Research Article

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# Eosin-5′-maleimide (EMA)-binding assay as a diagnostic method of hereditary spherocytosis

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**Abstract**

**Objectives:** Erythrocyte membrane disorders are caused by a deficiency of structural proteins in the erythrocyte membrane. Accurate differential diagnosis within this group of disorders (is essential for appropriate management. The eosin-5′-maleimide (EMA) binding assay is a novel test that is used for the differential diagnosis of erythrocyte membrane disorders. In this study, we have examined and reported blood counts, reticulocyte indices, and the EMA binding assay results with clinical findings of cases admitted to our laboratory for suspected red blood cell (RBC) membrane disorder.
**Methods:** We performed the EMA binding assay on the blood samples of 103 patients who were screened for hereditary erythrocyte membrane disorders at the Flow Cytometry Laboratory of Ankara Numune Training and Research Hospital. The total cohort was grouped as patients with hereditary spherocytosis (HS) (n=36) and control group (patients without erythrocyte membrane disorders (n=60), and non-HS patients with a preliminary diagnosis of hemolytic anemia (n=7). The control group included during data collection, the results of the EMA binding assay were recorded along with demographic features, clinical information, morphologic features, blood count parameters, RBC and reticulocyte indices, and a conventional osmotic fragility (OF) test. Receiver Operating Characteristics (ROC) analyses were performed to evaluate the diagnostic accuracy of the EMA binding assay and reticulocyte parameters.
**Results:** Both EMA testing and flow cytometric (FC) OF test were significantly lower in overall (n=36), ≤10-year-old (n=12), and >10-year-old (n=24) patients with HS than in healthy controls ($p<0.001$). The EMA binding assay had 100 % sensitivity and specificity in screening HS.
**Conclusions:** Combined with conventional blood tests, clinical findings, and medical history, the EMA binding assay is a reliable and convenient tool for screening for HS and differentiating hereditary erythrocyte membrane disorders.

**Keywords:** hereditary membrane disorders; eosin-5′-maleimide binding test; flow cytometry; hereditary spherocytosis; hereditary stomatocytosis

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## Introduction

Red blood cell (RBC) membrane disorders are caused by a deficiency of structural proteins of the erythrocyte membrane and include hereditary spherocytosis (HS), hereditary elliptocytosis, Southeast Asian ovalocytosis, and hereditary stomatocytosis (HSt) [1]. Loss of membrane surface area, morphological changes, tendency to splenic destruction, and hemolytic anemia of varying degrees are typical findings of all RBC membrane disorders [1, 2].

Hereditary spherocytosis (HS) is the most common among RBC membrane disorders. Cases are mainly inherited as autosomal dominant (75 %). Autosomal recessive

transmission occurs in approximately 25 % of cases, and the *de novo* mutation rate has not yet been determined [1, 2].

Typical HS is manifested by anemia, jaundice, and reticulocytosis. The severity of HS is determined by the degree of anemia and is classified as asymptomatic, mild, moderate, or severe [1].

Diagnoses of mild-moderate and atypical HS may be challenging. Most cases (20–30 %) are described as mild with a fully compensated hemolysis owing to the balance between hemolysis and reticulocyte production [2–5]. In the differential diagnoses of mild-moderate types and atypical HS, it is essential to distinguish, especially from congenital dyserythropoietic anemia type II and HSt. There are some cases reported in the literature that had been erroneously diagnosed as HS, and correct diagnosis became possible only after unresponsiveness to splenectomy [6]. HS cases are generally diagnosed without any need for further testing if they have anemia, jaundice, splenomegaly, reticulocytosis, cholelithiasis, and the presence of family history [3, 5]. RBC morphology, red cell, and reticulocyte indices, dependent on the complete blood count (CBC) device used, are accepted as positive predictors for HS, but they are not necessary for diagnosis [3].

Confirmatory tests for HS are indicated if the clinical properties do not meet diagnostic criteria and other causes of hemolysis are ruled out [3, 5]. The guidelines recommend Eosin-5′-maleimide (EMA) binding assay and cryohemolysis test for screening in the neonate and mild-moderate cases; routine osmotic fragility (OF) testing is no longer recommended. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and ektacytometry are expensive methods requiring fresh blood samples that are not widely available. That is why they are recommended to be utilized only in atypical cases [3, 5–7].

