

REVIEW

Overview on Hereditary Spherocytosis Diagnosis

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ABSTRACT

Introduction: Hereditary spherocytosis (HS) is a congenital haemolytic disorder, resulting from plasma membrane protein deficiency of red blood cells (RBCs). Typical pathological signs are anemia, jaundice, and splenomegaly; in newborns, jaundice is the main symptom.

Material and Methods: This study focused on the state of art about the HS diagnosis, from traditional to innovative methods, including diagnostic algorithms that can be applied for pediatric and adult patients, for different laboratory diagnostic levels.

Results: The first erythrocyte parameters used for HS diagnosis were the mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), and red blood cell distribution width (RDW); nowadays new parameters are used in blood cell counter. Advia analyzers (Siemens Medical Solutions) supply the hyper-dense cell percentage (% Hyper), which reflects the red blood cells hyperchromia. Sysmex instruments (i.e. XT-4000i, XE-5000, XN-Series) provide the MicroR, that is the percentage of erythrocytes smaller than 60 fL, Hypo-He, which is the percentage of erythrocytes with a content of hemoglobin less than 17 pg and % Hyper-He, which represents the percentage of RBC with cellular hemoglobin content higher than 49 pg. CELL-DYN Sapphire (Abbott Diagnostics) introduced the HPR parameter (% HPR), which represents the erythrocytes with hemoglobin > 410 g/L. Beckman Coulter instruments supply the mean spherized corpuscular volume (MSCV), which is the average volume of all erythrocytes, including mature erythrocytes and reticulocytes. Other reference tests for screening and diagnosis of HS are the acidified glycerol lysis test (AGLT), the eosin-5-maleimide (EMA) binding test and genetic testing by next-generation sequencing.

Conclusions: The diagnostic workup of hereditary spherocytosis could be improved thanks to all the available tests, including new molecular tools. However, it requires synergy between clinicians and laboratory staff, evaluating clinical manifestations, all available data related to the disease and the prognosis to fill the diagnostic gaps in the near future.

1 | Introduction

Hereditary spherocytosis (HS) is included among congenital hemolytic anemia. It is caused by plasma membrane protein deficiency, resulting in spherical-shaped red blood cells (RBCs), which

are prone to hemolysis [1–3]. There is little data regarding the epidemiology of HS: in North America and Northern Europe the incidence is 1/5000 [4]. The prevalence of the disease above two areas is 1/2000–1/5000. The prevalence of HS in Europe is 1–5/10000 [5], however, the disease is mostly common in Caucasians, whose

prevalence is 1–2/5000, although by Kutter it is much higher due to the lack of symptoms in the majority of the cases [6]. In China, the prevalence of HS estimated by Wang et al. [7] was 1.27/100.000 in males and 1.49/100.000 in females. About 75% of cases present an autosomal dominant inheritance [8, 9].

1.1 | Biochemical Abnormalities Associated With HS

The main cause of HS is based on erythrocyte membrane protein defects, with a resulting reduction in surface-to-volume ratio. To date, the main membrane protein mutations involve ankyrin-1, band 3, α , β -spectrin, and protein 4.2 [10]. RBCs thus become spheroidal and osmotically fragile cells, which are prone to be destroyed in the spleen [11]. According to the study of Mariani et al. [12], band 3 protein mutations were detected in approximately 54% of 300 European patients with HS. Park et al. [13] discovered that mutations in ankyrin protein are found in 52% of HS patients in South Korea.

1.2 | Clinical Features

According to the “guidelines for the diagnosis and management of HS” [14], the patients are clinically classified as “mild,” “moderate,” or “severe”: the clinical severity of the disease is linked to the lower spectrin content. Typical pathological signs include anemia, jaundice, and splenomegaly. The degree of anemia is associated with spleen size and hemolysis. As for the last one, severe patients lack single membrane protein or have multiple protein mutations (commonly affecting band 3 and ankyrin), consequently more severe hemolytic anemia [15]. Splenectomy can help reduce symptoms, however, patients with severe HS remain anemic also postsplenectomy. Jaundice is commonly found in the first 2 days of life: it may be so serious that an exchange transfusion is required. However, it is not yet known whether it is linked to the severity of HS. In children and adults with HS, spleen enlargement can be observed, but other than helping in making diagnosis, it has little clinical significance.

1.3 | Laboratory Investigations

The HS diagnosis is focused on laboratory data, above all red cell indices, erythrocyte morphology, and reticulocyte count, in addition to clinical features, physical examination, and family history. The amount of erythrocyte diseases is very large, such as acquired hemolytic anemia, hemoglobinopathies, and intra-erythrocyte enzyme deficiencies. For this reason, many screening and laboratory tests have been introduced to distinguish the different diseases and to rule out confounding blood pathologies. The main reference tests for screening and diagnosis of HS are complete blood count analysis, the eosine-5-maleimide (EMA) binding test, the acidified glycerol lysis test (AGLT), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Unfortunately, not all these tests are easy to perform. They often require specialized centers and highly qualified operators, and, above all, none of these tests, carried out individually, is able to diagnose all cases of HS [16, 17].

