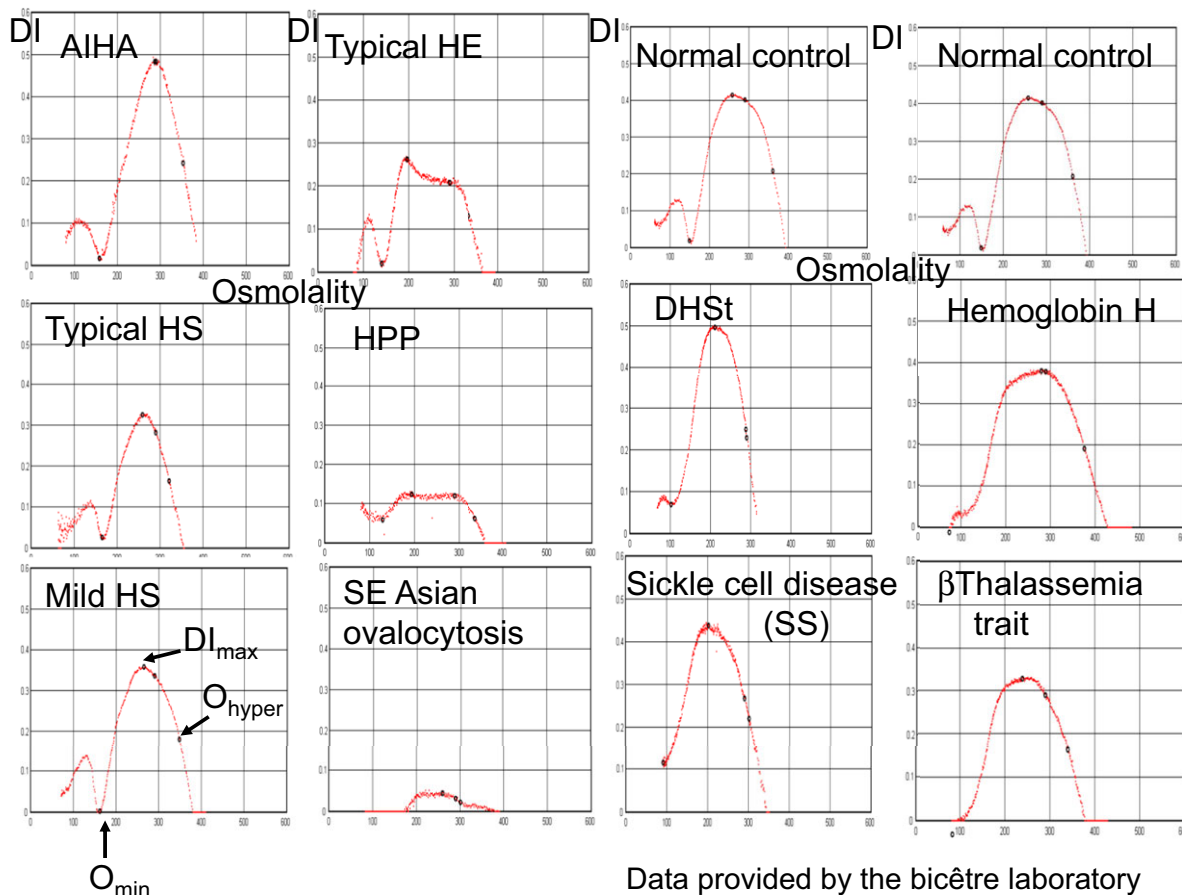


Figure 4. Osmotic deformability profiles of red cells from normal control, AIHA, HS, HE/HPP, DHSt, and hemoglobinopathies.

SAO red cells are rigid, and they do not deform in this assay. The DHSt curve has a normal  $ID_{max}$ , and its left-shifted DI profile indicates red cell dehydration (Figure 4). Although the DI profiles for DHSt and sickle cell disease look similar (Figure 4), ektacytometry is currently the only simple and reliable screening test for the diagnosis of DHSt.

Exclusion of hemoglobinopathy and iron deficiency should be considered when an abnormal DI profile is obtained. A 'flat-top' DI profile can be produced by either thalassemia (Figure 4) or iron deficiency. This kind of profile for thalassemia red cells is consistent with resistance to osmotic lysis.

The differentiation between HS and DHSt is straightforward due to the left shift of the DHSt curve. It can be challenging to distinguish between HS and CDAII using ektacytometry as some profiles for CDAII

can be interpreted as atypical HS. Therefore, SDS-PAGE is often performed in the absence of family history of HS for differential diagnosis. If necessary, there will be a follow-up appointment for family members and/or repeat testing of the proband after several (> 3) months.

Laser-assisted optical rotational red cell analyzer (LORCA) presumably works in a principle similar to ektacytometry although the way of generating the deformability index is different. An evaluation of both ektacytometry and LORCA by using red cells from a wider range of disorders (not just for HS, HE, overhydrated and dehydrated HSt) and clinical condition(s) (e.g. hyposplenism [98]) will establish whether abnormal deformability profiles are exclusive to those red cell disorders associated with defective intrinsic membrane proteins.

#### 6.2.4. The Eosin-5'-Maleimide (EMA) binding test

The EMA binding test [99] can detect HS red cells as well as SAO, cryohydrocytosis, and some cases of CDA type II [42, 99]. Their differentiation from HS [39, 40, 100] can be made using red cell morphology (Appendix A) and SDS-PAGE (Figure 2a). Furthermore, this technique can also detect HPP red cells (Appendix B.2) that give mean channel fluorescence (MCF) readings about 25% lower than that for HS [101] and high MCF results for the red cells with high MCV [102]. The EMA binding test can be used alone when adequate clinical and laboratory information on the patient are available [6]. The alternative approach is to use different combinations of screening tests, namely the OF test/the EMA binding test [6], AGLT/the EMA binding test [42], and ektacytometry/the EMA binding test [103].

As a general rule, whenever the MCF reading is within the range for typical HS, a membrane protein deficiency is usually detected in the patient's red cells (Figure 5). Exceptions to this were found in about 0.9% of patients (four in 403 cases) suspected with HS and unexplained hemolytic anemia (an audit in Bristol) to have given normal EMA results. Yet, their subsequent SDS-PAGE results showed a membrane protein deficiency: reduced  $\alpha$ -spectrin in three patients and one was band 3 deficient. All three spectrin deficient patients had presented with typical clinical

features namely, chronic mild hemolytic anemia (Hb 80–100 g/L), raised bilirubin, gallstones, reticulocytosis, and splenomegaly. By inference, using the EMA binding test together with a second screening test can probably detect all patients with HS [42, 104].

Table 4 shows the reference ranges for normal controls and patients with HS, which give the different formats of result presentation. Furthermore, these values can serve as a guide for other users when planning to establish this technique in a laboratory. However, these ranges from three laboratories (Bicêtre, Milan, and Mayo) have not taken into consideration of a gray area for HS (Figure 5). Interpretation of 'gray area' results requires the consideration of family history, clinical presentation with evidence of a hemolytic anemia as well as carrying out a family study to include proband, both parents and siblings [102]. A trend for HS can be identified based on results from the mildly affected family members [105].

