

Laboratorinė medicina.
2019, t. 21, Nr. 2(82), p. 56–64.

Laboratory Diagnosis of Hereditary Spherocytosis: Literature Review and Case Report

Eglė Petrik^{1,2}
Agnė Barzinskienė²
Rėda Matuzevičienė^{1,2}
Dovilė Karčiauskaitė^{1,2}

Summary

Hereditary spherocytosis is the most common inherited red blood cell membrane disorder in individuals of northern European ancestry with an incidence of 1 case per 2000–5000 individuals. Deficiency or dysfunction of erythrocyte membrane proteins (ankyrin, band 3, and spectrins, protein 4.2) result in weakened interactions between the lipid bilayer and membrane skeleton leading to formation of spherocytes with decreased deformability and increased osmotic fragility. Premature splenic destruction of abnormal spherocytes is a primary cause of hemolysis in hereditary spherocytosis. The disorder is typically characterised by anemia, jaundice and splenomegaly, however, the clinical presentation and course of disease is highly variable ranging from asymptomatic forms with fully compensated hemolysis to occasionally severe forms, requiring periodic blood transfusions and/or splenectomy. A diagnostic workout is based on a family history, physical examination, general laboratory tests (complete blood count, reticulocyte count, examination of erythrocyte morphology) and a combination of cause-oriented special diagnostic tests (osmotic fragility tests, flow cytometry, ektacytometry, cryohemolysis test, electrophoresis of red blood cell membrane proteins and molecular testing for genetic mutations within genes encoding erythrocyte plasma membrane proteins. This review highlights current understanding of disease pathophysiology, genetics and clinical features. Particular attention is given to a wide spectrum of laboratory assays (screening as well as confirmatory) emphasizing their advantages and disadvantages. Herein we also present a clinical case of aplastic crisis due to human parvovirus infection in a child with hereditary spherocytosis.

Keywords: hemolytic anemia, hereditary spherocytosis, laboratory diagnosis, flow cytometry, osmotic fragility, parvovirus.

¹Vilnius University, Faculty of Physiology, Biochemistry, Microbiology and Laboratory Medicine, M. K. Čiurlionio str. 21/27, LT-03101 Vilnius

²Vilnius University Hospital Santaros Clinics, Laboratory Medicine Centre, Laboratory of Hematology and Cytology, Santariškių str. 2, LT-08661 Vilnius
E-mail: danke100@gmail.com

INTRODUCTION

Red blood cell membrane disorders (so called membranopathies) comprise a heterogeneous group of congenital hemolytic anemias resulting from structural membrane defects. Historically, the classification of these disorders has been based on the alterations of erythrocyte morphology seen on the periph-

eral blood smear and include hereditary spherocytosis (HS), hereditary elliptocytosis (HE), hereditary stomatocytosis (HSt), hereditary pyropoikilocytosis (HPP) and Southeast Asian ovalocytosis (SAO) [1]. The major emphasis is given on hereditary spherocytosis (also known as Minkowski–Chauffard disease), the most frequently encountered red blood cell membranopathy by clinicians.

History

Hereditary spherocytosis was first described in 1871 by two Belgian physicians Vanlair and Masius, as microcythemia, referring to the small spherical cells in the peripheral blood smear. The disease was rediscovered by Wilson and Minkowski in the late 19th century, who described congenital type of icterus by reporting 8 cases of HS in three generations of one family. Minkowski was the first to show that icterus was associated with the hemolytic anemia and postulated that hemolysis was due to the splenic damage. Chauffard and co-workers were the first to describe the increased erythrocyte osmotic fragility and reticulocytosis in congenital hemolytic icterus. The 1934 studies of Haden proposed that structural abnormality of the erythrocyte membrane could be the basis for hemolysis. The next important contributions were reports of correction of hemolytic anemia by splenectomy and the studies of Ham and Castle involving the spleen in the conditioning of spherocytes. Later on various molecular defects of erythrocyte membrane proteins and their genes were identified [2, 3].

Epidemiology

Hereditary spherocytosis is considered to be the most common inherited hemolytic anemia in Northern European countries and Northern America, affecting approximately 1 in 2000 to 5000 individuals, although generally HS is a rare disease worldwide. In fact the real prevalence is likely to be even higher due to underdiagnosis of extremely mild or asymptomatic forms of HS [4]. Disease occurs worldwide in all ethnic and racial groups. However the incidence among Southeast Asian and African people is thought to be lower, although definitive information regarding the epidemiology of HS is unavailable. HS affects females and males equally and can present at any age, partly depending on the severity of disease [5].

Pathophysiological aspects

The erythrocyte membrane is a highly dynamic structure which is both extremely flexible and structurally resilient. During the typical lifespan of 120 days the red blood cell (RBC) passes through the circulation approximately 300 000–500 000 times and faces tremendous challenges – mechanical, biochemical as well as osmotic. A combination of membrane

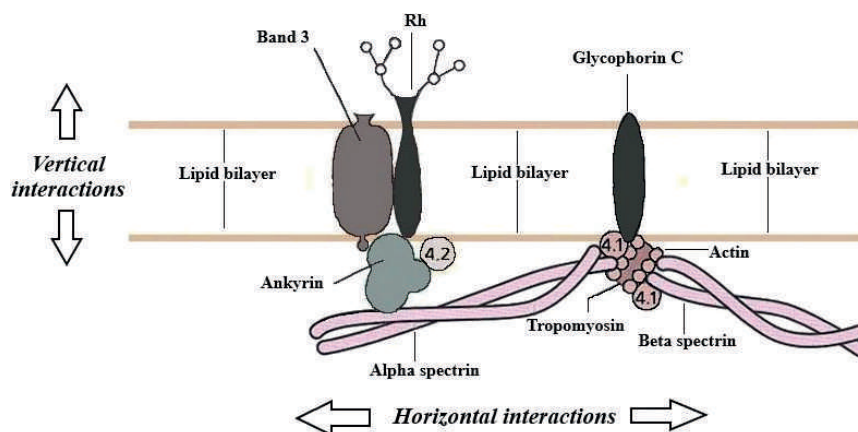


Fig. 1. A schematic representation of the red blood cell membrane structure

strength, deformability and stability allows erythrocytes to adapt to these challenges and withstand the stress of the circulation [6].

The RBC membrane is composed of three main components: the lipid bilayer punctuated with transmembrane proteins and an underlying spectrin-based membrane cytoskeleton, which is attached to the bilayer by linking proteins (Fig. 1).