EMA binding assay is an inexpensive screening test that results in a short time, and it is also applicable in stored blood samples (with 7-day stability at +4 °C). The main advantage of the EMA binding assay is that it analyzes blood samples at a single cell level without disrupting cellular integrity. The principle of this assay is measuring the fluorescence intensity, which is associated with the binding of EMA to RBC membrane proteins, such as Band 3, Rh protein, Rh glycoprotein, and CD47 [5, 8, 9]. Reference intervals may differ between different flow cytometry instruments in different laboratories [5]. For standardized results, the difference between mean channel fluorescence (MCF) values are reported as percentages or ratios. However, the number of healthy controls required, and the reference intervals used in the EMA binding assay are unclear. It is recommended that every laboratory should implement its reference intervals [5]. The EMA binding test is recommended as a first-line screening test for diagnosing HS in routine hematology laboratories [8]. Furthermore, it is accepted as a reliable technique to detect hereditary pyropoikilocytosis (HPP) as it can help differential diagnostic workup when MCF readings are below or borderline the lower limit for the HS reference range [10].

In this study, we have examined and reported blood counts, reticulocyte indices, and the EMA binding assay results with clinical findings of cases admitted to our laboratory for suspected RBC membrane disorder.

# Methods

## Patients and sampling

At the Flow Cytometry Laboratory of Ankara Numune Training and Research Hospital, the EMA binding assay was performed on 103 patients, who were screened for hereditary RBC membrane disorders. The patients' age range was 0–73 (mean; 28.17 ± 19.34) years. The total cohort was grouped as patients with hereditary spherocytosis (HS) (n=36) and control group (patients without erythrocyte membrane disorders (n=60), and non-HS patients with a preliminary diagnosis of hemolytic anemia (n=7)). Blood samples were taken into 4 mL K3 EDTA (BD Vacutainer®, CA) tubes for CBC and EMA binding assay. Clinical findings and laboratory test results regarding etiology (CBC, blood smear, direct antiglobulin test, FC-OF, vitamin B12, folate, hemoglobin (Hb) electrophoresis, glucose-6-phosphate dehydrogenase, pyruvate kinase, total and unbound bilirubin, serum iron, total iron binding capacity, and ferritin) were recorded.

The EMA binding assay was performed in 75 (72.8 %) cases diagnosed as hemolytic anemia, 7 (7.2 %) cases for anemia with unknown etiology, 9 (9.2 %) cases for cholelithiasis, 7 (7.2 %) cases for splenomegaly and 5 (5.1 %) cases for prolonged neonatal jaundice.

The local Ethical Committee of the Ankara Numune Training and Research Hospital approved the study (E-17-1265, 12.04.2017).

## Complete blood count

CBC analyses were performed with Sysmex XN-1000 (Sysmex Co., Kobe, Japan) with App PLT-F and App RET. New reticulocyte (RET) parameters (RET Hb equivalent [RET-He], microcytic RBC [MicroR] and macrocytic RBC [MacroR] and hypochromic-RBC [Hypo-He], hyperchromic-RBC [Hyper-He]) besides conventional complete blood count parameters i.e. RBC, Hb, hematocrit [Hct], mean corpuscular hemoglobin [MCH], mean corpuscular hemoglobin concentration [MCHC], mean corpuscular volume [MCV], RET absolute count (ARC).

[#] and present [%], immature reticulocyte fraction [IRF] were studied. Reticulocyte/IRF% ratio and MicroR/Hyper-He ratios were calculated.

The internal quality control (IQC) procedure and external quality assurance scheme ensured the quality of analytical results throughout the study period. Daily IQC procedure includes analyses of quality control material (XN-CHECK Streck Laboratories Inc., Omaha, NE, USA) with three levels. 0.97, 0.69, 0.49 for RBC CV%, 1.18, 0.48, 0.78 for Hb CV%, 0.97, 0.74, 0.86 for Hct CV%; 0.43, 0.15, 0.18 for MCV% was used. For external quality assurance, the Labquality External Quality Assessment program was used (z score range −2.0–2.0).

## The EMA binding assay with flow cytometry

The EMA binding assay was performed as defined by King et al. [8] with a minimal modification. The EMA binding test was conducted on EDTA-stabilised blood samples within a 48 h period following their collection. EMA dye (0.5 mg/mL, Sigma Aldrich, Germany) was dissolved in phosphate-buffered saline (PBS, Sigma Aldrich, Germany), and aliquots were stored at −80 °C (max. six months), and one fresh aliquot was thawed for each run.