The drawback of the EMA binding test is a lack of universal reference ranges for normal controls and HS as they are currently established by individual laboratories due to the different scales for mean fluorescence intensity (MFI) shown on flow cytometers from different manufacturers (Table 4). Harmonization of result presentation can facilitate comparison of patient results between laboratories [106]. This issue together with appropriate cutoff value for distinguishing between HS and normal controls was already raised by several testing laboratories [102, 107–109].

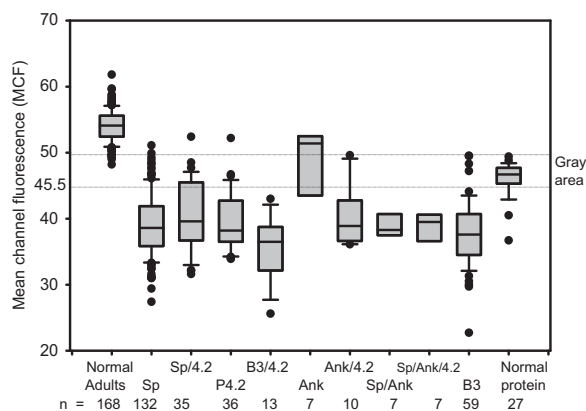


Figure 5. Box plot showing the MCF readings from the EMA binding test for patients with HS classified based on membrane protein deficiencies (unpublished data from Bristol).

## 7. MOLECULAR GENETIC ANALYSIS

Table 5 shows the wide genetic heterogeneity of hereditary spherocytosis, HE/HPP, and hereditary stomatocytosis as defects in some of the membrane proteins can cause more than one type of red cell disorders. Consideration for molecular analysis is a case-by-case decision, which is often the last option in a diagnostic plan for the patient. A general approach is that molecular analysis of membrane protein genes for HS and HE does not add extra information for the patient whose family is already known to have the red cell disorder. A recessive mode of inheritance, and a case of suspected *de novo* mutation or compound heterozygosity do warrant further investigation using DNA sequencing.

**Table 5.** Genes involved in red cell membrane defects

Protein ( <i>Gene</i> )	Chromosomal location	Exons (number)	mRNA length (bp)	Disease
Alpha spectrin ( <i>SPTA1</i> )	1q22-23	52	7999	HS, HE
Beta-spectrin ( <i>SPTB</i> )	14q23-24.1	35	10 063	HS, HE
Ankyrin ( <i>ANK1</i> )	8p11.2	43	8297	HS
Band 3 ( <i>SLC4A1</i> )	17q21-22	20	4965	HS, SAO, CHC dRTA
Protein 4.1R ( <i>EPB41</i> )	1p33-34.2	21	5930	HE
Protein 4.2 ( <i>EPB42</i> )	15q15-21	13	4553	HS
RhAG ( <i>RHAG</i> )	6p12-21	10	1912	OHSt
PIEZO1 ( <i>FAM38A</i> )	16q24.2-16qter	51	1912	DHSt
Glut1 ( <i>SLC2A1</i> )	1p31.3-35	10	3318	CHC
ABCB6 ( <i>ABCB6</i> )	2q35-36	19	3016	FP

ABCB6- ATP binding cassette (ABC) transporter in the B subfamily, also known as mitochondrial porphyrin transporter, which imports porphyrin into mitochondria during heme biosynthesis [120]. dRTA, distal renal tubular acidosis; HS, hereditary spherocytosis; HE, hereditary elliptocytosis; SAO, South East Asian ovalocytosis, DHSt, dehydrated hereditary stomatocytosis; OHSt, overhydrated hereditary stomatocytosis, CHC, cryohydrocytosis; FP, familial pseudo-hyperkalemia.

Websites for protein and gene sequences: DNA Data Bank of Japan <http://www.ddbj.nig.ac.jp/>

EMBL European Bioinformatics Institute <http://www.ebi.ac.uk>

National Center for Biotechnology Information <http://www.ncbi.nlm.nih.gov/>

Molecular assessment of the presence of several modifiers genes, such as UGT1A1 promoter polymorphisms and mutations in the HFE gene, could assist in the prediction of the course of disease development [18]. If available, such analysis can confirm the diagnosis of HS for patients with apparently normal parents. These patients could have either a genuine recessive pattern of inheritance (i.e. homozygosity or double heterozygosity for an  $\alpha$ -spectrin mutation) or an apparently recessive one caused by *de novo* mutation of ankyrin or  $\beta$ -spectrin genes (Table S1 in Appendix C). To discriminate between various possibilities, SDS-PAGE can be performed prior to quantitation of ankyrin or  $\beta$  spectrin gene expression (e.g. the reverse-transcribed amplified cDNA from the region of several polymorphisms). A *de novo* monoallelic expression of these genes is often the underlying cause of recessive inheritance pattern in patients with HS [110]. However, a reduction of ankyrin could be the result of a mutation in the *SLC4A1* or *ANK1*.

Finally,  $\alpha$  spectrin alleles in patients with isolated spectrin deficiency and a co-inheritance of defective  $\beta$  spectrin gene have to be investigated at the molecular level to distinguish between a mutation or a polymorphic allele (e.g. spectrin  $\alpha^{\text{LEPRA}}$ ) responsible for the recessive pattern of inheritance [25, 26, 111].

SDS-PAGE can detect the typical hypoglycosylated CDAlI band 3 (Figure 2a) if there is uncertainty between HS and CDAlI for a patient. The alternative option is to perform molecular analysis of the SEC23B gene for confirmation of CDA II [112, 113].

In some cases of HE that have a proband with a markedly severe clinical presentation and a milder HE phenotype in both parents, demonstration of a hypomorphic allele (such as spectrin  $\alpha^{\text{LELY}}$  allele) co-inherited *in-trans* to a Sp<sup>HE</sup> allele (i.e. a structural spectrin defect) in the proband will explain the clinical severity [58, 59]. The molecular basis for a protein 4.1R abnormality can be caused by one of the mutational events: gene deletions, decreased 4.1mRNA levels caused by promoter mutations or unstable mRNA [53, 54], or a decreased protein synthesis caused by translational defects [55]. SDS-PAGE can detect the protein 4.1 deficiency.

Reaching a preliminary diagnosis of hereditary stomatocytosis takes time due to a lack of specific disease indicators for testing in general laboratory. The basic information is an increase of MCV and MCHC, time- and temperature-dependent K<sup>+</sup> leak from red cells, stomatocytes, or target cells (Table 3). Using ektacytometry is the best option for OHSt and DHSt, albeit only available in a very few testing centers in

Europe. If hereditary stomatocytosis is suspected at presentation of a patient, confirmation of the diagnosis can be carried out by means of sequencing the appropriate candidate gene (Table 5). For instance, sequencing of the coding region for the C-terminal domain of *SLC4A1* will be able to confirm cyrohydrocytosis as nine amino acid substitutions in the region between aa-687 and aa-796 of band 3 protein have been reported for cryohydrocytosis [114].