1.4 | Complete Blood Count (CBC) Analysis

The oldest and most important erythrocyte indices used for HS diagnosis are the mean corpuscular volume (MCV), the mean corpuscular hemoglobin concentration (MCHC), and red blood cell distribution width (RDW) [18]. MCHC and RDW indices are increased in HS, MCHC could be normal in mild cases and elevated in moderate and severe ones. These findings can allow diagnosis with a good probability and interesting levels of sensitivity and specificity [19, 20]. At this time, many hematological analyzers provide additional erythrocyte markers, useful to the laboratory specialist to suspect the presence of spherocytes that, in the appropriate context, may lead to suspicion of HS diagnosis. Among them, the Advia analyzer (Siemens Medical Solutions Diagnostics, NY, USA) supplies a parameter obtained by a double-beam laser technology, that connects light scattering measurement from hemoglobin content (HC) and volume (V) per cell, obtaining a cytogram (V/HC) indicating the hyper-dense cell percentage (% Hyper): this parameter highlights the RBC hyperchromia [21]. Abbott Diagnostics (Abbott Diagnostics, Santa Clara, CA, USA) developed a new software for the Cell-Dyn Sapphire analyzer, which allowed the measurement of extended RBC and reticulocyte parameters, detecting hyperchromic RBC (HPR), which corresponds to erythrocytes with hemoglobin > 410 g/L, using a multi-angle light scatter technology [22] and correlates spherocytes with increased HPR.

Sysmex instruments (i.e., XT-4000i, XE-5000, XN-Series) introduced MicroR, which corresponds to the percentage of erythrocytes smaller than 60 fL and two other parameters, based on the mean HC of all the RBC analyzed in the reticulocyte channel: % Hypo-He, defined as the percentage of erythrocytes with a content of hemoglobin less than 17 pg and % Hyper-He, which represents the percentage of RBC with cellular HC higher than 49 pg [23]. On Sysmex analyzers spherocytes are characterized by an increased % Hyper-He and MicroR count, a decreased % Hypo-He count, and an increased MicroR/Hypo-He ratio. This is the consequence because the spherocytes are small, dense, and hyperchromic RBC and they have a reduced membrane surface area-to-volume ratio [24, 25]. Beckman Coulter instruments (Beckman Coulter Inc., Fullerton, CA, USA) offer a new and original parameter, the MSCV, which is an artificial volume measurement count [26, 27]. The MSCV represents the average volume of all erythrocytes, both mature erythrocytes and reticulocytes, thanks to a mild swelling in the hypo-osmotic solution, used in the reticulocyte count [2]. Normally MSCV should be higher than MCV, in patients with HS, instead, MSCV is lower than MCV, due to the rigidity of the membrane and the consequent inability of the spherocytes to swell and expand their volume in hypo-osmotic solution. Low MSCV has been demonstrated to be a sensitive parameter for HS detection; one parameter that has proven to be very interesting is the difference between MCV and MSCV (MCV-MSCV), also known as delta MCV-MSCV. This calculation, when increased, has an even higher discriminatory value for HS detection [28, 29]. When the delta (MCV-MSCV) value is greater than 9.6 fL, HS could be suspected. For this reason, each laboratory should validate its own cutoff point, because different hematological analyzers cannot be compared.

1.5 | Reticulocyte Count

Reticulocyte indices are very important for HS screening: the decrease in the surface-to-volume ratio is a characteristic that belongs to both reticulocytes and spherocytes. The reticulocyte parameters developed on the latest hematological analyzers are the absolute number of reticulocytes, the immature reticulocyte fraction (IRF), and mean reticulocyte volume (MRV or MCVR), which represent the average volume of all reticulocytes. A low MRV has been demonstrated to be a good parameter to discriminate between HS and non-HS subjects. However, it has a lower sensitivity and specificity than other reticulocyte indices [28]. Patients with HS are also characterized by a high reticulocyte count with a low immature reticulocyte fraction (IRF). The combination of reticulocyte count and immature reticulocyte fraction (Ret/IRF ratio) is another useful parameter for the screening of HS. Mullier et al. showed that mild cases of HS have a Ret/IRF ratio higher than 19, while severe cases of HS have a Ret/IRF ratio higher than 7.7 [30]. In the study of Lazarova et al., IRF compared to delta (MCV-MSCV) or MRV, proved effective diagnostic performances, lower specificity, lower positive predictive value, and likelihood ratio. Combining some parameters into diagnostic algorithms, such as $MCV-MSCV > 10$ with $MRV-MSCV < 25$, sensitivity and specificity have been improved [29].

1.6 | Which Method Is Better?

Anne-Sophie Adam et al. [31] evaluated and compared the effectiveness of six published algorithms for HS screening. The study was based on the use of the UniCel DxH800 (Beckman Coulter) and the XN-9000 (Sysmex) analyzers, with the aim of determining the most suitable algorithm in daily clinical practice (Table 1). The reticulocyte parameters used on the DxH800 were MRV, IRF, and MSCV. On the XN-9000 were hypohemoglobinized erythrocytes, microcytic erythrocytes, and IRF. Regarding the Beckman Coulter analyzer parameters, the algorithm proposed by Liao et al. [32] achieved the best performances, with the correct identification of all HS patients tested (specificity 94.9%). On the other hand, the other two algorithms published by Lazarova et al. [26] and Nair et al. [29], had poorer results. The three algorithms applied on the Sysmex instruments, that is, the algorithms published by Mullier et al. [30], Persjin et al. [33], and Bobee et al. [24] showed similar performances to the ones obtained with the Beckman Coulter analyzer.