RBCs from fresh samples with EDTA were transferred 143 to a 100 µL polypropylene tube and washed twice (2,500 g, 5 min) with PBS. A total of 25 µL of working EMA dye was added to 5 µL of washed red cells in a polypropylene tube. The mixture was gently vortexed and incubated for 20 min at room temperature (approximately 22–25 °C) in the dark to prevent photobleaching of the fluorochrome. After incubation, the cells were washed twice with 1 mL of phosphate-buffered saline (PBS) by centrifugation at 300 × g for 5 min to remove unbound dye before analysis.

The cells were acquired on BD FACS Canto II (Becton Dickinson, USA), and MCF values were determined for 30,000 events in the FL1 channel and analyzed with BD FACSDiva software. To minimize intra-assay errors, patient samples were compared with samples from six age-matched healthy controls per test run. The results of the EMA binding assay were expressed as MCF values. The results were interpreted as normal if there was a 0–15 % decrease in fluorescence (normal inter-individual variability; negative for HS) and abnormal if there was ≥20 % reduction in fluorescence (abnormality increased interindividual variability; probable HS). The results were calculated as ratios. The ratio of MCF values (MCF of patient/mean of MCF of five normal controls), and percentage of decrease in MCF values of patients compared to the mean of normal controls.

We examined EMA binding assay test results in our laboratory according to reference intervals determined according to the Clinical and Laboratory Institute (CLSI) C28-A3 Guideline [11]. Reference intervals were EMA ratio (EMA R):0.86–1.15 for healthy adults and children older than two years of age, EMA R: 0.82–1.10 for children aged 0–2. Cases with EMA R results below the lower limit of reference interval were considered HS, and cases with EMA R results higher than the upper limit of reference interval were considered as other types of anemia (megaloblastic anemia, congenital dyserythropoietic anemia type II, pyruvate kinase deficiency or HSt). The healthy adults were selected from blood bank donors, while the healthy children were selected who were attending the outpatient clinic for healthy children.

## Flow cytometric-osmotic fragility test

In conducting the flow cytometric osmotic fragility test, appropriate volume of peripheral blood collected in tube containing $K_3$EDTA was suspended in 1,100 µL 0.9 % NaCl. The volume of blood was calculated according to the equation that allowed collecting similar number of cells from every specimen. The equation was as follows: V (µL)=$\frac{130}{RBC}/10^6$, where *V* is the volume of blood collected for the analysis and RBC the number of red blood cells in 1 µL of the analyzed sample. Subsequently, 10 µL of the primary suspension was combined with 1,100 µL of saline in a cytometric tube. The secondary suspension was then analysed using a Cytomics FC500 flow cytometer (BD FACSDiva software) (BD Biosciences Coulter, Brea, CA, USA). RBC populations were identified and enumerated using a forward scatter/side scatter cytogram on a logarithmic scale. The time vs. forward scatter cytogram on a logarithmic scale was employed to record the events. Following a 30-s analysis period, the cytometer was paused, 900 µL of deionised water was added to the tube, and the analysis resumed [12].

$$\text{EMA Formula} = \frac{\text{Patient x} - \text{median}}{(\text{Control 1 x} - \text{median} + \text{Control 2 x} - \text{median})/2}$$

EMA reference range: 0.8<: This finding is consistent with the clinical picture of hereditary spherocytosis;0.8–0.0: Suspicious range;0.9–1.1: Normal range;>1.1: High.

The daily performance and reproducibility of the flow cytometer were ensured using the BD Cytometer Setup and Tracking Beads Kit (Cat. No: 642412) and the BD FACSDiva™ software (v8.0.3). Daily measurements were performed by preparing a bead mixture with one drop of beads in 0.35 mL of diluent. The software recorded median fluorescence intensity (MFI) and %robust CV values, which were monitored using Levey-Jennings plots to ensure consistency. Measurements were compared against predefined target

values, with a tolerance range of ±5 %. The BD FACSDiva™ software automatically optimized the cytometer settings, ensuring measurement reproducibility. These procedures guaranteed the instrument's accuracy, sensitivity, and reliability throughout the study.

### Statistical analysis

Qualitative data were summarized using numbers and percentages, while quantitative data were summarized using medians and quartiles. Independent two-sample comparisons were made with the Mann-Whitney U test regarding quantitative data. To evaluate the diagnostic accuracy of EMA binding assay and reticulocyte parameters, the receiver operating characteristic (ROC) analyses were performed. The area under the ROC curve was calculated. ROC curves were compared to determine the diagnostic performance. The statistical significance level was accepted as 5 %, and all analyses were performed with the IBM SPSS Statistics for Windows Version 26 (IBM Corp., Chicago, IL, USA).