The structural integrity of the erythrocyte membrane is determined by vertical interactions between the lipid bilayer together with its transmembrane proteins and underlying cytoskeleton as well as by horizontal interactions between various cytoskeletal proteins. The most important vertical linkages in the pathogenesis of HS include: ankyrin-spectrin-band 3-protein 4.2 interactions and spectrin-protein 4.1-glycophorin C-p55 interactions [7]. Deficiency or dysfunction of one or more previously mentioned membrane proteins is responsible for the loss of cohesion between lipid bilayer and spectrin-based skeleton. As a result, membrane is destabilized, leading to release of skel-

eton-free lipid vesicles and reduction of both membrane surface area and the surface area to volume ratio (SA/V). Such mechanism leads to the changes in erythrocyte shape and formation of spherical RBCs [5, 8].

The spleen plays very important role in the pathogenesis of HS. Because of the membrane surface area reduction and impaired flexibility erythrocytes are unable to effectively pass through constricted regions of the circulation. In addition to trapping spherocytes, the spleen also “conditions” such cells (so called splenic conditioning) (Fig. 2) by causing further damage on the erythrocyte membrane. Ultimately abnormal red blood cells are exposed to splenic macrophages that eventually lead to erythrophagocytosis. This currently proposed mechanism of premature extravascular erythrocyte destruction is responsible for anemia and splenomegaly [8].

Molecular genetics

The molecular defects responsible for qualitative and/or quantitative abnormalities of erythrocyte membrane pro-

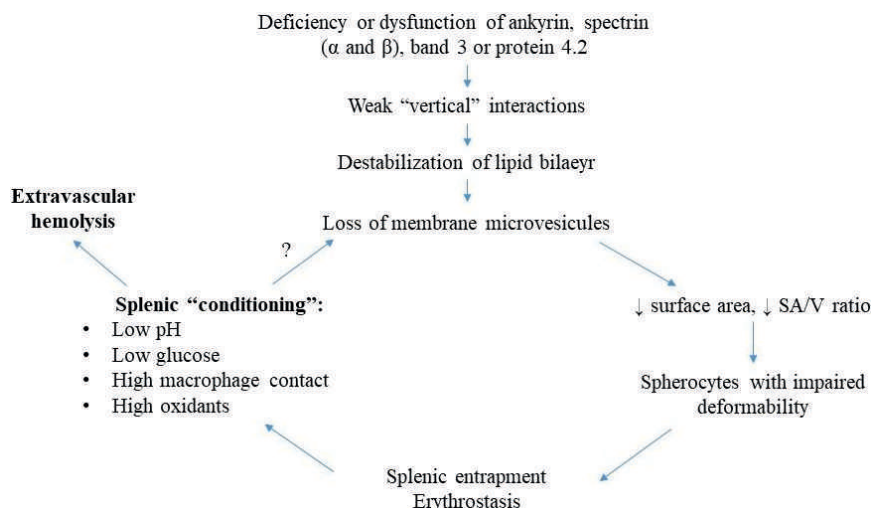


Fig. 2. Pathophysiological effects of erythrocyte destruction

Table 1. Molecular classification of hereditary spherocytosis

Type	Gene	Location	Membrane protein	Prevalence in patients with HS	Inheritance	Severity of disease	OMIM
1	ANK1	8p11.21	Ankyrin-1	Europe and USA 40–65% 5–10% in Japan	AD, AR, <i>de novo</i>	Mostly moderate	#182900
2	SPTB	14q23.3	-spectrin	15–30%	AD, <i>de novo</i>	Mild to moderate	#182870
3	SPTA1	1q23.1	-spectrin	~5%	AR	Mostly severe	#270970
4	SLC4A1	17q21.31	Band 3	20–35%	AD	Mild to moderate	#109270
5	EPB42	15q15.2	Protein 4.2	Europe and USA <5% Japan 40–50%	AR	Mild to moderate	#612690

AD – autosomal dominant, AR – autosomal recessive, HS – hereditary spherocytosis, OMIM – Online Mendelian Inheritance in Man

Table 2. Classification of hereditary spherocytosis

	Mild	Moderate	Moderate to severe	Severe
GENERAL INFORMATION				
Prevalence	20–30%	60–70%	~10%	3–5%
Inheritance	AD	AD, <i>de novo</i> mutation	AD, <i>de novo</i> mutation	AR
Main clinical features	Usually asymptomatic	Fatigue, pallor, (intermittent) jaundice		
Splenomegaly	Absent or mild	Modest	Significant	
LABORATORY DATA				
Hemoglobin (g/L)	Normal	80–110	60–80	<60
Reticulocyte count (%)	<6%	>6%	>10%	>10%
Bilirubin (mol/L)	17.1–34.2	>34.2	>34.2–51.3	>51.3
Blood smear	Mild spherocytosis	Spherocytes	Spherocytes	Abundant spherocytes, poikilocytosis, anisocytosis
MANAGEMENT				
Splenectomy	Usually not necessary	In some cases	Necessary >5 years-old	Necessary >2–3 years-old
Transfusions	0–1	0–2	Intermittent need (>2)	Regular

AD – autosomal dominant, AR – autosomal recessive

teins show significant heterogeneity and are typically caused by their corresponding gene mutations. There are 5 HS-related genes: ANK1, SPTB, SPTA1, SLC4A1 and EPB42 (Table 1) [9]. It is being reported that most of the gene mutations are ‘private’, meaning that nearly every family has an isolated mutation or it appears in a few families from different countries. In approximately three-thirds of HS patients, mutations are inherited in an autosomal dominant manner (*e.g.* a single mutant allele is sufficient for HS to appear) [10]. According to the guidelines for the diagnosis and management of HS, primary mutations are found most often in the genes encoding ankyrin, -spectrin or band 3 with mutations in the ANK1 gene being the most common cause of HS in the Europe and USA [11]. In the remaining 25% of cases, inheritance is non-dominant and the parents of these patients are both clinically and hematologically normal. Non-dominant HS (nd-HS) cases can be further divided in two groups. About half of them are caused by spontaneous new mutations (*de novo*), particularly in the ANK1 and SPTB genes. The rest of the cases are due to recessively inherited HS typi-

cally associated with abnormalities in the -spectrin or protein 4.2 genes. The highly variable clinical manifestations depend partly on the mode of inheritance. In general, hemolytic anemia is more severe and sometimes even transfusion-dependent in patients with non-dominant HS [5, 9, 12].

LABORATORY INVESTIGATION

According to the current guidelines for the diagnosis and management of HS from the British Committee for Standards in Haematology, the diagnostic workout of hereditary spherocytosis should be based on a combination of family and clinical histories, typical clinical presentation (*e.g.* pallor, jaundice, splenomegaly) and laboratory findings (*e.g.* spherocytes on blood smear, elevated MCHC, reticulocytosis) with no further testing needed. In case of equivocal diagnosis, two tests with a high predictive value are recommended – flow cytometric EMA-binding test and cryohemolysis test both presenting grade 1 recommendation and grade A evidence [11].