On the other hand, Rooney et al. [22] tested the hyperchromic RBC (HPR) in pediatric patients using the Cell-Dyn Sapphire analyzer: the ROC analysis showed that HPR had high sensitivity (96.4%) and specificity (99.1%), with an optimal cutoff value of 4.9% [22].

1.7 | Morphology Analysis

According to the first “guidelines for the diagnosis and management of HS” [14] well-defined cases characterized by clinical details, such as the presence of spherocytes and reticulocytosis or a negative direct antiglobulin test (DAT), will support the

diagnosis of HS. When there is no family history, an important differential diagnosis is autoimmune hemolytic anemia (AIHA), which is rare in children. AIHA can sometimes be excluded by a negative DAT. Nonetheless, the absence of spherocytes on the blood smear, or normal red cell indices with typical reticulocyte count, then a ‘carrier’ condition should be considered. If the morphology is atypical, other membrane abnormalities must be considered, especially, for congenital dyserythropoietic anemia type II (CDAII).

1.8 | Osmotic Fragility (OFT) Test

OFT is a semiquantitative test that assesses erythrocyte hemolysis as a consequence of hypotonic solutions of sodium chloride (NaCl) treatment. Spherocytes hemolyze after exposure to NaCl, because of their lower osmotic resistance compared to normal RBC. Positive results are reported in many diseases such as HS, hereditary elliptocytosis, and autoimmune hemolytic anemia [34]. Shim et al. [35] compared HS patients with non-HS controls. In patients with HS, the OFT showed a diagnostic sensitivity and specificity of 66% and 81.8%, respectively. Overall, a normal result cannot rule out the diagnosis of HS, this condition represents 10%–20% of HS cases.

In the same way, a newborn could be considered as a false negative due to the high level of fetal hemoglobin (HbF), which gives erythrocytes more resistance to hemolysis.

1.9 | AGLT

The AGLT is based on the affinity of glycerol and membrane lipids for a hypotonic salt buffer, that chemically reacts with erythrocyte membrane, causing hemolysis of RBC. As cell lysis increases, the absorbance of the solution declines gradually to half the initial absorbance level [36]. Even if the AGLT test has a high sensitivity, it has reduced specificity, and it is time-consuming.

1.10 | EMA

This is a flow cytometric test, which is based on the binding of the Eosin-5'-maleimide to band 3 protein. EMA provides quantitative results: it is interpreted as positive for HS when the grade of fluorescence is $> 21\%$, negative if fluorescence is $< 16\%$ [37, 38], and equivocal if the percentage is included between 16% and 21% [39]. EMA has a high sensitivity (92.7%–96.6%) and specificity (99.1%) for HS diagnosis. EMA test is considered the most accurate screening test, with a relatively short time-around-time (TAT), high sensitivity, economical, and feasibility also in laboratories furnished with a basic flow cytometer. Furthermore, the EMA test can discriminate between different red cell populations, so that the results are not skewed in patients undergoing transfusion [40].

1.11 | Plasma Membrane Protein Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), different from other tests, consents to the qualitative and quantitative identification of abnormal plasma membrane

TABLE 1 | Algorithms for diagnosis of HS.

Author	Instrument	Algorithm (Ret μ L) (Hb g/L) (MicroR %)		Sensitivity (%)	Specificity (%)
Mullier et al. (2011) [30]	Sysmex XE5000	Hb > 120 Ret/ IRF ≥ 19	Ret $\geq 80\,000$ e Ret/IRF > 7.7 80 \leq Hb ≤ 120 MicroR ≥ 3.5 e MicroR/Hypo-He ≥ 2.5	100.0	99.3
Persijn et al. (2012) [33]	Sysmex XE5000	Trait and Mild HS	Moderate HS	Severe HS	
		Hb > 120 Ret/ IRF ≥ 19	Ret $\geq 100\,000$ e Ret/IRF > 7.7 80 \leq Hb ≤ 120 MicroR ≥ 2.6 e MicroR/Hypo-He ≥ 2.5	84.0	99.0
Bobee et al. (2018) [24]	Sysmex XE5000	Trait and mild HS	Moderate HS	Severe HS	
		Ret > 80 000 and Ret/IRF > 9.1 and MicroR (if Hb < 120) > 2.2 and MicroR/Hypo-HE (if Hb > 120) ≥ 3.5		100.0	92.1
Lazarova et al. (2014) [26]	Beckman Coulter UniCel DxH800	(MCV-MSCV-MRV fL)			
		MCV-MSCV > 10.4 and/or MCV < 70.2 and/or MRV < 96.7		100	74
				92	90
Nair et al. (2015) [29]	Beckman Coulter LH755	MCV-MSCV > 10 and MRV-MSCV < 25		100	88
Liao et al. (2019) [32]	Beckman Coulter LH780	MSCV-MCV < 0.6		84.2	94.7
Rooney et al. (2015) [22]	Abbott Cell-Dyn Sapphire	HPR%		95.5	94.9
				96.4	99.1

Abbreviations: Hb, hemoglobin concentration; HPR%, hyperchromic RBC; Hypo-He, percentage of erythrocytes with a content of hemoglobinaemoglobin less than 17 pg; IRF, immature reticulocyte fraction; MCV, mean corpuscular volume of erythrocytes; MicroR, percentage of erythrocytes smaller than 60 fL; MRV, mean reticulocyte volume; MCV, mean spheroid corpuscular volume of erythrocytes; Ret, reticulocyte count.

protein content. Differently from EMA or the OFT test, which are much more common [16], the SDS technique can only be performed in specialized diagnostic centers and is time-consuming. Unfortunately, the SDS test in 10%–20% of cases, could not detect deficient plasma membrane.