## Results

Of the study cohort, 14 cases (from seven different families) had an EMA binding assay test for family screening. 14 cases tested for EMA binding assay. Eight cases were infants (0–5 months), and seven out of 103 cases had splenectomy. The most common hospital admission causes were hemolytic anemia, splenomegaly, and cholelithiasis. Among 36 cases with HS (34.9 %), 4 had moderate (7–10 g/dL), and 30 had mild (Hb>10 g/dL) disease, while only 2 cases had severe HS (Hb<6 g/dL). The demographic characteristics of definite diagnostic groups are presented in Table 1.

**Table 1:** Demographic characteristics of hereditary spherocytosis and control group.

| | HS n, % | Controls[a] n, % |
|---|---|---|
| Age groups | | |
| 0–24 months | 4 (11.1) | 6 (10.0) |
| 3–5 years | 3 (8.3) | 4 (6.7) |
| 6–10 years | 5 (13.9) | 2 (3.3) |
| 11–18 years | 1 (2.8) | 7 (11.7) |
| 19–30 years | 23 (63.9) | 41 (68.3) |
| Sex | | |
| Female | 20 (55.6) | 26 (43.3) |
| Male | 16 (44.4) | 34 (56.7) |

HS, hereditary spherocytosis. [a]Controls were selected among patients without red blood cell (RBC) membrane disorders.

The median of EMA ratio was 0.65 (0.56–0.77) in HS cases. The EMA ratio was over 1.20 in 4.8 % (n=5) of HS cases, which had macrocytosis (MCV>98 fL) and normal MCHC (28.8–33.9) values. Further investigations for megaloblastic anemia revealed that one patient had pyruvate kinase deficiency, and another had vitamin B12 deficiency. The other 3 cases had neither enzyme/vitamin deficiency nor chronic alcoholism that could explain macrocytosis. They were previously splenectomized but had ongoing hemolysis. Family screening was performed for the two cases with recurrent thromboembolism; the EMA binding assay results were >1.2 in the father of the first case and the mother of the second. Family screening in both cases revealed asymptomatic high MCVs and normal MCHC values. Among 60 cases with normal EMA-binding assay (EMA R: 0.92), one had clinical, and laboratory results consistent with HS despite normal EMA R.

Both EMA R and flow cytometric (FC) OF test results of subjects diagnosed with HS were statistically significantly lower than those of the normal subjects (p<0.001) (Table 2). The results of red cell distribution width (Rdw), RET, RET/IRF, and Macro-R test were significantly higher in the HS group than the normal subjects (p<0.001, p<0.001, p<0.001,

**Table 2:** Comparisons of diagnostic workup parameters between the study patients grouped based on definite diagnosis.

| | HS n=36 Median ($Q_1$-$Q_3$) | Normal n=60 Median ($Q_1$-$Q_3$) | p-Value[a] |
|---|---|---|---|
| EMA ratio | 0.65 (0.56–0.77) | 0.97 (0.92–1.01) | **<0.001** |
| FC OF test | 12 (8–19) | 60 (45–72) | **<0.001** |
| Hemoglobin | 11.8 (10.35–12.4) | 11.15 (8.75–12.8) | 0.451 |
| Rdw | 20.7 (18.4–23.45) | 16.3 (14.95–18.6) | **<0.001** |
| MCV | 85.4 (81.85–90.5) | 85.45 (79.35–90.3) | 0.422 |
| MCHC | 33.2 (30.25–34.6) | 31.95 (30.15–33.25) | 0.109 |
| RET | 380.5 (289–485) | 75 (36.5–165) | **<0.001** |
| IRF | 18.2 (10.55–21.85) | 12.95 (7.7–21.8) | 0.272 |
| Ret/IFR | 21.89 (17.27–27.92) | 5.93 (3.16–10.08) | **<0.001** |
| Ret-He | 30 (28.1–31.05) | 28.85 (27.2–30.1) | 0.070 |
| MicroR | 8.7 (3.5–11.9) | 3.6 (2.35–9.5) | 0.061 |
| MacroR | 5.55 (4.7–8) | 4.9 (4.2–6.75) | **0.032** |
| RBC-He | 27.2 (25.85–28.2) | 26.7 (26.2–27.9) | 0.462 |
| HYPO-He | 0.9 (0.3–3.35) | 0.7 (0.4–4) | 0.707 |
| HYPER-He | 0.3 (0.2–0.4) | 0.4 (0.3–0.5) | 0.147 |
| MicroR/HYPO-He | 5.28 (2.09–17.95) | 4.7 (1.8–12.58) | 0.378 |