GENERAL DIAGNOSTIC TESTS

Complete blood count

All patients with suspected HS should have a complete blood count (CBC) with reticulocyte count and erythrocyte indices. On the basis of anemia extent and markers of hemolysis (bilirubin and reticulocyte count) severity of the disease is classified as mild, moderate, moderately severe and severe (Table 2). In a majority of HS cases anemia varies from mild to moderate (Hb 80–120 g/L) or there is no anemia at all (because of compensated hemolysis due to balance between erythrocyte destruction and reticulocyte production). The elevation of both mean corpuscular hemoglobin concentration (MCHC >360 g/L) and red blood cell distribution width (RDW >14%) is of high relevance to identifying HS because of the satisfactory sensitivity and specificity [5, 13]. On the other hand, mean corpuscular volume (MCV) do not have significant diagnostic value as it is often low normal or only slightly decreased. Reticulocyte count and automated reticulocyte indices might be helpful in the diagnosis

of HS. According to the guidelines for RBC membrane disorders from the International Council for Standardization in Haematology (ICSH), high reticulocyte count without an equally elevated immature reticulocyte fraction (IRF), e. g. high RET/IRF ratio, has a high predictive value for HS [13, 14]. Currently, automated hematology analyzers are able to provide new data concerning RBC and reticulocyte analysis, e.g. mean sphered corpuscular volume (MSCV), hyperchromic RBC percentage (% Hyper), mean reticulocyte volume (rMCV or MRV), reticulocyte MCHC (rMCHC), reticulocyte hemoglobin equivalent (Ret-Hb), reticulocyte distribution width (rRDW), etc. [13, 15, 16]. Although these parameters might be of great interest for HS screening, they are still unfamiliar and rarely used by laboratories. For a correct interpretation of red cell and reticulocyte indices the dependence on the technologies used by different hematological analyzers should be taken into account [15].

Blood smear microscopic examination

Erythrocyte morphology in HS is quite diverse. Typically round, dense, hyperchromic red cells without a central pallor (so called spherocytes) are found on peripheral blood smear (Fig. 3). The number of spherocytes is highly dependent on the form of HS and varies from only a few cells in mild forms to large numbers of spherocytes as well as bizarre morphology with poikilocytosis and anisocytosis in severe cases of disease. Varying degrees of polychromasia, reflecting reticulocytosis, is also noted. Molecular studies have also identified the association between abnormal erythrocyte shapes and some membrane protein defect, e.g. spherocytic acanthocytes (spherocytes), "mushroom" shaped red cells (band 3), spherostomatocytes (protein 4.2). However, many other hemolytic anemias can show spherocytes on the peripheral blood smear, therefore a diagnosis of HS cannot be established by their identification alone [5, 8, 17].

Biochemical markers of hemolysis

Laboratory findings of ongoing extravascular hemolysis in HS include variably increased lactate dehydrogenase (LDH) as well as indirect bilirubin and decreased or absent serum haptoglobin. It should be noted, that

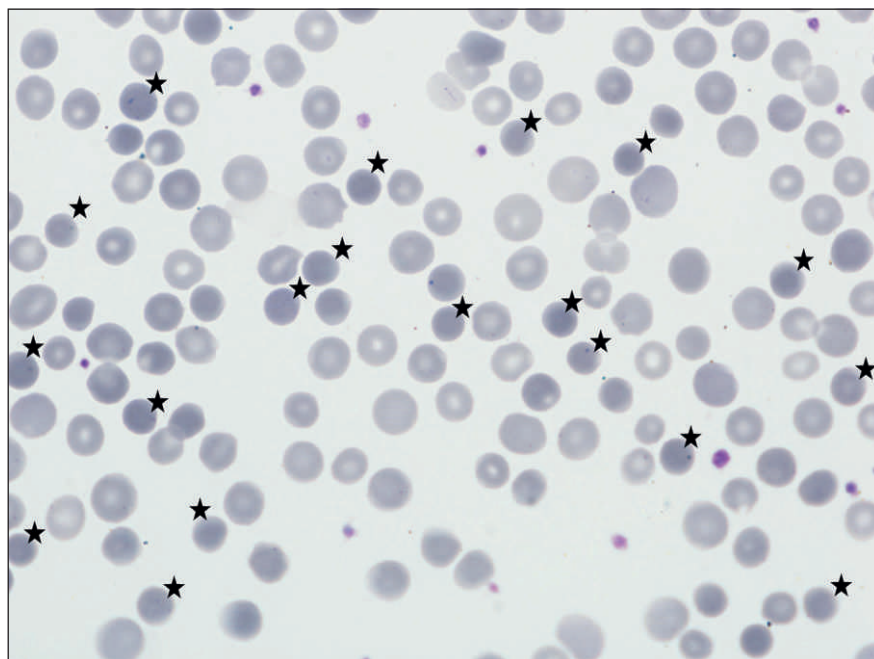


Fig. 3. Peripheral blood smear of the patient with hereditary spherocytosis

such abnormalities can be absent in patients with a mild form of the disease.

CAUSE-ORIENTED SPECIAL DIAGNOSTIC TESTS

EMA-binding test

A rapid flow cytometric analysis for the diagnosis of HS was presented almost two decades ago by King *et al.* EMA (eosin-5-maleimide) is an eosin-based fluorescent dye that covalently binds band 3, a most abundant integral protein of red cell membrane. Therefore a quantitative decrease of band 3 protein results in a reduction of fluorescence intensity. To a lesser extent EMA also binds other RBC membrane proteins, particularly Rh-related transmembrane proteins and CD47. If HS is due to defects in other membrane proteins, e.g. spectrin or protein 4.2, EMA-binding test can also be useful. A decrease in fluorescence intensity is thought to appear because of modified EMA binding site on band 3. On the other hand, test is less effective in cases of HS caused by ankyrin defects partly due to distal ankyrin location in relation to the EMA binding site [17, 18]. Classically, a comparison between mean fluorescence intensity (MFI) of patient erythrocytes and MFI of red cells from 5–6 healthy age- and MCV-matched controls is made. Primary gating for both – controls and patient – is based on side scatter *vs.* forward scatter. Red

blood cells are gated in gate "Erythrocytes" (Fig. 4 A and B). As EMA bound cells' fluorescence is measured, it is presented as mean fluorescence intensity (Fig. 4 C and D). Two ways of result presentation are in use. The first one, the cut-off value of MFI ratio that is calculated by the following formula: MFI patient / mean of 5–6 MFI's for normal controls. MFI ratio of 0.8 is considered to be definitely positive for HS [19, 20]. The second one, used at Vilnius University Hospital Santaros Clinics, is a percentage of MFI reduction (% reduction) between suspected HS patient and normal controls. The reduction in MFI is calculated by the following formula:

$$\frac{MFI_{controls} - MFI_{patient}}{MFI_{controls}} \times 100$$

The EMA-binding test is considered to be positive for HS when reduction is 21%, negative when 16% [15, 17, 19]. Percentage of MFI reduction from 16% to 21% (so called "gray zone") is probable for HS, but a thorough correlation between family history, clinical presentation, family study and additional tests are needed either to confirm or to rule out the diagnosis of HS [14, 17]. However, despite the high predictive value of the test, the lack of universal reference ranges for both normal controls and HS patients as well as standardization of the test across different laboratories, is an ongoing issue. The discrepancies are most likely to be associated with the different MFI scales from different flow cytometer models and unstandardised software, used for the