1.12 | Genetic Testing

The genetic expression study of HS is used in association with the standard screening tests in order to enhance the reliability of HS diagnosis. Actually, some laboratories use the high-resolution melting curve technique, even if it has some limitations, such as poor identification of A/T or G/C base changes. For this reason, it was partially replaced by next-generation sequencing, which is the modern technology for genetic screening and diagnosis. The latter is a time- and cost-effective technology, characterized by high throughput and with appropriate phenotyping, which significantly increases the sensitivity (from 70% to 100%) [41]. In order to sequence and to screen potential variants deletion and/or duplication analysis at once, an NGS panel targeting *ANK1*, *SPTB*, *SLC4A1*, *SPTA1*, and *EPB42* gene has been created: *ANK1*, *SLC4A1*, or *SPTB* mutations are common in patients with an autosomal dominant profile for HS [3].

The last guidelines suggest the use of genetic testing in association with other screening tests, when the diagnosis of HS is difficult, especially in the presence of mild clinical symptoms [42].

This is applied in a particular way to estimate the disease development, treatment options (splenectomy or not), and most of all, to allow genetic counseling for patients and their families [43]. In addition, genotyping is also useful to individuate a recessive inheritance or de novo variant, especially in HS cases without a clear family history. Finally, genotyping is useful in conditions of suspected HS, also with markedly different phenotypes: as it can be possible to see in cases of coexistent conditions that could decrease the severity of the HS [44].

1.13 | Diagnostic Protocol for HS

According to the first version of British guidelines for the diagnosis and management of HS [4], in the case of recently diagnosed patients, with family and clinical history of HS, as well as confirmatory diagnostic laboratory tests, no further investigations are required. However, if the diagnosis is equivocal, it is recommended to do screening tests, such as cryo-hemolysis, and the EMA binding test. In case the screening tests are borderline or inaccurate, more specific tests are required, particularly gel electrophoresis analysis of erythrocyte membranes, like SDS-PAGE. The latter is recommended in the following cases:

1. when the clinical appearance is more severe than the RBC morphology;
2. when the red cell morphology is more severe than the one observed in family history;
3. in case of the uncertainty of the diagnosis before the splenectomy.

The last update, published in 2021 [42], suggests a simple protocol for the diagnosis of HS, with the addition of some tests, such as MRV and MSCV. Furthermore, it takes into consideration symptoms, family history, diagnostic tests for hemolytic anemia, genetic testing, and other laboratory surrogate tests.

1.13.1 | Screening Tests

- Hb level in trait form is normal; mild 110–150 g/L; moderate 80–120 g/L; severe 60–80 g/L.
- Reticulocyte count in trait form is < 3%; in mild cases 3%–6%; moderate > 6%; severe: > 10%.
- MCHC > 355 g/L is used as the cutoff for HS diagnosis.
- High number of spherocytes.
- Total serum bilirubin increased, especially for unconjugated bilirubin.
- MRV is decreased (cutoff: ≤ 95.77 fL); MSCV < MCV.

Differently from the previous guidelines, the last one suggests the use of analytical methods, such as OFT, AGLT, Coombs test, and G6PD level determination, in addition to genetic testing. On the other hand, ICSH proposed an algorithm in case of HS suspicion [2]. To verify the hemolysis, RDW-CV and parameters obtained from the reticulocyte channel are suggested. In order to analyze the presence of spherocytes or other morphological abnormalities, a blood smear is performed. If the HS diagnosis is still suspicious, cryo-hemolysis and EMA tests are suggested. In this case, there are two possibilities. Both negative tests: no further testing is required. Positive or doubtful results: the ektacytometry test is conducted.

Furthermore, if the patient's anamnesis is typical for HS, the diagnosis can be preserved. In case of doubtful anamnesis, SDS-PAGE is performed to exclude CDA II, to confirm the diagnosis of HS, and to determine the protein mutation. Molecular analysis is only performed in cases of chronic transfusion or mismatch between clinical profile and analytical results.

1.14 | Making the Diagnosis of HS in a Newborn

About 65% of newborns with HS have a parent with the same pathology [45]. The clinical profile of HS through the perinatal period varies from severe fetal anemia with hydrops fetalis to the absence of symptoms. In newborns, jaundice is the most common symptom [46]. Approximately in one-third of newborns with HS, spherocytes are not detected in the blood smear, but pyknocytes: distorted densely stained red cells. According to the guidelines for the diagnosis and management of hereditary spherocytosis, in case of suspected HS [3], in newborns without symptoms, analytical tests can be rescheduled until the sixth month of age or later, as the morphology could be less confusing. Typically, a newborn with HS presents low MCV and elevated MCHC. New studies demonstrate that when MCHC > 360 g/dL in newborns, it suggests the presence of the disease (82% sensitivity and 98% specificity) [46]. Based on this data, the HS ratio in the neonatal period could be calculated

by dividing the MCHC by the MCV. According to a recent review, when the HS ratio > 0.36 is predictive for identifying the disease, with 97% sensitivity and $> 99\%$ specificity [47]. HS could be suspected when the HS ratio intermediate [38, 39] or normal (< 35) is present. Using this ratio, an overlap may occur, whereby it is possible to differentiate HS newborns from healthy ones, through additional tests, such as elution of anti-A or anti-B from neonatal erythrocytes or indirect Coombs test. Since the HS diagnosis may be still uncertain, EMA binding or OFT testing could be performed [48]. Sequencing of the main genes can be done as a confirmatory test [49]. In this scenario, evaluating a newborn for HS, when a parent is known to have HS, according to the American Academy of Pediatrics' guidelines [50], it is important to treat the newborn as if they have HS, starting with the evaluation during the birth hospitalization. Among the exams, peripheral smear examination and a reticulocyte count are recommended, as well as bilirubin monitoring at birth and within 24 h before the hospital discharge.