[a]Mann Whitney U test, $Q_1$: first quartile, $Q_3$: third quartile. HS, hereditary spherocytosis; EMA, eosin-5′-maleimide; FC OF, flow cytometric osmotic fragility; Rdw, red cell distribution width; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RET, reticulocyte; IRF, immature reticulocyte fraction; RET-He, reticulocyte hemoglobin equivalent; MicroR, microcytic red cell population; MacroR, macrocytic red cell population; RBC-He, hemoglobin content of mature red blood cell; Hypo-He, hypochromic-RBC (percentage of red blood cells with cellular hemoglobin content lower than 17 pg); Hyper-He, hyperchromic-RBC (percentage of red blood cells with cellular hemoglobin content higher than 49 pg). Bold values are statistically significant.

**Table 3:** Comparisons of diagnostic workup parameters between study patients (aged ≤10) based on definite diagnosis.

| | HS n=12 Median ($Q_1$-$Q_3$) | Normal n=12 Median ($Q_1$-$Q_3$) | p-Value[a] |
|---|---|---|---|
| EMA ratio | 0.65 (0.56–0.76) | 0.99 (0.92–1.03) | **<0.001** |
| FC OF test | 14 (9–19.5) | 54.5 (42.5–65.5) | **<0.001** |
| Hemoglobin | 11.75 (9.75–13.05) | 11.2 (7.2–12.1) | 0.259 |
| Rdw | 19.4 (16.4–22.4) | 16.35 (15.15–28.05) | 0.729 |
| MCV | 83.25 (80.1–88.9) | 85.9 (79.35–92.15) | 0.665 |
| MCHC | 31.35 (29.4–33.55) | 32.3 (31.5–34.2) | 0.194 |
| Ret | 305 (197–468.5) | 159 (51–290.5) | **0.021** |
| IRF | 20.35 (9.35–24) | 19.2 (9.1–35.2) | 0.817 |
| Ret/IFR | 20.1 (15.17–25.13) | 6.34 (4.22–11.66) | **0.001** |
| Ret-He | 29.8 (27.2–30.55) | 27.05 (25.65–28.15) | **0.046** |
| MicroR | 10.55 (3.85–13.95) | 5.7 (2.3–8.9) | 0.133 |
| MacroR | 5.65 (4.45–7.1) | 4.8 (4.15–7.25) | 0.419 |
| RBC-He | 27.3 (25.6–28.2) | 26.2 (25.3–27.15) | 0.214 |
| Hypo-He | 0.65 (0.35–3.6) | 1 (0.45–3.65) | 0.684 |
| Hyper-He | 0.35 (0.2–0.4) | 0.3 (0.2–0.45) | 0.953 |
| MicroR/Hypo-He | 16 (1.75–20.34) | 3.96 (1.8–12.06) | 0.248 |

[a]Mann Whitney U test, $Q_1$: first quartile, $Q_3$: third quartile. HS, hereditary spherocytosis; EMA, eosin-5′-maleimide; FC OF, flow cytometric osmotic fragility; Rdw, red cell distribution width; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RET, reticulocyte; IRF, immature reticulocyte fraction; RET-He, reticulocyte hemoglobin equivalent; MicroR, microcytic red cell population; MacroR, macrocytic red cell population; RBC-He, hemoglobin content of mature red blood cell; Hypo-He, hypochromic-RBC (percentage of red blood cells with cellular hemoglobin content lower than 17 pg); Hyper-He, hyperchromic-RBC (percentage of red blood cells with cellular hemoglobin content higher than 49 pg). Bold values are statistically significant.