Recent advances in the next generation sequencing (NGS) technologies have transformed the genetics study of human diseases [115]. This is an era of unprecedented productivity. Exome sequencing, the targeted sequencing of the protein-coding portion of the human genome, has been shown to be a powerful and cost-effective method for detection of disease variants underlying Mendelian disorders. There are several new attempts to use this technology to diagnosing in one reaction all the possible causes of red cell cytoskeleton defects. This method could be certainly useful mainly for autosomal dominant cases. However for autosomal recessive inheritance, the lack of capability of NGS to diagnose mutations in intronic sequence as well in regulatory sequence and deletion-al forms could impair this methodology [116, 117].

Application of NGS in the diagnosis of red cell membrane disorders has recently been reported [118, 119]. Presented herein is a brief summary on the experience of the Naples laboratory in their validation of various molecular techniques using cases of known specific membrane protein defects. After identifying the underlying protein gene mutation in a proband using the NGS, other family members (e.g. parents and affected relatives) will always be tested by other less expensive molecular techniques (namely PCR and sequencing) to confirm the inherited mutation(s). In a patient, more than one membrane protein gene can be identified to have a mutation. Verification of the gene mutation directly responsible for the clinical phenotype is based on functional assay using the *Xenopus* oocyte expression system. As a general estimate, a wide genome search can identify causative mutation(s) in about 60% of cases. The correct assignment rate (i.e. matching the diagnosis with the membrane protein gene) increases to 80% with whole exome sequencing. A 90% assignment rate is feasible when using targeted sequencing. The added advantage of targeted sequencing is the concomitant detection of

mutations in nonmembrane protein genes that can have a modifying effect on the clinical phenotype.

## 8. RECOMMENDATIONS FOR LABORATORY DIAGNOSIS OF RED CELL MEMBRANE DISORDERS

Aim to reduce the turnaround time due to request for unnecessary additional tests; e.g. SDS-PAGE of erythrocyte membrane proteins and molecular testing:

- A testing laboratory can make an informed decision when a test requisition form gives adequate patient information and the reason for the test(s) required. Useful information includes a tentative diagnosis, the patient's clinical presentation, a DAT-negative result, reticulocyte count, and markers for hemolysis (Figure 6), and family history.
- State clearly if the reason for test request is for exclusion of membrane defect.
- Send a pretransfusion blood specimen or wait for complete clearance of residual transfused red cells before sending a blood specimen to testing laboratory. It takes 8 weeks for complete clearance of transfused cells from circulation.

For hereditary spherocytosis:

- Spherocytes on blood smear has a good positive predictive value for HS in a patient with a family history and compatible red cells indices. In the absence of a family history, DAT and a screening test for HS should both be performed.
- Signs for mild HS: occasional spherocytes in the peripheral blood smear, together with borderline reticulocytosis, slightly abnormal biochemical markers of hemolysis with or without splenomegaly. These observations may be diagnostic of mild HS after exclusion of other causes of spherocytosis namely, pregnancy, autoimmune hemolysis, CDAIL, systemic hematological diseases, and some enzyme deficiencies.
- All the screening tests presented can detect typical HS. If in doubt, use two screening tests (Table 4). Equally important is an awareness of when a screening test can give false-positive results with other red cell disorders and false-negative results with isolated HS cases.
- If available, molecular testing is considered when all screening tests give negative or equivocal results for a



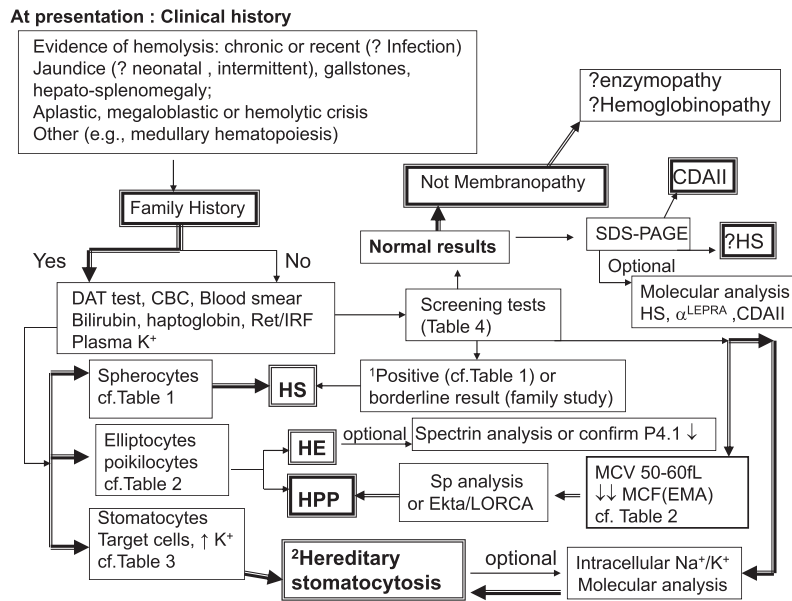


Figure 6. Diagnostic flow chart for HS, HE, and HSt. <sup>1</sup>Exclusion of CDAII when test results are within in HS but the patient's clinical presentation is not typical of HS. Exclusion of hemoglobinopathy (e.g. unstable Hb) when the test result is mildly HS-like for a patient whose MCV is < 65 fL and the blood smear shows anisocytosis and poikilocytic red cells. <sup>2</sup>: exclusion of HS.

proband presenting with characteristic features of membrane disorder, whereas the family members are hematologically well and healthy. Identifying the molecular basis of the hemolytic anemia facilitates genetic counseling or when considering for splenectomy.

- Detection of  $Sp\alpha^{\text{LEPRA}}$  allele by PCR technique (cf. Table S1 in Appendix C) can explain the more severe clinical phenotype in a proband than in other HS family members.

For hereditary elliptocytosis:

- In the absence of a family history of HE and only a few elliptocytes on the blood smear, it is advised to confirm the diagnosis of HE for the proband [51].
- If available, carry out SDS-PAGE for quantitation of protein 4.1 and spectrin analysis (spectrin dimer content and spectrin variant) (Figure 3). Or, use ektacytometry/LORCA to obtain characteristic DI profile (Figure 4).
- In HPP, detection of  $Sp\alpha^{\text{LELY}}$  allele *in trans* to a  $Sp^{\text{HE}}$  allele by PCR technique can explain the severe clinical phenotype in a majority of patients. New mutational events can also cause HPP-like phenotype (cf. Table S1 in Appendix C).

For hereditary stomatocytosis:

- Important to make a firm diagnosis as splenectomy is not beneficial to a patient with dehydrated HSt, and overhydrated HSt [7, 121]. The confirmatory tests include ion flux measurement, ektacytometry, or LORCA for overhydrated HSt, dehydrated HSt, and DNA sequencing for specific genes (Table 5).
- If none of the above is available, use screening tests for differential diagnosis of HS and other rarer red cell disorders (Table 4). If available, use flame photometry to quantitate plasma  $K^+$ , intracellular  $[K^+]$  and  $[Na^+]$  in fresh and stored specimens from patient and a paired normal control being kept at room temperature and in the refrigerator [72].
- If a patient does not respond to splenectomy (which was performed based on a diagnosis of HS) [9], re-examine the patient for the likelihood of HSt.