Differently in the case of a jaundiced newborn with HS, whose parents are healthy, there is no need to wait for anemization to start the evaluation. However, in the case of hemolytic jaundice, it could be useful to perform a complete blood cell count with peripheral blood smear, blood type of mother and infant, DAT, reticulocyte count, and glucose-6-phosphate dehydrogenase deficiency testing. If the diagnosis of HS is doubtful, EMA binding or OFT testing could be performed or additional diagnostic testing, by NGS or a hematology consultant.

1.15 | Confounding Factors in HS Diagnosis

The main factors involved in misdiagnosis or missed diagnosis of HS are:

- a. heterogeneous clinical manifestations of HS;
- b. the sensitivity or specificity of some parameter, such as MCHC, or diagnostic tests, such as OFT or red cell morphology;
- c. insufficient knowledge of HS.

Several common confounders include vitamin/iron deficiency, and the coexistence of other diseases, such as mild myelodysplasia in advanced age, which could hinder bone marrow compensation. Likewise, the presence of hemolysis associated with mechanical injury, toxic agents, and infections, which could be found in healthy subjects and many other hematologic diseases should be considered [51,52]. Cases of HS misdiagnosis were reported by Deng et al. [53]. Overall, confounding factors should be taken into consideration, keeping an open-minded view when analyzing different pathological conditions characterized by hemolysis.

2 | Conclusions

The HS diagnosis may be challenging due to the need for specialized tests and staff, including the symptom heterogeneity, which overall could cause a delayed diagnosis, misdiagnosis, or inappropriate therapies. Currently, HS diagnosis could be improved, thanks to all the new and traditional available tests. The

characteristic morphology of spherocytes, consequently the abnormal concentration of hemoglobin, and the typical surface-to-volume ratio have prompted the technology to find methods to accurately measure these features. The first parameters used in the diagnosis of HS were MCHC and RDW, together with reticulocyte count and IRF. MCHC and RDW indices are increased in HS, particularly MCHC could be normal in mild cases and elevated in moderate and severe ones, reaching good levels of sensitivity and specificity. Regarding reticulocyte parameters, patients with HS are characterized by a high reticulocyte count with a low IRF: the combination of reticulocyte count and immature reticulocyte fraction (Ret/IRF ratio) is a useful parameter for the screening of HS. Some instruments have introduced innovative parameters, which are altered in the HS, such as the MSCV and the associated delta MCV-MSCV: in case delta MCV-MSCV > 10 there are good specificity and sensibility. In other cases, the great knowledge of physical principles has allowed us to discover new systems for correlating standard parameters and finding new strategies, such as the hyperchromic RBC (HPR) measurement, based on multi-angle laser scatter flow cytometry. Other technological systems have exploited the characteristic of erythrocytes that in HS are small, dense, and hyperchromic. Some authors found a very good statistical correlation with the diagnosis of HS, relating erythrocytes smaller than 60 fL (MicroR) to erythrocytes with hemoglobin concentration below 17 pg (Hypo-HE). Furthermore, an EMA binding assay or OF test allows for confirming or rejecting the presence of the disease. In the same way, further defects affecting RBC, such as acquired hemolytic anemia, hemoglobinopathies, and intra-erythrocyte enzyme deficiencies, are identified in the laboratory with all the available technologies and methods, from morphology to molecular biology, allowing the successful identification of individual atypical erythrocyte subset. However, the pathway of HS diagnosis, as well as other defects, primarily needs the synergy between clinicians and laboratory staff, evaluating clinical manifestations, all available data linked to the disease, and the prognosis. Furthermore, the availability of new molecular tools, such as NGS, could fill the diagnostic gaps in the near future, thus giving more value to genetic counseling, which is most in demand by affected families.

Author Contributions

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The authors have nothing to report.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data and information used for the drafting of this paper are available on request, writing to Dr. Alessandro Polizzi at Operative Unit of Clinical Pathology of "R. Guzzardi" Hospital in Vittoria, A.S.P. of Ragusa.