**Table 4:** Comparisons of diagnostic workup parameters between study patients (aged >10) based on definite diagnosis.

| | HS n=24 Median ($Q_1$-$Q_3$) | Normal n=48 Median ($Q_1$-$Q_3$) | p-Value[a] |
|---|---|---|---|
| EMA ratio | 0.66 (0.57–0.78) | 0.97 (0.91–1.01) | **<0.001** |
| FC OF test | 11.5 (7–19) | 60 (46.5–74.5) | **<0.001** |
| Hb | 11.8 (10.45–12.2) | 11.05 (8.85–13.05) | 0.756 |
| Rdw | 21.55 (18.4–24.85) | 16.3 (14.85–18.25) | **<0.001** |
| MCV | 87.7 (84.2–91.6) | 85.35 (79.35–90.3) | 0.177 |
| MCHC | 33.95 (30.85–34.85) | 31.7 (30–33.2) | **0.009** |
| Ret | 384 (315.5–494) | 69 (32.5–120) | **<0.001** |
| IRF | 17.5 (13–21.6) | 11.85 (7.3–21.7) | 0.173 |
| RET/IFR | 24.15 (19.41–36.32) | 5.92 (3.11–10.08) | **<0.001** |
| RET-He | 30.05 (28.3–31.5) | 29.45 (27.9–30.4) | 0.287 |
| MacroR | 5.55 (5–9.85) | 4.9 (4.2–6.75) | **0.033** |
| RBC-He | 27.1 (26–28.1) | 26.95 (26.4–28.15) | 0.806 |
| Hyper-He | 0.3 (0.25–0.45) | 0.4 (0.3–0.5) | 0.115 |
| MicroR | 7.9 (3.25–9.8) | 3.55 (2.35–9.6) | 0.220 |
| Hypo-He | 1.85 (0.3–3.35) | 0.65 (0.4–4) | 0.549 |
| MicroR/Hypo-He | 4.8 (2.26–16.25) | 5.17 (1.71–12.58) | 0.756 |

[a]Mann Whitney U test, Me: median, $Q_1$: first quartile, $Q_3$: third quartile. HS, hereditary spherocytosis; EMA, eosin-5′-maleimide; FC OF, flow cytometric osmotic fragility; Rdw, red cell distribution width; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RET, reticulocyte; IRF, immature reticulocyte fraction; RET-He, reticulocyte hemoglobin equivalent; MicroR, microcytic red cell population; MacroR, macrocytic red cell population; RBC-He, hemoglobin content of mature red blood cell; HYPO-He, hypochromic-RBC (percentage of red blood cells with cellular hemoglobin content lower than 17 pg); Hyper-He, hypochromic-RBC (percentage of red blood cells with cellular hemoglobin content higher than 49 pg). Bold values are statistically significant.

p<0.001, respectively). Comparisons in EMA R, FC OF test, Rdw, RET, RET/IRF, and Macro-R measurements between HS and normal groups are presented in Table 2.

The study cohort was divided into two age groups: subjects ≤10 years old and >10 years old (2 study cohort were determined as child age and below and adolescent age and above.) Then, the diagnostic workup parameters for RBC membrane disorders were compared within ≤10-year (Table 3) and >10-year age (Table 4) groups between subjects with HS and the normal group.

Among the subjects ≤10 years, the EMA R and FC OF test results were significantly lower in patients with HS than in those in the normal group (p<0.001) (Table 3). RET, RET/IRF, and RET-He measurements were significantly higher in the HS group than in the control (p=0.021, p<0.001, and p=0.046, respectively).

Among the subjects >10 years of age, the EMA R and FC OF test results were significantly lower in patients with HS than those in the normal group (p<0.001) (Table 4). RET and RET/IRF measurements were significantly higher in the HS group than in the control (p<0.001) (Table 4).

Comparison of normal and abnormal results of EMA and FC-OF tests is shown in the Figure 1.

The ROC analysis indicated that both EMA R and FC OF tests had 100 % diagnostic performance in HS (The area under the curve (AUC), 0.997) (Figure 2).

## Discussion

The findings of the present study indicate that the EMA binding assay is highly sensitive and specific for diagnosing HS. Additionally, it is a resourceful technique in differential diagnosis between erythrocyte membrane disorders because it enabled us to determine 5 cases with HSt in our screening cohort.