## 9. ACKNOWLEDGEMENTS

The Hematology Departments in the <sup>1</sup>Foundation IRCCS Ca' Granda Ospedale Maggiore of Milan (Italy), <sup>2</sup>CHU Bicêtre (France), and the <sup>3</sup>Mayo Clinic

Rochester (USA) formed a Harmonization Group. The purpose was to retrieve their respective archival data for the EMA binding test carried out using normal controls and patients with HS in the last 3–6 years. Statistical analysis was carried out individually to establish the reference ranges for normal controls and patients with HS. We thank <sup>1</sup>Elisa Fermo PhD, <sup>2</sup>Caroline Mayeur-Rousse MD, <sup>2</sup>Aurore Gerstenmey-

er, <sup>3</sup>Adam J. Wood, MD, <sup>3</sup>Jennifer L. Oliveira, MD, and <sup>3</sup>Michelle E. Savedra for their assistance in this work.

## 10. CONFLICT OF INTEREST

All the contributors of this Guideline have declared no conflict of interest.

## 11. REFERENCES

1. Brugnara C, Mohandas N. Red cell indices in classification and treatment of anemias: from M.M. Wintrobe's original 1934 classification to the third millennium. *Curr Opin Hematol* 2013;20:222–30.
2. Stewart GW, Turner EJ. The hereditary stomatocytoses and allied disorders: congenital disorders of erythrocyte membrane permeability to Na and K. *Baillieres Clin Haematol* 1999;12:707–27.
3. Gallagher PG. Disorders of red cell volume regulation. *Curr Opin Hematol* 2013;20:201–7.
4. Van der Harst P, Zhang W, Leach IM, Rendon A, *et al*. Seventy-five genetic loci influencing the human red blood cell. *Nature* 2012;492:369–75.
5. Guitton C, Garçon L, Cynober T, Gauthier F, Tchernia G, Delaunay J, Leblanc T, Thuret I, Bader-Meunier B. Sphérocytose héréditaire: recommandations pour le diagnostic et la prise en charge chez l'enfant (Hereditary spherocytosis: guidelines for the diagnosis and management in children). *Arch Pédiatr* 2008;15:1461–73.
6. Bolton-Maggs P, Langer JC, Iolascon A, Tittensor P, King M-J. Guidelines for the diagnosis and management of hereditary spherocytosis- 2011 update. *Br J Haematol* 2012;156:37–49.
7. Stewart GW, Amess JAL, Eber SW, Kingswood C, Lane PA, Smith BD, Mentzer WC. Thrombo-embolic disease after splenectomy for hereditary stomatocytosis. *Br J Haematol* 1996;93:303–10.
8. Delaunay J. The hereditary stomatocytoses: genetic disorders of the red cell membrane permeability to monovalent cations. *Sem Hematol* 2004;41:165–72.
9. Lock SP, Smith RS, Hardisty RM. Stomatocytosis: a hereditary red cell anomaly associated with haemolytic anaemia. *Br J Haematol* 1961;7:303–14.
10. Zarkowsky HS, Oski FA, Sha'afi R, Shohet SB, Nathan DG. Congenital hemolytic anemia with high sodium, low potassium red cells. *New Eng J Med* 1968;278:573–81.
11. Glader BE, Fortier N, Albala MM, Nathan DG. Congenital hemolytic anemia associated with dehydrated erythrocytes and increased potassium loss. *New Eng J Med* 1974;291:491–6.
12. Flatt J, Bruce LJ. The hereditary stomatocytosis. *Haematologica* 2009;94:1039–41.
13. Salomao M, Zhang X, Yang Y, Lee S, Hartwig JH, Chasis JA, Mohandas N, An X. Protein 4.1R-dependent multiprotein complex: new insights into the structural organization of the red blood cell membrane. *Proc Nat Acad Sci USA* 2008;105:8026–31.
14. Giorgi M, Cianci CD, Gallagher G, Morrow JS. Spectrin oligomerization is cooperatively coupled to membrane assembly: a linkage targeted by many hemolytic anemias? *Exp Mol Pathol* 2001;70:215–30.
15. Delaunay J. The molecular basis of hereditary red cell membrane disorders. *Blood Rev* 2007;21:1–20.
16. Molnar Z, Rappaport H. Fine structure of the red pulp of the spleen in hereditary spherocytosis. *Blood* 1972;39:81–98.
17. Christensen RD, Henry E. Hereditary spherocytosis in neonates with hyperbilirubinemia. *Pediatrics* 2012;125:120–5.
18. Gallagher PG. Abnormalities of the erythrocyte membrane. *Pediatr Clin North Am* 2013;60:1349–62.
19. Pajor A, Lehoczy D, Szakács Z. Pregnancy and hereditary spherocytosis. Report of 8 patients and a review. *Arch Gynecol Obstet* 1993;253:37–42.
20. Friedman EW, Williams JC, van Hook L. Hereditary spherocytosis in the elderly. *Am J Med* 1988;84:513–6.
21. Kimberling WJ, Taylor RA, Chapman RG, Lubs HA. Linkage and gene localization of hereditary spherocytosis (HS). *Blood* 1978;52:859–67.
22. Bass EB, Smith SW, Stevenson RE, Rosse WF. Further evidence for location of the spherocytosis gene on chromosome 8. *Ann Int Med* 1983;99:192–3.
23. Chilcote RR, Le Beau MM, Dampier C, Pergament E, Verlinsky Y, Mohandas N, Frischer H, Rowley JD. Association of red cell spherocytosis with deletion of short arm of chromosome 8. *Blood* 1987;69:156–9.
24. Eber SW, Armbrust R, Schroter W. Variable clinical severity of hereditary spherocytosis: relation to erythrocytic spectrin concentration, osmotic fragility, and autohemolysis. *J Pediatrics* 1990;117:409–16.
25. Wichterle H, Hanspal M, Palek J, Jarolim P. Combination of two mutant alpha spectrin alleles underlies a severe spherocytic hemolytic anemia. *J Clin Invest* 1996;98:2300–7.
26. Dhermy D, Steen-Johnsen J, Bournier O, Hietet G, Cynober T, Tchernia G, Grandchamp B. Coinheritance of two  $\alpha$ -spectrin gene defects in a recessive spherocytosis family. *Clin Lab Haem* 2000;22:329–36.
27. Boivin P, Galand C, Devaux L, Lecomte MC, Garbarz M, Dhermy D. Spectrin alpha IIa variant in dominant and non-dominant spherocytosis[published erratum appears in *Human Genetics* 1994 May; 93, 614.]. *Hum Genet* 1993;92:153–6.
28. Alloisio N, Maillat P, Carré G, Texier P, Vallier A, Baklouti F, Philippe N, Delaunay J. Hereditary spherocytosis with band 3 deficiency. Association with a non-sense mutation of the band 3 gene (Allel Lyon), and aggravation by a low-expression allele occurring in trans (Allele Genas). *Blood* 1996;88:1062–9.
29. Whitfield CF, Follweiler JB, Lopresti-Morrow L, Miller BA. Deficiency of  $\alpha$ -spectrin synthesis in burst-forming unit-erythroid in lethal hereditary spherocytosis. *Blood* 1991;78:3043–51.
30. Ribeiro ML, Alloisio N, Almeida H, Gomes C, Texier P, Lemos C, Mimoso G, Morlé L, Bey-Cabet F, Rudigoz RC, Delaunay J, Tamagini G. Severe hereditary