References

1. S. Manciu, E. Matei, and B. Trandafir, "Hereditary Spherocytosis—Diagnosis, Surgical Treatment and Outcomes. A Literature Review," *Chirurgia (Bucur)* 112 (2017): 110–116, 116, <https://doi.org/10.21614/chirurgia.112.2.110>.
2. M. J. King, L. Garcon, J. D. Hoyer, et al., "ICSH Guidelines for the Laboratory Diagnosis of Nonimmune Hereditary Red Cell Membrane Disorders," *International Journal of Laboratory Hematology* 37 (2015): 304–325, <https://doi.org/10.1111/ijlh.12335>.
3. P. H. Bolton-Maggs, J. C. Langer, A. Iolascon, General Haematology Task Force of the British Committee for Standards in Haematology, et al., "Guidelines for the Diagnosis and Management of Hereditary Spherocytosis—2011 Update," *British Journal of Haematology* 156 (2012): 37–49, <https://doi.org/10.1111/j.1365-2141.2011.08921>.
4. P. H. Bolton-Maggs, "Hereditary Spherocytosis; New Guidelines," *Archives of Disease in Childhood* 89, no. 9 (2004): 809–812, <https://doi.org/10.1136/adc.2003.034587>.
5. B. Gulbis, A. Eleftheriou, M. Angastiniotis, et al., "Epidemiology of Rare Anaemias in Europe," *Advances in Experimental Medicine and Biology* 686 (2010): 375–396, https://doi.org/10.1007/978-90-481-9485-8_22.
6. D. Kutter, "Hereditary Spherocytosis Is More Frequent Than Expected: What to Tell the Patient?," *Bulletin de la Société des Sciences Médicales du Grand-Duché de Luxembourg* 1 (2005): 7–22, <https://pubmed.ncbi.nlm.nih.gov/16042049/>.
7. C. Wang, Y. Cui, Y. Li, X. Liu, and J. Han, "A Systematic Review of Hereditary Spherocytosis Reported in Chinese Biomedical Journals From 1978–2013 and Estimation of the Prevalence of the Disease Using a Disease Model," *Intractable & Rare Disease Research* 4, no. 2 (2015): 76–81, <https://doi.org/10.5582/iridr.2015.01002>.
8. A. Iolascon, I. Andolfo, and R. Russo, "Advances in Understanding the Pathogenesis of Red Cell Membrane Disorders," *British Journal of Haematology* 187 (2019): 13–24, <https://doi.org/10.1111/bjh.16126>.
9. J. Narla and N. Mohandas, "Red Cell Membrane Disorders," *International Journal of Laboratory Hematology* 39, no. Suppl 1 (2017): 47–52.
10. S. Perrotta, P. G. Gallagher, and N. Mohandas, "Hereditary Spherocytosis," *Lancet* 372 (2007): 1411–1426, <https://doi.org/10.1016/j.blre.2006.03.005>.
11. N. Mohandas and P. G. Gallagher, "Red Cell Membrane: Past, Present, and Future," *Blood* 112 (2008): 3939–3948, <https://doi.org/10.1182/blood-2008-07-161166>.
12. M. Mariani, W. Barcellini, C. Vercellati, et al., "Clinical and Hematologic Features of 300 Patients Affected by Hereditary Spherocytosis Grouped According to the Type of the Membrane Protein Defect," *Haematologica* 93, no. 9 (2008): 1310–1317, <https://doi.org/10.3324/haematol.12546>.
13. J. Park, D. C. Jeong, J. Yoo, et al., "Mutational Characteristics of ANK1 and SPTB Genes in Hereditary Spherocytosis," *Clinical Genetics* 90, no. 1 (2016): 69–78, <https://doi.org/10.1111/cge.12749>.
14. P. H. Bolton-Maggs, R. F. Stevens, N. J. Dodd, G. Lamont, P. Tittensor, and M. J. King, "Guidelines for the Diagnosis and Management of Hereditary Spherocytosis," *British Journal of Haematology* 126 (2004): 455–474, <https://doi.org/10.1111/j.1365-2141.2010.08166>.
15. S. Rocha, E. Costa, P. Rocha-Pereira, et al., "Erythrocyte Membrane Protein Destabilization Versus Clinical Outcome in 160 Portuguese Hereditary Spherocytosis Patients," *British Journal of Haematology* 149 (2010): 785–794, <https://doi.org/10.1111/j.1365-2141.2010.08166>.
16. M. J. King, "Zanella A Hereditary Red Cell Membrane Disorders and Laboratory Diagnostic Testing," *International Journal of Laboratory Hematology* 35, no. 3 (2013): 237–243, <https://doi.org/10.1111/ijlh.12070>.