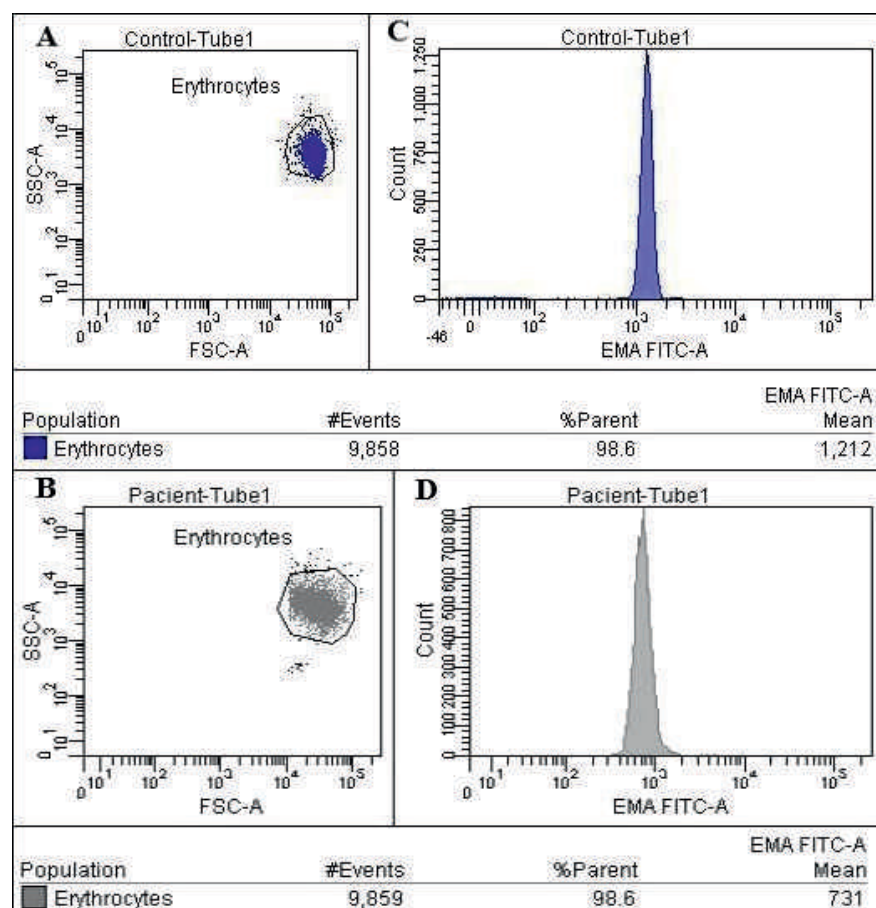


Fig. 4. EMA-binding test

evaluation of the results [14, 15, 18]. Nevertheless, EMA binding test is characterized by a high degree of specificity (99.1%) and sensitivity (92.7–96.6%). Other advantages of this test include its short turnaround time (app. 2–3 h), minimal amount of blood (~5 L) (especially important in neonate testing), possibility of delayed testing (up to 6–7 days) as the ability of RBCs to bind an EMA dye is not affected or the fact that test results are not affected by recent blood transfusions [15, 17]. It is also independent from the clinical manifestation of HS being positive even in compensated forms of disease [17]. It is important to note that in case of abnormal EMA-binding test results other red blood cell membrane disorders should be taken into account, namely Southeast Asian ovalocytosis (SAO), cryohydrocytosis (one type of hereditary stomatocytosis), some cases of congenital dyserythropoietic anemia type II (CDA II) and hereditary pyropoikilocytosis (a severe form of hereditary elliptocytosis). The differentiation of previously mentioned disorders from HS should include clinical features as well as red cell morphology and SDS-PAGE of red blood cell proteins. Main disadvantages of the test are the lack of universal reference ranges and

possibility of false negative results in mild forms of HS that should be taken into account [14, 15, 18, 19].

The osmotic fragility tests (OFT)

Osmotic fragility (OF) is tested by the measurement of red blood cell hemolysis rate in a series of hypotonic saline solutions of various osmolarities. The deformability of a healthy discoid shape erythrocyte is determined by the abundant surface area relative to cell volume (so called SA/V ratio). Placed in hypotonic solution swelling of such erythrocyte takes place leading to cell lysis when a critical volume is reached. On the other hand, spherocytes have decreased SA/V ratio, resulting in a disability to absorb hypotonic solution and expand. As a consequence, they are prone to hemolysis at higher concentrations of NaCl (are os-

motically more fragile) [13, 20, 21]. Evaluation of red blood cell osmotic fragility can be made by means of different methods.

Conventional or classical OFT (cOFT) was first described in 1947 by Parpart *et al.* Briefly, patients erythrocytes are suspended in increasing solutions of NaCl (concentrations ranging from 0.1% to 0.8%). Spherocytes, being osmotically fragile, burst releasing hemoglobin that is measured spectrophotometrically. The test results show the concentration of NaCl needed to lyse 50% of RBCs. As the percent of hemolysis for each of the solutions is measured, it is plotted against saline concentrations and OF curve is drawn (Fig. 5) [13, 20, 21]. It is estimated that up to 25% patients with HS have normal OFT results when freshly collected blood is tested [5, 15]. To increase the sensitivity of the test, **incubated OFT** (iOFT) should be performed – blood sample is pre-incubated for 24 h at 37 °C in order to enhance the degree of RBCs hemolysis [15, 21]. According to the current guidelines for the diagnosis and management of HS, OF test is no longer recommended for routine practice [11]. The main disadvantage of the test is its relatively low sensitivity and specificity. On the one hand, a test result can be positive not only in HS but also in cases of secondary spherocytosis associated with other conditions, *e.g.* autoimmune hemolytic anemia (AIHA) [14, 15]. In addition, a false negative test result should be taken into account in case of mild HS forms or when a newborn patient is tested (due to high concentrations of fetal Hb red blood cells are less prone to hemolysis) [15, 20].