- spherocytosis and distal renal tubular acidosis associated with the total absence of band 3. *Blood* 2000;96:1602–4.
31. Saada V, Cynober T, Brossard Y, Schischmanoff PO, Sender A, Cohen H, Delaunay J, Tchernia G. Incidence of hereditary spherocytosis in a population of jaundiced neonates. *Pediatr Hematol Oncol* 2006;23:387–97.
  32. Delhommeau F, Cynober T, Schischmanoff PO, Rohrlisch P, Delaunay J, Mohandas N, Tchernia G. Natural history of hereditary spherocytosis during the first year of life. *Blood* 2000;95:393–7.
  33. Cynober T, Mohandas N, Tchernia G. Red cell abnormalities in hereditary spherocytosis: relevance to diagnosis and understanding of the variable expression of clinical severity. *J Lab Clin Med* 1996;128:259–69.
  34. Broséus J, Visomblain B, Guy J, Maynadié M, Girodon F. Evaluation of mean sphered corpuscular volume for predicting hereditary spherocytosis. *Int J Lab Hematol* 2010;32:519–23.
  35. Mullier F, Lainey E, Fenneteau O, Da Costa L, Schillinger F, Bailly N, Cornet Y, Chatelain C, Dogne JM, Chatelain B. Additional erythrocytic and reticulocytic parameters helpful for diagnosis of hereditary spherocytosis: results of a multicentre study. *Ann Hematol* 2011;90:759–68.
  36. Lazarova E, Pradier O, Cotton F, Gulbis B. Automated reticulocyte parameters for hereditary spherocytosis screening. *Ann Hematol* 2014;93:1809–18.
  37. Da Costa L, Mohandas N, Sorette M, Grange MJ, Tchernia G, Cynober T. Temporal differences in membrane loss lead to distinct reticulocyte features in hereditary spherocytosis and in immune hemolytic anemia. *Blood* 2001;98:2894–9.
  38. Danise P, Amendola G, Nobili B, Perrotta S, Miraglia del Giudice E, Matarese SMR, Iolascon A, Brugnara C. Flow-cytometric analysis of erythrocytes and reticulocytes in congenital dyserythropoietic anaemia type II (CDA II): value in differential diagnosis with hereditary spherocytosis. *Clin Lab Haem* 2001;23:7–13.
  39. Heimpel H, Anselstetter V, Chrobak L, Denecke J, Einsiedler B, Gallmeier K, Griesshammer A, Marquardt T, Janka-Schaub G, Kron M, Kohne E. Congenital dyserythropoietic anemia type II: epidemiology, clinical appearance, and prognosis based on long-term observation. *Blood* 2003;102:4576–81.
  40. Dukka S, King M-J, Hill Q. The diagnosis challenge of congenital dyserythropoietic anaemia: two cases of 'CDA type II'. *J Clin Path* 2014;67:367–9.
  41. Russo R, Gambale A, Langella C, Andolfo I, Unal S, Iolascon A. Retrospective cohort study of 205 cases with congenital dyserythropoietic anemia type II: definition of clinical and molecular spectrum and identification of new diagnostic scores. *Am J Hematol* 2014;89:E169–75.
  42. Bianchi P, Fermo E, Vercellati C, Marcello AP, Porretti L, Cortelezzi A, Barcellini W, Zanella A. Diagnostic power of laboratory tests for hereditary spherocytosis: a comparison study on 150 patients grouped according to the molecular and clinical characteristics. *Haematologica* 2012;97:516–23.
  43. Eyssette-Guerreau S, Bader-Meunier B, Garçon L, Guitton C, Cynober T. Infantile pyknocytosis: a cause of haemolytic anaemia of the newborn. *Br J Haematol* 2006;133:439–42.
  44. Bianchi P, Fermo E, Alfinito F, Vercellati C, Baserga M, Ferraro F, Guzzo I, Rotoli B, Anella A. Molecular characterization of six unrelated Italian patients affected by pyrimidine-5'-nucleotidase deficiency. *Br J Haematol* 2003;122:847–51.
  45. Facer CCA. Erythrocytes carrying mutations in spectrin and protein 4.1 show differing sensitivities to invasion by *Plasmodium falciparum*. *Parasitol Res* 1995;81:52–7.
  46. Dhermy D, Carnevale O, Blot I, Zohoun I. Hereditary elliptocytosis in Africa. *Lancet* 1989;1:225.
  47. Yawata Y. Characteristics of red cell membrane disorders in the Japanese population. *Rinsho Byori* 1997;45:367–76.
  48. Gallagher PG, Weed SA, Tse WT, Benoit L, Morrow JS, Marchesi SL, Mohandas N, Forget BG. Recurrent fatal hydrops fetalis associated with a nucleotide substitution in the erythrocyte  $\beta$ -spectrin gene. *J Clin Invest* 1995;95:1174–82.
  49. Alanio-Bréchet C, Schischmanoff PO, Fénéant-Thibaut M, Cynober T, Tchernia G, Delaunay J, Garçon L. Association between deficit in myeloid malignancies and acquired protein 4.1R deficiency: a retrospective analysis of 6 patients. *Am J Hematol* 2008;83:275–8.
  50. Telen MJ, Le Van Kim C, Chung A, Cartron JP, Colin Y. Molecular basis for elliptocytosis associated with glycophorin C and D deficiency in the Leach phenotype. *Blood* 1991;78:1603–6.
  51. Féo C, Dhermy D. Confirming or ruling out hereditary elliptocytosis. *Acta Haematol* 1988;79:116.
  52. Conboy JG, Chasis JA, Winardi R, Tchernia G, Kan YW, Mohandas N. An isoform-specific mutation in the protein 4.1 gene results in hereditary elliptocytosis and complete deficiency of protein 4.1 in erythrocyte but not in nonerythroid cells. *J Clin Invest* 1993;91:77–82.
  53. Conboy JG. Structure, function, and molecular genetics of erythroid membrane skeletal protein 4.1 in normal and abnormal red blood cells. *Sem Hematol* 1993;30:58–73.
  54. Baklouti F, Morinère M, Haj-Khéilil A, Fénéant-Thibault M, Gruffat H, Couté Y, Ninot A, Guitton C, Delaunay J. Homozygous deletion of EP41 genuine AUG-containing exons results in mRNA splicing defects, NMD inactivation and protein 4.1R complete deficiency in hereditary elliptocytosis. *Blood Cells Mol Dis* 2011;47:158–65.
  55. Morinère M, Ribeiro L, Venezia ND, Deguillien M, Maillet P, Cynober T, Delhommeau F, Almeida H, Tamagnini G, Delaunay J, Baklouti F. Elliptocytosis in patients with C-terminal domain mutations of protein 4.1 correlates with encoded messenger RNA levels rather than with alterations in primary protein structure. *Blood* 2000;95:1834–41.
  56. Delaunay J, Dhermy D. Mutations involving the spectrin heterodimer contact site: clinical expression and alterations in specific function. *Sem Hematol* 1993;30:21–33.
  57. Silveira P, Cynober T, Dhermy D, Mohandas D, Tchernia G. Red blood cell abnormalities in hereditary elliptocytosis and their relevance to variable clinical expression. *Am J Clin Pathol* 1997;108:391–9.
  58. Alloisio N, Morlé L, Maréchal J, Roux AF, Ducluzeau MT, Guetarni D, Pothier B, Baklouti F, Ghanem A, Kastally R, Delaunay J. Sp $\alpha^{V41}$ : a common spectrin polymorphism at the  $\alpha$ IV- $\alpha$ V domain junction. Relevance to the expression level of hereditary elliptocytosis due to  $\alpha$ -spectrin variants located in trans. *J Clin Invest* 1991;87:2169–2177.
  59. Wilmotte R, Maréchal J, Morlé L, Baklouti F, Philippe N, Kastally R, Kotula J, Delaunay J, Alloisio N. Low expression allele  $\alpha$ LELY of red cell spectrin is associated with mutations in exon 40 ( $\alpha^{V41}$  polymorphism) and intron 45 and with partial skipping of exon 46. *J Clin Invest* 1993;91:2091–6.
  60. Stewart GW. Hemolytic disease due to membrane ion channel disorders. *Curr Opin Hematol* 2004;11:244–50.
  61. Darghouth D, Koehl B, Hellier JF, Madalinski G, Bovee P, Bosman G, Delaunay J, Junot C, Roméo P-H. Alterations of red blood cell metabolome in overhydrated hereditary stomatocytosis. *Haematologica* 2011;96:1861–5.
  62. Bruce LJ, Robinson H, Guizouarn H, Borgese F, Harrison P, King M-J, Goede JS, Coles SE, Gore DM, Lutz HU, Ficarella R, Layton M, Iolascon A, Ellory JC, Stewart GW. Monovalent cation leaks in human red cells are caused by single amino acid substitutions in the transport