17. P. Bianchi, E. Fermo, C. Vercellati, et al., "Diagnostic Power of Laboratory Tests for Hereditary Spherocytosis: A Comparison Study in 150 Patients Grouped According to Molecular and Clinical Characteristics," *Haematologica* 97, no. 4 (2012): 516–523, <https://doi.org/10.3324/haematol.2011.052845>.
18. S. C. Steward, A. R. Chauvenet, and C. O'Suoi, "Hereditary Spherocytosis: Consequences of Delayed Diagnosis," *SAGE Open medicine* 2 (2014): 2050312114547093, <https://doi.org/10.1177/2050312114547093>.
19. R. D. Christensen, H. M. Yaish, and P. G. Gallagher, "A Pediatrician's Practical Guide to Diagnosing and Treating Hereditary Spherocytosis in Neonates," *Pediatrics* 135 (2015): 1107–1114, <https://doi.org/10.1542/peds.2014-3516>.
20. M. G. Farias, "Advances in Laboratory Diagnosis of Hereditary Spherocytosis," *Clinical Chemistry and Laboratory Medicine* 55 (2017): 944–948, <https://doi.org/10.1515/cclm-2016-0738>.
21. M. G. Farias and P. A. C. Freitas, "Percentage of Hyperdense Cells: Automated Parameter to Hereditary Spherocytosis Screening," *Clinical Biochemistry* 48, no. 18 (2015): 1341–1343, <https://doi.org/10.1016/j.clinbiochem.2015.07.025>.
22. S. Rooney, J. J. Hoffmann, O. M. Cormack, and C. McMahon, "Screening and Confirmation of Hereditary Spherocytosis in Children Using a CELL-DYN Sapphire Haematology Analyser," *International Journal of Laboratory Hematology* 37 (2015): 98–104, <https://doi.org/10.1111/ijlh.12245>.
23. S. M. Larsson, T. Ulinder, A. Rakow, et al., "Hyper High Haemoglobin Content in Red Blood Cells and Erythropoietic Transitions Postnatally in Infants of 22 to 26 Weeks' Gestation: A Prospective Cohort Study," *Archives of Disease in Childhood Fetal Neonatal Edition* 108 (2023): F612–F616, <https://doi.org/10.1136/archdischild-2022-325248>.
24. V. Bobée, S. Daliphard, A. Schräpp, et al., "Screening of Hereditary Spherocytosis and Pyruvate Kinase Deficiency by Automated Blood Count Using Erythrocytic and Reticulocytic Parameters," *International Journal of Laboratory Hematology* 40, no. 6 (2018): 697–703, <https://doi.org/10.1111/ijlh.12906>.
25. J. Y. Sottiaux, J. Favresse, C. Chevalier, B. Chatelain, H. Jacqmin, and F. Mullier, "Evaluation of a Hereditary Spherocytosis Screening Algorithm by Automated Blood Count Using Reticulocytes and Erythrocytic Parameters on the Sysmex XN-Series," *International Journal of Laboratory Hematology* 42, no. 2 (2020): e88–e91, <https://doi.org/10.1111/ijlh.13125>.
26. E. Lazarova, O. Pradier, F. Cotton, and B. Gulbis, "Automated Reticulocyte Parameters for Hereditary Spherocytosis Screening," *Annals of Hematology* 93, no. 11 (2014): 1809–1818, <https://doi.org/10.1007/s00277-014-2127-8>.
27. L. Liao, Z. F. Deng, Y. L. Qiu, P. Chen, W. Q. Chen, and F. Q. Lin, "Values of Mean Cell Volume and Mean Sphered Cell Volume Can Differentiate Hereditary Spherocytosis and Thalassemia," *Hematology* 19 (2014): 393–396, <https://doi.org/10.1179/1607845413Y.0000010149>.
28. O. Ciepiela, A. Adamowicz-Salach, A. Radgowska, K. Żbikowska, and I. Kotuła, "Usefulness of Reticulocyte Parameters for Diagnosis of Hereditary Spherocytosis in Children," *Indian Journal of Hematology and Blood Transfusion* 33 (2017): 239–247, <https://doi.org/10.1007/s12288-016-0680-0>.
29. S. C. Nair, N. Arora, S. Jain, D. Inbakumar, J. Mammen, and U. Sitaram, "Mean Reticulocyte Volume Enhances the Utility of Red Cell Mean Sphered Cell Volume in Differentiating Peripheral Blood Spherocytes of Hereditary Spherocytosis From Other Causes," *Indian Journal of Pathology & Microbiology* 58 (2015): 307–309, <https://doi.org/10.4103/0377-4929.162836>.