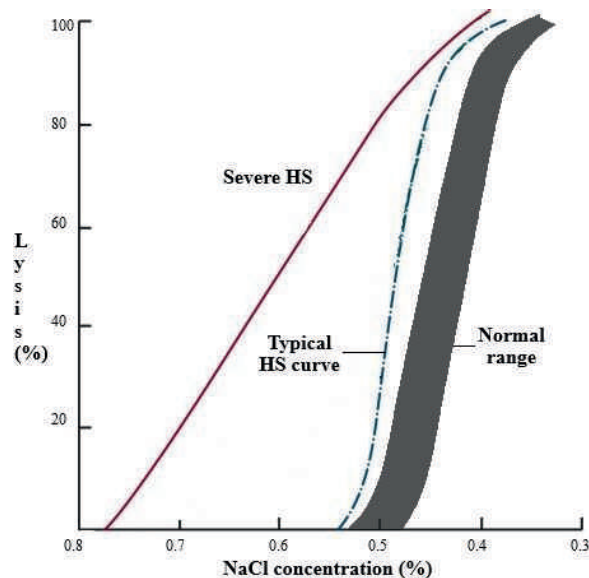


Fig. 5. The osmotic fragility curve

Subsequently, three modifications of the classical OFT were introduced, e.g. **glycerol lysis test (GLT)**, **acidified glycerol lysis test (AGLT)** and **the Pink test**. Based on a similar principle as OFT, they measure the time needed for 50% of erythrocytes to lyse in a buffered solution of hypotonic saline and glycerol [20, 21]. A large study of 150 HS patients by Bianchi *et al.* compared performances of glycerol-based erythrocyte fragility tests and NaCl-OF on fresh and incubated blood together with EMA-binding test. Researchers found out that AGLT and Pink test were more sensitive than classical OFT or GLT with sensitivities of 95% and 91% respectively. However, it was confirmed that each of the tests performed alone fails to diagnose a portion of HS cases due to complicated differentiation from secondary spherocytosis associated mainly with AIHA. Researchers concluded that the combinations of AGLT + EMA-binding test and Pink + EMA-binding test were reported to identify nearly all patients with HS with the sensitivity of 100% and 99% respectively [22].

Flow cytometric osmotic fragility test

A decade ago the most recent osmotic fragility test was introduced by Won and Suh [23]. The flow cytometry-based method measures the proportion of residual red blood cells (% RRC) in a 0.9% NaCl solution after the induction of hemolysis with deionized water (DW). The assessment of red cell count in real-time takes place twice – before and after DW was spiked to a red cell suspension during flow cytometric acquisition. Increased osmotic fragility is indicated by lower % RRC values. The key advantages of the test are as follows: 1) high sensitivity and specificity up to 85.7% and 97.2% respectively; 2) quantitative and objective; 3) cost-effective; 4) no pre-incubation of blood samples needed; 5) time and labor-saving [15, 23, 24]. Moreover, Shim and Won showed that flow cytometric osmotic fragility test (FCM OFT) results do correlate with the clinical severity of disease [25]. In spite still being under research, FCM OF test could be used as a screening tool for HS in hematology laboratories with the flow cytometric equipment.

Cryohemolysis test

Osmotic fragility tests also include the cryohemolysis test (CHT) proposed by

Streichman *et al.* in 1990. Measurement of red blood cell hemolysis is based on the greater fragility of spherocytes suspended in a hypertonic saline solution and exposed to sudden cooling from 37 °C to 0 °C. Originally, the sensitivity and specificity of CHT for the diagnosis of HS was announced to be higher in comparison with conventional OFT as assay is likely to be influenced by RBC membrane defect rather than spherocytic shape and surface to volume ratio decrease. Whereas the sensitivity and specificity of an assay reported in many studies over the years varied greatly, the CHT is now considered to be very specific but moderately sensitive. It is worth noting that false positive results of CHT in patients with widely spread iron deficiency anemia should be taken into account [20, 26].

Erythrocyte membrane protein electrophoresis

The underlying defect in hereditary spherocytosis is an isolated or combined deficiency of main erythrocyte membrane proteins, e.g. ankyrin, spectrin (α and β), band 3, protein 4.1 and/or protein 4.2. Identification of a defective protein is based on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed on disintegrated RBC ghosts followed by densitometric quantification [13, 27].

According to the latest guidelines for the diagnosis and management of HS, if both recommended screening tests, i.e. EMA-binding test and cryohemolysis, produce uncertain results, the recommended method of choice is SDS-PAGE. It is also recommended in cases of unclear diagnosis prior to

splenectomy as surgical management is contraindicated in patients with some forms of hereditary stomatocytosis [11]. Unfortunately, false negative results in up to 20% of cases should be taken into consideration, especially in patients with asymptomatic or very mild forms of disease [27]. Detection of the ankyrin deficiency can be also challenging due to the presence of reticulocytosis as young red blood cells have higher amounts of ankyrin than mature erythrocytes [17].

Osmotic gradient ektacytometry

A precise determination of osmotic fragility caused mainly by the reduction of spherocyte membrane surface area is available by means of osmotic gradient ektacytometry (OGE). Despite the fact, that for many years OGE has been the reference method for diagnosis of hereditary spherocytosis as well as other red blood cell membranopathies, it has not been included in the guidelines because of limited availability in the routine laboratory practice. Recently, the broader clinical application of the test has become possible with the introduction of the next generation osmotic gradient ektacytometry (NG-OGE). Ektacytometry determines erythrocyte deformability under a known shear stress but gradually changing osmolality. Briefly, red blood cell deformability depends on SA/V ratio, total membrane surface area and cytoplasmic viscosity, all reflected by 3 important parameters of the OGE profiles: Omin, deformability index DImax and O' or Ohyper (Fig. 6). The **Omin** point corresponds to the osmolality at the minimal