- domain of the band 3 chloride-bicarbonate exchanger, AE1. *Nat Genet* 2005;37:1258–63.
63. Lima PRM, Gontijo JAR, Lopes de Faria JB, Costa FF, Saad STO. Band 3 campinas: a novel splicing mutation in the band 3 gene (AE1) associated with hereditary spherocytosis, hyperactivity of Na<sup>+</sup>/Li<sup>+</sup> countertransport and an abnormal renal bicarbonate handling. *Blood* 1997;90:2810–8.
  64. Guizouarn H, Borgese F, Gabillat N, Harrison P, Geode JS, McMahon C, Stewart GW, Bruce L. Southeast Asian ovalocytosis and the cryohydrocytosis form of hereditary stomatocytosis show virtually indistinguishable cation permeability defects. *Br J Haematol* 2011;152:655–64.
  65. Bruce LJ, Guizouarn H, Burton NM, Gabillat N, Poole J, Flatt JF, Brady RL, Borgese F, Delaunay J, Stewart G. The monovalent cation leak in overhydrate stomatocytic red blood cells results from amino acid substitutions in the Rh-associated glycoprotein. *Blood* 2009;113:1350–7.
  66. Basu AP, Carey P, Cynober T, Chetty M, Delaunay J, Stewart GW, Richmond S. Dehydrated hereditary stomatocytosis with transient perinatal ascites. *Arch Dis Child Fetal Neonatal Ed* 2003;88:F438–9.
  67. Zarychanski R, Schulz VP, Houston BL, Maksimova Y, Houston DS, Smith B, Rinehart J, Gallagher PG. Mutations in the mechanotransduction protein PIEZO1 are associated with hereditary xerocytosis. *Blood* 2012;120:1908–15.
  68. Andolfo I, Alper SL, De Franceschi L, Auriemma C, Russo R, De Falco L, Vallefuoco F, Esposito MR, Vanderpe DH, Shmukler BE, Narayan R, Montanaro D, D'Armiento M, Vetro A, Limongelli I, Zuffardi O, Glader BE, Schrier SL, Brugnara C, Stewart GW, Delaunay J, Iolascon A. Multiple clinical forms of dehydrated hereditary stomatocytosis arise from mutations in *PIEZO1*. *Blood* 2013;121:3925–35, S1–S12.
  69. Bae C, Gnanasambandam R, Nicolai C, Schs F, Gottlieb PA. Xerocytosis is caused by mutations that alter the kinetics of the mechanosensitive channel PIEZO1. *Proc Natl Acad Sci USA* 2013;110:E1162–8.
  70. Albuissou J, Murthy SE, Bandell M, Coste B, Louis-dit-Picard H, Mathur J, Féneant-Thibault M, Tertian G, de Jaureguiberry JP, Syfuss PY. Dehydrated hereditary stomatocytosis is linked to gain-of-function mutations in mechanically activated PIEZO1 ion channels. *Nat Commun* 2013;4:1884.
  71. Platt OS, Lux SE, Nathan DG. Exercise-induced hemolysis in xerocytosis. Erythrocyte dehydration and shear sensitivity. *J Clin Invest* 1981;68:631–8.
  72. Haines PG, Jarvis HG, King S, Noor-mohamad FH, Chetty M, Fisher J, Hill P, Nicolaou A, Stewart GW. Two further British families with the 'cryohydrocytosis' form of hereditary stomatocytosis. *Br J Haematol* 2001;113:932–7.
  73. Flatt JF, Guizouarn H, Burton N, Borgese F, Tomlinson RJ, Forsyth RJ, Baldwin SA, Levinson BE, Quittl P, Aguilar-Martinez P, Delaunay J, Stewart GW, Bruce LJ. Stomatatin-deficient cryohydrocytosis results from mutations in *SLC2A1*: a novel form of GLUT1 deficiency syndrome. *Blood* 2011;118:5267–77.
  74. Beaurain G, Mathieu F, Grootenboer S, Fiquet B, Cynober T, Tchernia G, Delaunay J, Jeunemaitre X. Dehydrated hereditary stomatocytosis mimicking familial hyperkalaemic hypertension: clinical and genetic investigation. *Eur J Haematol* 2007;78:253–9.
  75. Gordon RD. Syndrome of hypertension and hyperkalemia with normal glomerular filtration rate. *Hypertension* 1986;8:93–102.
  76. Rees D, Iolascon A, Carella M, O'Marraig A, Kendra JR, Jowitt SN, Wales JK, Vora A, Makris M, Manning N, Nicolaou A, Fisher J, Mann A, Machin SJ, Clayton PT, Gasparini P, Stewart GW. Stomatocytic haemolysis and macrothrombocytopenia (Mediterranean stomatocytosis/macrothrombocytopenia) is the haematological presentation of phytosterolaemia. *Br J Haematol* 2005;130:297–309.