The prevalence of overhydrated HSt is one in a million births, whereas dehydrated HSt is one in 10,000 births [5]. While splenectomy is included in the treatment of HS, it has no place in the treatment of HSt [13]. Differential diagnosis of these two items is essential, as recent guidelines also emphasize [5, 7]. The European Network for Rare and

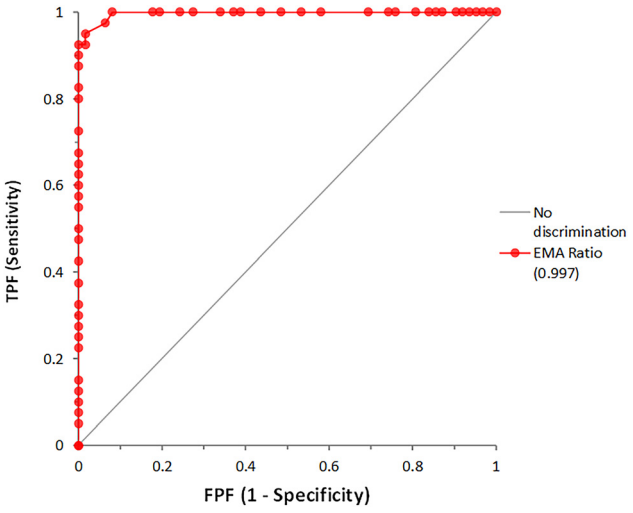


**Figure 1:** Receiver operating characteristics (ROC) analysis of eosin-5′-maleimide (EMA) binding test assay in hereditary spherocytosis. (a) Normal and abnormal results of EMA a ratio. HS (n=24) median ($Q_1$-$Q_3$) =0.66 (0.57–0.78). Normal (n=48) median ($Q_1$-$Q_3$)=0.97 (0.91–1.01) p=<0.001. (b) Normal and abnormal results of FC OF test. HS (n=24) median ($Q_1$-$Q_3$)=11,5 (7–19). Normal (n=48) median ($Q_1$-$Q_3$)=60 (46.5–74.5) p=<0.001.

Congenital Anemias (ENERCA) indicates that the prevalence of this rare syndrome may be higher than reported as the screening tests are insufficient (www.enerca.org). The EMA binding test is easily accessible for HS and HSt. We believe that the EMA binding test is easily accessible for HS and HSt.

The laboratory hallmark of HS is the presence of spherocytes in the peripheral blood smear; however, it is not a specific finding [5]. Thus, other laboratory tests examining the surface area-to-volume ratio are employed during the diagnostic workups [14]. Unfortunately, they do not differentiate HS from other secondary spherocytosis etiologies, especially from autoimmune hemolytic anemia [14]. Advances in hematology analyzers and accumulated data on new hematologic parameters, mostly confined to erythroid lineage, improved the definite diagnosis of HS, which is rather heterogeneous [15, 16]. In this context, the EMA binding assay, in which membrane molecules are evaluated before erythrocyte hemolysis at the single cell level, has evolved as an optimum technique for screening a much larger population for HS with no additional cost [7, 10, 17].

Morphology and conventional erythrocyte parameters in CBC in suspicion of HS are insufficient for mild and asymptomatic cases [5, 7]. Erythrocyte morphology was non-specific in peripheral blood smears in our HS cases because most of our cases were mild and moderate. We detected typical stomatocytes in only HSt suspected cases [15]. We found decreased MCHC and increased MCV (95–98 fL) values in our HSt cases, which is opposite to HS cases. MCV and MCHC give equivocal information in HSt [16, 18]. Erythrocyte morphology, MCV, and MCHC may only support findings but can't be diagnostic when used alone. Automated reticulocyte indices have been recommended in the differential diagnosis of