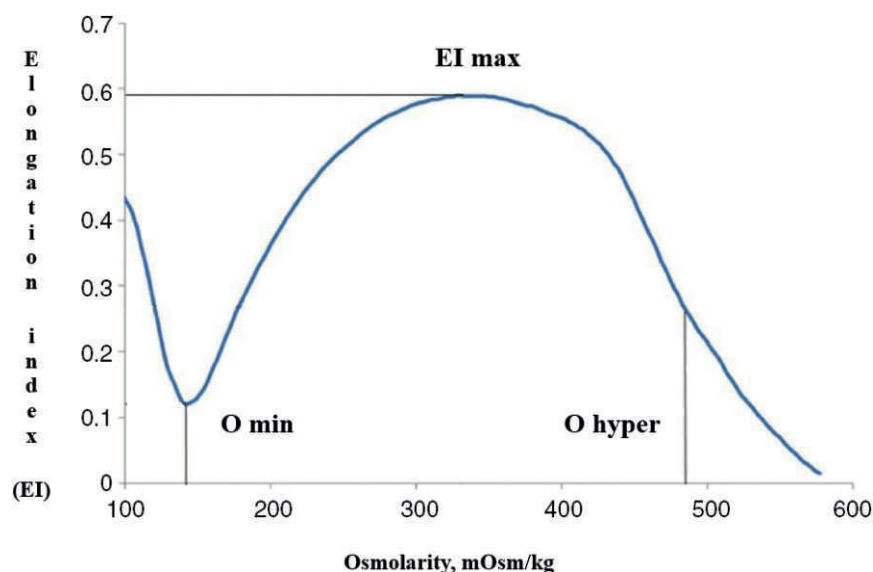


Fig. 6. The ektacytometry curve

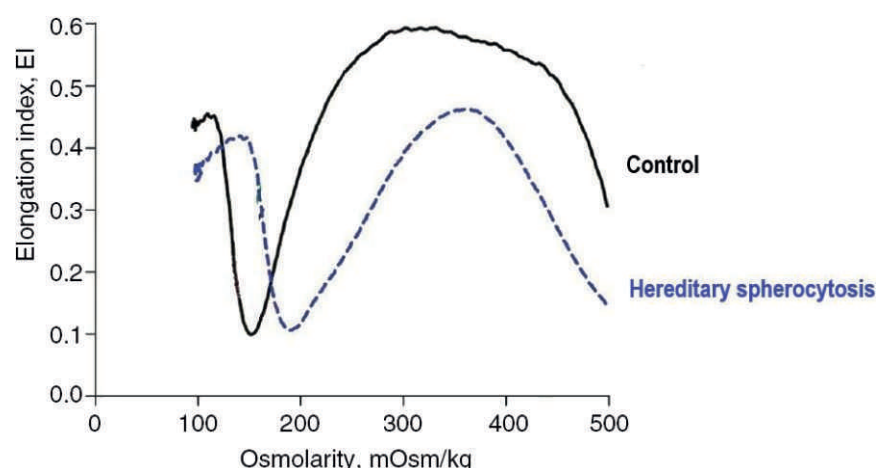


Fig. 7. Ektacytometry indicating typical curves for control and patient with hereditary spherocytosis

deformability region where $\frac{1}{2}$ of the erythrocytes are hemolyzed in the conventional OFT providing with the information on the RBCs SA/V ratio. Maximal deformability index (**DI_{max}**) or elongation index (**EI_{max}**) represents the maximal erythrocyte deformability near 300 mOsm/kg and depends mostly on the cell surface. **O'** or **hyper point** corresponds to the osmolality at 50% **DI_{max}** at the hypertonic region providing with the information about intracellular viscosity, i.e. hydration status of the red cells. An ektacytometry profile in the case of HS shows following characteristic features: **DI_{max}** with **O_{min}** (reflects osmotic fragility) and/or

O_{hyper} (reflects RBC dehydration) (Fig. 7). Importantly, by generating different ektacytometry profiles, NG-OGE is a powerful tool in the differential diagnosis of inherited RBC membrane disorders [14, 28]. Recently Llaudet-Planas *et al.* pointed out that achieving 100% sensitivity for diagnosis of HS is possible with the combination of two diagnostic tests, namely NG-OGE and EMA-binding test [29]. However, the main drawback of the test remains the limited availability of the instrumentation.

Molecular diagnostics

Determination of the underlying genetic mutations within HS-related genes (ANK1, SPTA1, SPTB, SLC4A1 and EPB42) allows appropriate genetic counselling or prenatal diagnosis and is of great importance prior to splenectomy. Until recently, molecular approach for mutation screening in HS included conventional Sanger sequencing based on single gene sequencing. However, the utility of this method in diagnosing HS was considered to be limited because of signifi-

cant heterogeneity of possible molecular defects. In order to overcome diagnostic shortcomings, the advanced genomic survey – next-generation sequencing (NGS) including exome sequencing, whole genome sequencing and gene panels – has been introduced [30–32]. Currently particular attention is paid to NGS panels including large numbers of common HS causative genes. Being accurate, time- and cost-effective method it could be applied to routine molecular testing [10]. Nevertheless, according to the guidelines for RBC membrane disorders from the International Council for Standardization in Haematology (ICSH), molecular testing should be a last option test in the diagnostics of HS, reserved for the cases when family history is uninformative or routine screening tests give either negative or equivocal results [14].

CONCLUSION

The specific survey in 26 European centers was carried out by ENERCA (European Network for Rare Red Cell Anemias) in order to assess which laboratory diagnostic parameters and/or tests were used for the diagnosis of hereditary spherocytosis. It was revealed, that next to red blood cell morphology and reticulocytes, the most frequently used tests are EMA-binding test (performed in 60% of centers) as well as OFT and AGLT (performed in 50% of centers). The majority of centers preferred to use a combination of three previously mentioned diagnostic tests rather than one as no single test can identify all cases of HS. In fact a consensus on which diagnostic test(s) have highest sensitivity as well as specificity is still a matter of debate. Cryohemolysis test despite the recom-

mendation submitted in the guidelines for HS is used in less than 20% of centers. Specific confirmatory assays for HS (SDS-PAGE, ektacytometry or molecular testing) were mostly performed in atypical cases by few centers [4, 33]. In conclusion, the diagnosis of hereditary spherocytosis is a stepwise process including patient's family history, clinical features, physical examination, laboratory tests and the exclusion of other causes of secondary spherocytosis, e.g. autoimmune hemolysis, systemic hematological diseases, congenital dyserythropoietic anemia (CDA) type II, etc.

CASE REPORT

An 11 year-old girl was admitted to Vilnius University Hospital Santaros Clinics Pediatric hospital with general complaints of febrile fever, drowsiness, yellowish discoloration of skin and sclerae. She also presented with nausea, vomiting and abdominal pain since 3 days. Three weeks prior admission to the hospital she had an acute respiratory tract infection, which was complicated by purulent otitis afterwards. Patient has also had past medical history of microspherocytic anemia for approximately 6 years. Her family history was positive as older sister was diagnosed with hereditary spherocytosis and underwent splenectomy at the age of 8 years because of frequent hemolytic crises.

The patient was conscious on an arrival to the hospital, but her general condition was considered to be severe because of anemia, fluid and electrolyte imbalance.

On **physical examination**, the temperature was 37.3 °C, the arterial blood pressure 107/67 mmHg, the heart rate 120 beats/min, the respiratory rate 13 breaths/min, lung sounds represent vesicular breathing. The patient was pale with mild skin and scleral icterus present. Abdominal examination revealed that liver and spleen were both enlarged and palpable 2 cm and 6 cm below the costal margin respectively.