30. F. Mullier, E. Lainey, O. Fenneteau, et al., "Additional Erythrocytic and Reticulocytic Parameters Helpful for Diagnosis of Hereditary Spherocytosis: Results of a Multicentre Study," *Annals of Hematology* 90 (2011): 759–768, <https://doi.org/10.1007/s00277-010-1138-3>.
31. A. S. Adam, F. Cotton, B. Cantinieaux, S. Benyaich, and B. Gulbis, "Screening for Hereditary Spherocytosis in Daily Practice: What Is the Best Algorithm Using Erythrocyte and Reticulocyte Parameters?," *Annals of Hematology* 101 (2022): 1485–1491, <https://doi.org/10.1007/s00277-022-04845-4>.
32. L. Liao, Y. Xu, H. Wei, et al., "Blood Cell Parameters for Screening and Diagnosis of Hereditary Spherocytosis," *Journal of Clinical Laboratory Analysis* 33, no. 4 (2019): e22844, <https://doi.org/10.1002/jcla.22844>.
33. L. Persijn, C. Bonroy, V. Mondelaers, A. Vantilborgh, J. Philippé, and V. Stove, "Screening for Hereditary Spherocytosis in Routine Practice: Evaluation of a Diagnostic Algorithm With Focus on Non-Splenectomised Patients," *Annals of Hematology* 91, no. 2 (2012): 301–302, <https://doi.org/10.1007/s00277-011-1243-y>.
34. P. M. T. Zhu, *The Clinical Test of Blood and Body Fluid*, 1st ed. (Beijing: People's Medical Publishing House, 2017).
35. Y. J. Shim and D. I. Won, "Flow Cytometric Osmotic Fragility Testing Does Reflect the Clinical Severity of Hereditary Spherocytosis," *Cytometry. Part B, Clinical Cytometry* 86, no. 6 (2014): 436–443, <https://doi.org/10.1002/cyto.b.21143>.
36. O. Ciepiela, "Old and New Insights Into the Diagnosis of Hereditary Spherocytosis," *Annals Translational Medicine* 6, no. 17 (2018): 339, <https://doi.org/10.21037/atm.2018.07.35>.
37. F. Girodon, L. Garçon, E. Bergoin, et al., "Usefulness of the Eosin-5'-Maleimide Cytometric Method as a First-Line Screening Test for the Diagnosis of Hereditary Spherocytosis: Comparison With Ektacytometry and Protein Electrophoresis," *British Journal of Haematology* 140 (2008): 468–470, <https://doi.org/10.1111/j.1365-2141.2007.06944>.
38. P. Joshi, A. Aggarwal, M. Jamwal, et al., "A Comparative Evaluation of Eosin-5'-Maleimide Flow Cytometry Reveals a High Diagnostic Efficacy for Hereditary Spherocytosis," *International Journal of Laboratory Hematology* 38 (2016): 520–526, <https://doi.org/10.1111/ijlh.12533>.
39. G. Stoya, B. Gruhn, H. Vogelsang, E. Baumann, and W. Linss, "Flow Cytometry as a Diagnostic Tool for Hereditary Spherocytosis," *Acta Haematologica* 116 (2006): 186–191, <https://doi.org/10.1159/000094679>.
40. O. Ciepiela, A. Adamowicz-Salach, I. Zdziechowicz, and I. Kotuła, "Delayed Measurement of Eosin-5'-Maleimide Binding May Affect the Test Results of Highly Hemolyzed Samples In Vivo and In Vitro: A Case Study," *Journal of Pediatric Hematology/Oncology* 38 (2016): e303–e306, <https://doi.org/10.1097/MPH.0000000000000652>.
41. P. Bianchi, C. Vercellati, and E. Fermo, "How Will Next Generation Sequencing (NGS) Improve the Diagnosis of Congenital Hemolytic Anemia?," *Annals Translational Medicine* 8 (2020): 268, <https://doi.org/10.21037/atm.2020.02.151>.
42. Y. Wu, L. Liao, and F. Lin, "The Diagnostic Protocol for Hereditary Spherocytosis-2021 Update," *Journal of Clinical Laboratory Analysis* 35 (2021): e24034, <https://doi.org/10.1002/jcla.24034>.
43. F. Häuser, H. Rossmann, A. Adenauer, et al., "Hereditary Spherocytosis: Can Next Generation Sequencing of the Five Most Frequently Affected Genes Replace Time Consuming Functional Investigations?," *International Journal of Molecular Sciences* 24, no. 23 (2023): 17021, <https://doi.org/10.3390/ijms242317021>.
44. M. Chen, Y. P. Ye, L. Liao, et al., "Hereditary Spherocytosis Overlooked for 7 Years in a Pediatric Patient With B Thalassemia Trait and Novel Compound Heterozygous Mutations of SPTA1 Gene," *Hematology* 25, no. 1 (2020): 438–445, <https://doi.org/10.1080/16078454.2020.1846874>.
45. P. G. Gallagher, "Abnormalities of the Erythrocyte Membrane," *Pediatric Clinics of North America* 60, no. 6 (2013): 1349–1362, <https://doi.org/10.1016/j.pcl.2013.09.001>.
46. R. D. Christensen and E. Henry, "Hereditary Spherocytosis in Neonates With Hyperbilirubinemia," *Pediatrics* 125, no. 1 (2010): 120–125, <https://doi.org/10.1542/peds.2009-0864>.
47. R. Christensen, H. Yaish, E. Henry, V. Baer, and S. Bennett, "A Simple Method of Screening Newborn Infants for Hereditary Spherocytosis," *Journal Applied Hematology* 4, no. 1 (2013): 27–32, <https://doi.org/10.1542/peds.2014-3516>.
48. R. D. Christensen, A. M. Agarwal, R. H. Nussenzweig, N. Heikal, M. A. Liew, and H. M. Yaish, "Evaluating Eosin-5-Maleimide Binding as a Diagnostic Test for Hereditary Spherocytosis in Newborn Infants," *Journal of Perinatology* 35, no. 5 (2015): 357–361, <https://doi.org/10.1038/jp.2014.202>.
49. R. D. Christensen, R. H. Nussenzweig, H. M. Yaish, E. Henry, L. D. Eggert, and A. M. Agarwal, "Causes of Hemolysis in Neonates With Extreme Hyperbilirubinemia," *Journal of Perinatology* 34, no. 8 (2014): 616–619, <https://doi.org/10.1038/jp.2014.68>.
50. American Academy of Pediatrics Subcommittee on Hyperbilirubinemia, "Management of Hyperbilirubinemia in the Newborn Infant 35 or More Weeks of Gestation," *Pediatrics* 114, no. 1 (2004): 297–316, <https://doi.org/10.1542/peds.114.1.297>.
51. B. Fattizzo, J. Giannotta, A. Zaninoni, A. Kulasekararaj, L. Cro, and W. Barcellini, "Small Paroxysmal Nocturnal Hemoglobinuria Clones in Autoimmune Hemolytic Anemia: Clinical Implications and Different Cytokine Patterns in Positive and Negative Patients," *Frontiers in Immunology* 11 (2020): 1006, <https://doi.org/10.3389/fimmu.2020.01006>.
52. B. Fattizzo, R. Ireland, A. Dunlop, et al., "Clinical and Prognostic Significance of Small Paroxysmal Nocturnal Hemoglobinuria Clones in Myelodysplastic Syndrome and Aplastic Anemia," *Leukemia* 11 (2021): 1006, <https://doi.org/10.1038/s41375-021-01190-9>.
53. Z. Deng, L. Liao, W. Yang, and F. Lin, "Misdiagnosis of two cases of hereditary spherocytosis in a family and review of published reports," *Clinica Chimica Acta* 441 (2015): 6–9, <https://doi.org/10.1016/j.cca.2014.12.002>.