  77. Fuchareon S, Winichagoon P. Haemoglobinopathies in Southeast Asia. *Indian J Med Res* 2011;134:498–506.
  78. Laosombat V, Viprakasit V, Dissaneevate S, Leetanaporn R, Chotsampancharoen T, Wongchanchailert M, Kodchawan S, Thongnoppakun W, Duangchu S. Natural history of Southeast Asian ovalocytosis during the first 3 years of life. *Blood Cells Mol Dis* 2010;45:29–32.
  79. Yenichitsomanus PT, Kittanakom S, Rungrøj N, Cordat E, Reithmeier RAF. Molecular mechanisms of autosomal dominant and recessive distal renal tubular acidosis caused by *SLC4A1* (*AE1*) mutations. *J Mol Genet Med* 2005;1:49–62.
  80. Khositseth S, Bruce LJ, Walsh SB, Bawazir WM, Ogle GD, Unwin RJ, Thong MK, Sinha R, Choo KE, Chartapisak W, Kingwatanakul P, Sunboonnanonda A, Vasuvattakul S, Yenichitsomanus P, Wrong O. Tropical distal renal tubular acidosis: clinical and epidemiological studies in 78 patients. *Q J Med* 2012;105:861–77.
  81. Chiu C, Woods N, Sawasdee N, Guizouarn H, Pellissier B, Borgese F, Yenichitsomanus PT, Gowrishankar M, Cordat E. Band 3 Edmonton I, a novel mutant of the anion exchange 1 causing spherocytosis and distal renal tubular acidosis. *Biochem J* 2010;426:379–88.
  82. Sinha R, Agarwal I, Bawazir WM, Bruce LJ. Distal renal tubular acidosis with hereditary spherocytosis. *Indian Pediatr* 2013;50:693–5.
  83. Mariani MG, Barcellini W, Vercellati C, Marcello AP, Fermo E, Pedotti P, Boschetti C, Zanella A. Clinical and hematologic features of 300 patients affected by hereditary spherocytosis grouped according to the type of the membrane protein defect. *Haematologica* 2008;93:1310–7.
  84. Iolascon A, Avvisati RA. Genotype/phenotype correlation in hereditary spherocytosis. *Haematologica* 2008;93:1283–8.
  85. Miraglia del Giudice E, Francese M, Polito R, Nobili B, Iolascon A, Perrotta S. Apparently normal ankyrin content in unsplenectomized hereditary spherocytosis patients with the inactivation of one ankyrin (*ANK1*) allele. *Haematologica* 1997;82:332–3.
  86. Speicher DW, Morrow JS, Knowles WJ, Marchesi VT. Identification of proteolytically resistant domains of human erythrocyte spectrin. *Proc Natl Acad Sci USA* 1980;77:5673–7.
  87. Alloisio N, Morlé L, Pothier B, Roux AF, Maréchal J, Ducluzeau MT, Benhadji-Zouaoui Z, Delaunay J. Spectrin Oran ( $\alpha$ II/21), a new spectrin variant concerning the  $\alpha$ II domain and causing severe elliptocytosis in the homozygous state. *Blood* 1988;71:1039–47.
  88. Alloisio N, Wilmotte R, Morlé L, Baklouti F, Maréchal J, Ducluzeau M-T, Denoroy L, Féo C, Forget BG, Kastally R, Delaunay J. Spectrin Jendouba: an  $\alpha$ II/31 spectrin variant that is associated with elliptocytosis and carried a mutation distant from the dimer self-association site. *Blood* 1992;80:809–15.
  89. Fournier CM, Nicolas G, Gallagher PG, Dhermy D, Grandchamp B, Lecomte MC. Spectrin St Claude, a splicing mutation of the human  $\alpha$ -spectrin gene associated with severe poikilocytic anemia. *Blood* 1997;89:4584–90.
  90. Tse WT, Lecomte MC, Costa FF, Garbarz M, Féo C, Boivin P, Dhermy D, Forget BG. Point mutation in the beta-spectrin gene associated with alpha I/74 hereditary elliptocytosis. Implications for the mechanism of spectrin dimer self-association. *J Clin Invest* 1990;86:909–16.
  91. Dhermy D, Galand C, Bournier O, King M-J, Cynober T, Roberts I, Kanyike F, Adekile A. Co-inheritance of  $\alpha$ - and  $\beta$ -spectrin gene mutations in a case of hereditary elliptocytosis. *Blood* 1998;92:4481–2.
  92. Parpart AK, Lorenz PB, Parpart ER, Gregg J, Chase AM. The osmotic resistance (fra-