The **laboratory data** were as follows: WBC $3.16 \times 10^9/L$ () (the differential count showed no significant changes), RBC $1.76 \times 10^{12}/L$ (), Hb 46 g/L (), MCV 73.9 fL (), MCH 26.1 pg, MCHC 354 g/L, RDW 21.8% (), PLT $109 \times 10^9/L$ (), RET 0.54% (), RET $0.01 \times 10^{12}/L$ (), IRF 1.4% (). The peripheral blood smear was not performed. The patient's liver function tests were as follows: LDH 332 U/L (upper limit of nor-

mal), total bilirubin 39.2 mol/L () with direct bilirubin of 14.7 mol/L (). CRP level 15 mg/L.

On the basis of familial and clinical history, physical and laboratory investigations, the patient was finally diagnosed as having hereditary spherocytosis. To confirm the diagnosis, an EMA-binding test was performed. Flow cytometric determination of EMA-labeled red blood cells revealed decreased mean fluorescence intensity by 35% (Fig. 4), which definitely confirmed the diagnosis of hereditary spherocytosis.

The patient has received red blood transfusions, fluid infusions and antipyretics. She recovered well and was discharged 8 days after admission with recommendations of regular follow-up.

COMMENTS

Hereditary spherocytosis, as well as other chronic hemolytic disorders, may be complicated by three types of crises, namely aplastic, hemolytic or megaloblastic [5]. Our case report illustrates a typical presentation of aplastic crisis in a case of hereditary spherocytosis.

Aplastic crises are mainly due to virally-mediated bone marrow suppression. The most frequent etiologic agent of aplastic crises in patients with HS is human parvovirus B19 (HPV B19) (the cause of *erythema infectiosum* in general). It selectively infects bone marrow erythroid progenitor cells resulting in their apoptosis and severe red cell aplasia. Individuals with chronic hemolytic anemia, being highly dependent on the accelerated erythropoiesis, can experience a profound fall in hemoglobin level (in this case Hb 46 g/L) due to bone marrow inability to compensate for hemolysis. Thus the characteristic laboratory finding is a low reticulocyte

count (in this case Ret 0.54%, Ret $0.01 \times 10^{12}/L$) despite severe hemolytic anemia [3, 34]. Regardless of the predilection for erythroid precursors, less often parvovirus B19 can also affect other cell lineages (e.g. neutrophils, lymphocytes, platelets) leading to pancytopenia on peripheral blood (also seen in this case) [35].

Parents should be advised to watch for the following parvovirus B19 infection related symptoms such as fever, chills, malaise, nausea, vomiting, abdominal pain with occasional diarrhea, respiratory symptoms or characteristic maculopapular rash (slapped cheek appearance) [5].

The diagnosis of parvovirus B19 induced aplastic crises is often presump-

tive (as in this case), based on previously mentioned laboratory data e.g. profound decrease of Hb value with reticulocytopenia. Other laboratory means of diagnosing parvovirus B19 infection include either identification of HPV B19 DNA using PCR in the blood sample or aspirate of bone marrow or serological tests for anti-HPV B19 IgM and/or IgG antibodies [34].

Usually being a self-limiting, aplastic crisis requires mainly a supportive care and blood transfusions to fulfil the interval between bone marrow suppression and recovery (typically 1–2 weeks) [34]. ♦

Gauta: 2019 05 27
Priimta spaudai: 2019 06 28

Santrauka

PAVELDIMOS SFEROCITIZĖS LABORATORINĖ DIAGNOSTIKA: LITERATŪROS APŽVALGA IR ATVEJO PRISTATYMAS

Eglė Petrik, Agnė Barzinskienė, Rėda Matuzevičienė, Dovilė Karčiauskaitė

Hemolizinė anemija dėl eritrocitų membranos anomalijų dažniausiai pasireiškia sergant paveldimąja sferocitoze. Didžiausias sergamumas yra registruojamas Šiaurės Amerikoje ir šiaurinėje Europos dalyje, kur 2000–5000 gyventojų tenka vienas sergantis asmuo. Eritrocitų membranos baltyminių komponentų (ankirino, band 3 komplekso, ir spektrino, baltymo 4.2) stoka arba defektas susilpnina ryšius tarp lipidų dvilauksnio ir membranos citoskeleto, lemia osmosiškai trapių ir rigidiškų sferocitų formavimąsi. Sergant paveldimąja sferocitoze, pirminė hemolizės priežastis yra prieš laikiną sferocitų destrukciją blužnyje. Liga dažniausiai pasireiškia anemija, gelta ir splenomegalija. Klinikiniai simptomai ir ligos eiga gali įvairuoti nuo besimptomų formų, kai vyrauja visiškai kompensuota hemolizė, iki labai sunkių formų, kurioms gydyti reikia eritrocitų masės transfuzijų ir (ar) splenektomijos. Paveldimosios sferocitozės diagnostika grindžiama šeimine anamneze, ligonio apžiū-

ros duomenimis, bendraisiais laboratoriniais tyrimais (automatizuotu kraujo tyrimu hematologiniu analizatoriumi, retikulocitų skaičiaus, eritrocitų morfologijos nustatymu) ir įvairių specializuotų diagnostinių testų (osmosinio trapumo testo, tėkmės citometrijos, ektacitometrijos, kriohemolizės testo, eritrocitų membranos baltymų elektroforezės ir eritrocitų membranos baltymų koduojančių genų mutacijų nustatymo) deriniu. Straipsnyje apžvelgiama paveldimosios sferocitozės patologinė fiziologija, genetiniai aspektai ir ligos simptomai. Išskirtinis dėmesys tenka laboratorinės diagnostikos metodams – bendriesiems ir ligą patvirtinantiems, šių metodų privalumams ir trūkumams. Straipsnyje aprašomas klinikinis atvejis – parvoviruso sukelta ap-lazinė krizė vaikui, sergančiam paveldimąja sferocitoze.

Reikšminiai žodžiai: hemolizinė anemija, paveldimoji sferocitozė, laboratorinė diagnostika, tėkmės citometrija, osmosinis trapumas, parvovirusas.