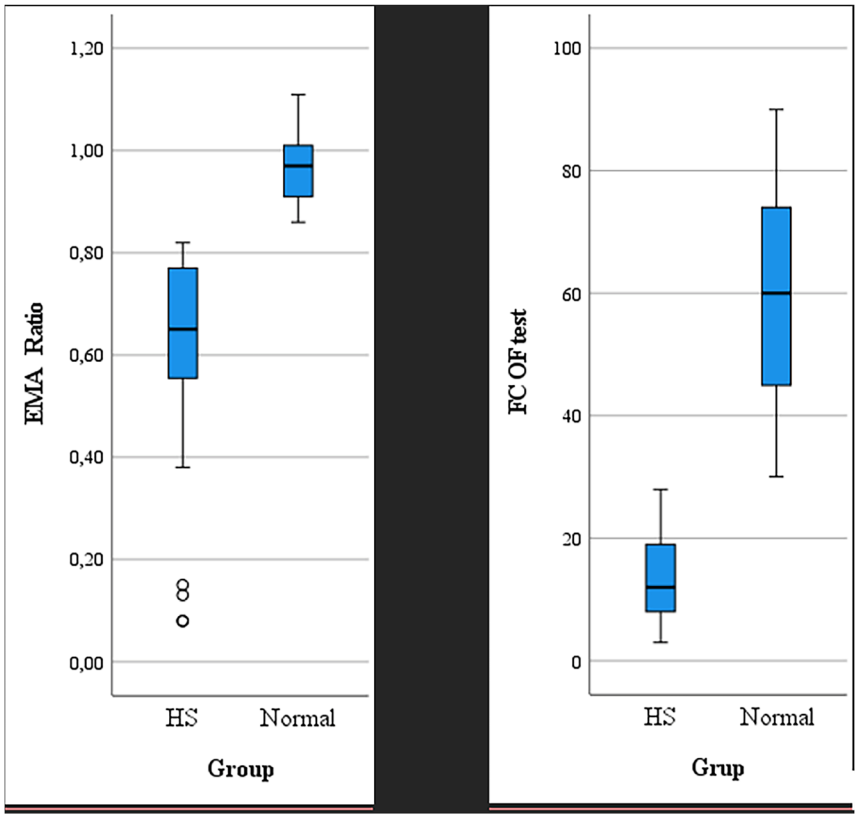


**Figure 2:** Receiver operating characteristics (ROC) analysis of eosin-5′-maleimide (EMA) binding test assay in hereditary spherocytosis. TPF, true positive fraction; FPF, false positive fraction (the area under the curve (AUC), 0.997).

hereditary and secondary spherocytosis in recent years [19, 20]. As membrane remodeling becomes at reticulocyte level in HS, reticulocyte volume is less than 100 fL classically except in neonates [20]. In a study conducted to diagnose HS and pyruvate kinase deficiency on a patient cohort with erythrocyte membrane disorders, Sysmex's unique parameters for erythrocytes were investigated in addition to the automated counts [15]. It was reported that new red cell parameters included in the Sysmex analyzer had 100 % sensitivity and 92.1 % specificity for HS. Recent studies propose algorithms for HS diagnosis by using reticulocyte indices and RET/IRF and MicroR/Hypo-He ratios with hypochromic cells detected in the Sysmex analyzer [16], EMA binding assay (MCF readings) combined with MCV values [10], reticulocytes and erythrocytic parameters associated with clinical data on Sysmex XN-series [16, 21]. In the present study, the RET/IR and MicroR/Hypo-He algorithm were not used, as most cases had mild HS. The EMA R and FC OF test results were significantly decreased in the overall group and the subgroups, which were categorized according to age (Tables 2–4).

In line with the method described by King et al. [9], blood samples from six healthy controls were used for FC analysis of each patient. According to previous studies, the MCF results of the EMA binding test differ between different age groups, especially for neonates and early childhood [22–24]. Megaloblastic anemia, chronic alcoholism, and erythrocyte enzyme deficiencies should be ruled out in cases with high EMA binding test results before examining for HSt [11]. The EMA binding results were higher than normal in five suspected HSt cases, one with vitamin B12 deficiency and one with pyruvate kinase deficiency. Family screening was performed in the three cases with high EMA binding, and two asymptomatic cases with HSt were diagnosed. Although the number of cases in our cohort is small, our results for HSt patients indicate that high EMA binding results may be informative for clinicians in asymptomatic cases.

The major limitation of the present study is that the definite diagnosis of HSt has not been confirmed by performing advanced tests; instead, it has been confirmed by clinical findings, direct antiglobulin negativity, stomatocytes in peripheral blood smears, and high MCV and EMA binding assays. The current literature underlines that the EMA binding assay is efficient, reliable, and cheap for a mass screening test that is used for the differential diagnosis of erythrocyte membrane disorders [7, 8, 10, 15, 19, 21]. Confirmatory tests, such as SDS-PAGE, molecular analysis, and ektacytometry, may be preserved for a small patient population that requires further investigation due to heterogeneity or the severity of the disorders. Furthermore, we believe nonspecific tests, such as the osmotic fragility test, may be helpful when an EMA test indicates suspicious results. Moreover, the EMA binding test will help clinicians make rapid decisions during differential diagnosis of hemolytic anemias and determine the incidence and prevalence of rare diseases.

**Research ethics:** The local Institutional Review Board deemed the study exempt from review.
**Informed consent:** Informed consent was obtained from all individuals included in this study.
**Use of Large Language Models, AI and Machine Learning Tools:** None declared.
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