- gility) of human red cells. *J Clin Invest* 1947;26:636–40.
93. Larson CJ, Scheidt R, Fairbanks VF. The Osmotic Fragility Test for Hereditary Spherocytosis: use of EDTA-anticoagulated blood stored at 4° C for up to 96 hours. *Am J Clin Pathol* 1988;90:507.
  94. Park SH, Park C-J, Lee B-R, Cho Y-U, Jang SS, Kim NY, Koh KN, Im HJ, Seo JJ, Park ES, Lee JW, Yoo KH, Jung HL. Comparison study of the eosin-5'-maleimide binding test, flow cytometric osmotic fragility tests, and cryohemolysis test in the diagnosis of hereditary spherocytosis. *Am J Clin Pathol* 2014;142:474–84.
  95. Gottfried EL, Robertson NA. Glycerol lysis time of incubated erythrocytes in the diagnosis of hereditary spherocytosis. *J Lab Clin Med* 1974;84:746–51.
  96. Zanella A, Izzo C, Rebulli P, Zanuso F, Perroni L, Sirchia G. Acidified glycerol lysis test: a screening test for spherocytosis. *Br J Haematol* 1980;45:481–6.
  97. Bucx MJ, Breed WP, Hoffmann JJ. Comparison of acidified glycerol lysis test, Pink test and osmotic fragility test in hereditary spherocytosis: effect of incubation. *Eur J Haematol* 1988;40:227–31.
  98. Prendki V, Ndour PA, Jais X, Ciceron L, Settegrna C, Buffet P. Reduced deformability of circulating erythrocytes: a marker for hyposplenism. *Amer J Hematol* 2012;87:E81–2.
  99. King MJ, Behrens J, Rogers C, Flynn C, Greenwood D, Chambers K. Rapid flow cytometric test for the diagnosis of membrane cytoskeleton-associated haemolytic anaemia. *Br J Haematol* 2000;111:924–33.
  100. CLSI. Red Blood Cell Diagnostic Testing Using Flow Cytometry, Approved Guideline –Second Edition. CLSI document H52-A2. Wayne, PA: Clinical and Laboratory Standards Institute; 2014. Section 8: Red Blood Cell Shape Diseases: Cellular Defects and Clinical Implication (pp 29-41) and Appendix C: Differential Diagnosis using the Eosin-5-Maleimide Binding Test (pp 89-93).
  101. King M-J, Telfer P, MacKinnon H, Langabeer L, McMahon C, Darbyshire P, Dhermy D. Using the eosin-5-maleimide binding test in the differential diagnosis of hereditary spherocytosis and hereditary pyropoikilocytosis. *Cytometry B* 2008;74B:244–50.
  102. Hunt L, Greenwood D, Heimpel H, Noel N, Whiteway A, King M-J. Toward the harmonization of result presentation for the eosin-5-maleimide binding test in the diagnosis of hereditary spherocytosis. *Cytometry B* 2015;88B:50–7.
  103. Da Costa L, Galimand J, Fenneteau O, Mohandas N. Hereditary spherocytosis, elliptocytosis, and other red cell membrane disorders. *Blood Rev* 2013;27:167–78.
  104. Crisp RL, Solari L, Vota D, Garcia E, Miguez G, Chamorro ME, Schwartzman GA, Alfonso G, Gammella D, Caldrola S, Riccheri C, Vittori D, Venegas B, Nesse A, Donat H. A prospective study to assess the predictive value for hereditary spherocytosis using five laboratory tests (cryohemolysis test, eosin-5'-maleimide flow cytometry, osmotic fragility test, auto-hemolysis test, and SDS-PAGE) on 50 hereditary spherocytosis families in Argentina. *Ann Hematol* 2011;90:625–34.
  105. King M-J, Bruce LJ, Dhermy D. Hemolytic anemias associated with disorders of erythrocyte membrane and cytoskeleton proteins. In: Laboratory Hematology Practice, Kotte-Marchant K, Davis B. (eds). Chichester, UK: Wiley Blackwell; 2010: 602–14.
  106. Miller GW, Myers GL, Gantzer ML, Kahn SE, Schönbrunner ER, Thienpont LM, Bunk DM, Christenson RH, Eckfeldt JH, Lo SF, Nübling CM, Sturgeon CM. Roadmap for harmonization of clinical laboratory measurement procedures. *Clin Chem* 2011;57:1108–17.
  107. Girodon F, Garçon L, Bergoin E, Largier M, Delaunay J, Feneant-Thibault M, Maynadié M, Couillaud G, Moreira S, Cynober T. Usefulness of the eosin-5- maleimide cytomeric method as a first-line screening test for the diagnosis of hereditary spherocytosis: comparison with ektacytometry and protein electrophoresis. *Br J Haematol* 2008;140:468–70.
  108. Mayeur-Rousse C, Gentil M, Botton J, Thibaut MF, Guitton C, Picard V. Testing for hereditary spherocytosis: a French experience. *Haematologica* 2012;97:e48–9. (Bianchi P, Fermo E, Zanella A. Reply to 'Testing for hereditary spherocytosis: a French experience' *Haematologica* 2012; 97(12): e52.)
  109. Mackiewicz G, Bailly F, Favre B, Guy J, Maynadié M, Girodon F. Flow cytometry test for hereditary spherocytosis. *Haematologica* 2012;97:e47. (Bianchi P, Fermo P, Zanella A. Reply to 'Flow cytometry test for hereditary spherocytosis'. *Haematologica*, 2012; 97 (12): e50-51.)
  110. Miraglia del Giudice E, Lombardi C, Francese M, Nobili B, Conte ML, Amendola G, Cuttillo S, Iolascon A, Perrotta S. Frequently *de novo* monoallelic expression of  $\beta$  spectrin gene (*SPTB*) in children with hereditary spherocytosis and isolated spectrin deficiency. *Br J Haematol* 1998;101:251–4.
  111. Iolascon A, Avvisati RA, Piscopo C. Hereditary spherocytosis. *Transfus Clin Biol* 2010;17:138–42.
  112. Schwarz K, Iolascon A, Verissimo F, Tredde N, Horsley W, Chen W, Par BH, Kopfner KP, Holzmann K, Russo R, Eposito RM, Spano D, Falco LD, Heinrich K, Joggorst B, Rojewski MT, Perrotta S, Dennecke J, Pannicke U, Delaunay J, Pepperkok R, Heimpel H. Mutations affecting the secretory COPII coat protein SEC23B cause congenital dyserythropoietic anemia type II. *Nat Genet* 2009;41:936–40.
  113. Bianchi P, Fermo E, Vercellati C, Boschetti C, Barcellini W, Lurlo A, Marcello AP, Righetti PG, Zanella A. Congenital dyserythropoietic anemia type II (CDAI) is caused by mutations in the SEC23B gene. *Hum Mutat* 2009;30:1292–8.
  114. Barneaud-Rocca D, Pellissier B, Borgese F, Guizourn H. Band 3 missense mutations and stomatocytosis: insight into the molecular mechanism responsible for monovalent cation leak. *Int J Cell Biol* 2011;2011:13682.
  115. Solomon BD. Obstacles and opportunities for the future of genomic medicine. *Mol Genet Genomic Med* 2014;2:205–9.
  116. Khincha PP, Savage SA. Genomic characterization of the inherited bone marrow failure syndromes. *Semin Hematol* 2013;50:333–47.
  117. Gerrard G, Valgañón M, Foong HE, Kasperaviciute D, Iskander D, Game L, Müller M, Aitman TJ, Roberts I, de la Fuente J, Foroni L, Karadimitris A. Target enrichment and high-throughput sequencing of 80 ribosomal protein genes to identify mutations associated with Diamond-Blackfan anaemia. *Br J Haematol* 2013;162:530–6.
  118. Christensen RD, Nussenzveig RH, Yaish HM, Henry E, Eggert LD, Agarwal AM. Causes of hemolysis in neonates with extreme hyperbilirubinemia. *J Perinatol* 2014;34:616–9.
  119. Van Wijk R, Vogel M, Elferink M, Nijman I, Cuppen E, van Solinger W, van Amstel HKP, van der Zwaag B. Next generation sequencing in the diagnosis of red cell membrane disorders. *Int J Lab Hematol* 2014;36(Suppl. 1):18, abstract O45.
  120. Krishnamurthy PC, Du G, Fukuda Y, Sun D, Sampath J, Mercer KE, Wang J, Sosa-Pineda B, Murti KG, Schuetz JD. Identification of a mammalian mitochondrial porphyrin transporter. *Nature* 2006; 443:586–9.
  121. Smith BD, Segel GB. Abnormal erythrocyte endothelial adherence in hereditary stomatocytosis. *Blood* 1997;89:3451–6.

## 12. Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1** Blood smears of different red cell disorders.

**Appendix S2** Screening tests for hereditary spherocytosis.

**Appendix S3** Identification of specific mutations in membrane protein genes.