LITERATURE

- Wintrobe MM, Greer JP. Wintrobe's clinical hematology. 12th ed. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins, 2009.
- Freedman J. Autoimmune hemolysis: a journey through time. Transfus Med Hemother 2015; 42: 278–85.
- Kaushansky K, Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Prchal JT. Williams hematology. 8th ed. McGraw-Hill, 2010.
- Vives-Corrons JL, Manó Pereira MM, Romeo-Casabona C, Nicolás P, Gulbis B, Eleftheriou A, et al. ENERCA recommendations for centers of exper-
- tise in rare anaemias. A White Book 2013. Internet access: www.enerca.org
- Perrotta S, Gallagher PG, Mohandas N. Hereditary spherocytosis. Lancet 2008; 372(9647): 1411–26.
- Benz EJ, Berliner N, Schiffman FJ. Anemia. Pathophysiology, diagnosis and management. Cambridge University Press, 2018.
- An X, Mohandas N. Disorders of red cell membrane. Br J Haematol 2008; 141: 367–75.
- Gallagher PG. Red cell membrane disorders. Hematology Am Soc Hematol Educ Program 2005; 13–8.
- He BJ, Liao L, Deng ZF, Tao YF, Xu YC, Lin FQ. Molecular genetic mechanisms of hereditary spherocytosis: current perspectives. Acta Haematol 2018; 139: 60–6.
- Kim Y, Park J, Kim M. Diagnostic approaches for inherited hemolytic anemia in the genetic era. Blood Res 2017; 52: 84–94.
- Bolton-Maggs PH, Langer JC, Iolascon A, Tittensor P, King MJ. Guidelines for the diagnosis and management of hereditary spherocytosis – 2011 update. Br J Haematol 2012; 156(1): 37–49.

12. Iolascon A, Avvisati RA. Genotype/phenotype correlation in hereditary spherocytosis. *Haematologica* 2008; 93(9): 1283–8.
13. Ciepiela O. Old and new insights into the diagnosis of hereditary spherocytosis. *Ann Transl Med* 2018; 6(17): 339.
14. King MJ, Garçon L, Hoyer JD, Iolascon A, Picard V, Stewart G, et al. ICSH guidelines for the laboratory diagnosis of nonimmune hereditary red cell membrane disorders. *Int J Lab Hematol* 2015; 37(3): 304–25.
15. Farias MG. Advances in laboratory diagnosis of hereditary spherocytosis. *Clin Chem Lab Med* 2017; 55(7): 944–8.
16. Lazarova E, Pradier O, Cotton F, Gulbis B. Automated reticulocyte parameters for hereditary spherocytosis screening. *Ann Hematol* 2014; 93: 1809–18.
17. Da Costa L, Galimand J, Fenneteau O, Mohandas N. Hereditary spherocytosis, elliptocytosis, and other red cell membrane disorders. *Blood Rev* 2013; 27(4): 167–78.
18. Agarwal AM, Liew MA, Nussenzveig RH, Sangle N, Heikal N, Yaish H, et al. Improved harmonization of eosin-5-maleimide binding test across different instruments and age groups. *Cytometry Part B* 2016; 90B: 512–6.
19. Hunt L, Greenwood D, Heimpel H, Noel N, Whiteway A, King MJ. Toward the harmonization of result presentation for eosin-5'-maleimide binding test in the diagnosis of hereditary spherocytosis. *Cytometry Part B* 2015; 88B: 50–7.
20. Ledesma Achem E, Haro C, Teran MM, Monaco ME, Issé BA, Lazarte SS. Cryohemolysis, erythrocyte osmotic fragility and supplementary hematimetric indices in the diagnosis of hereditary spherocytosis. *Blood Res* 2018; 53(1): 10–7.
21. Paleari R, Mosca A. Controversies on the osmotic fragility test. Internet access: www.enerca.org
22. Bianchi P, Fermo E, Vercellati C, Marcello AP, Porretti L, Cortelezzi A, et al. Diagnostic power of laboratory tests for hereditary spherocytosis: a comparison study in 150 patients grouped according to molecular and clinical characteristics. *Haematologica* 2012; 97(4): 516–23.
23. Won DI, Suh JS. Flow cytometric detection of erythrocyte osmotic fragility. *Cytometry Part B* 2009; 76B: 135–41.
24. Arora RD, Dass J, Maydeo S, Arya V, Radhakrishnan N, Sachdeva A, et al. Flow cytometric osmotic fragility test and eosin-5'-maleimide dye-binding tests are better than conventional osmotic fragility tests for the diagnosis of hereditary spherocytosis. *Int J Lab Hem* 2018; 40: 335–42.
25. Shim YJ, Won DI. Flow cytometric osmotic fragility testing does reflect the clinical severity of hereditary spherocytosis. *Cytometry Part B* 2014; 86B: 436–43.
26. Park SH, Park CJ, Lee BR, Cho YU, Jang S, Kim N, et al. Comparison study of the eosin-5'-maleimide binding test, flow cytometric osmotic fragility test and cryohemolysis test in the diagnosis of hereditary spherocytosis. *Am J Clin Pathol* 2014; 142: 474–84.
27. Debaugnies F, Cotton F, Boutique C, Gulbis B. Erythrocyte membrane protein analysis by sodium dodecyl sulphate-capillary gel electrophoresis in the diagnosis of hereditary spherocytosis. *Clin Chem Lab Med* 2011; 49(3): 485–92.
28. Da Costa L, Suner L, Galimand J, Bonnel A, Pascreau T, Couque N, et al. Diagnostic tool for red blood cell membrane disorders: assessment of a new generation ektacytometer. *Blood Cells Mol Dis* 2016; 56(1): 9–22.
29. Llaudet-Planas E, Vives-Corrons JL, Rizzuto V, Gómez-Ramírez P, Sevilla Navarro J, Coll Sibina MT, et al. Osmotic gradient ektacytometry: a valuable screening test for hereditary spherocytosis and other red blood cell membrane disorders. *Int J Lab Hem* 2018; 40(1): 94–102.
30. He Y, Jis S, Dewan RK, Liao N. Novel mutations in patients with hereditary red blood cell membrane disorders using next-generation sequencing. *Gene* 2017; 627: 556–62.
31. Del Orbe Barreto R, Arrizabalaga B, De la Hoz AB, García-Orad Á, Tejada MI, García-Ruiz JC, et al. Detection of new pathogenic mutations in patients with congenital haemolytic anaemia using next-generation sequencing. *Int Jnl Lab Hem* 2016; 38(6): 629–38.
32. Agarwal AM, Nussenzveig RH, Reading NS, Patel JL, Sangle N, Salama ME, et al. Clinical utility of next-generation sequencing in the diagnosis of hereditary haemolytic anemias. *Br J Haematol* 2016; 174: 806–14.
33. Bianchi P. Current diagnostic approach and screening methods for hereditary spherocytosis. *Thalassemia Reports* 2013; 3(1s): e32.
34. Balaji D, Vinay V, Shankar Vangalpudi V, et al. Aplastic crisis in a child with hereditary spherocytosis with a strong family history resolving with supportive care. *Int J Contemp Pediatr* 2018; 5(3): 1150–2.
35. Frotscher B, Salignac S, Morlon L, Bonmati C, Jeulin H, Venard V, et al. Neutropénie et/ou thrombopénie associée(s) à une infection aiguë par le parvovirus B19. *Ann Biol Clin* 2009; 67: 343–8.