Laboratory Indices in Patients with Positive and Borderline Flow Cytometry Eosin-5-Maleimide-Screening Test Results for Hereditary Spherocytosis

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Objective To evaluate laboratory indices in patients with hereditary spherocytosis, with positive and borderline flow cytometry eosin-5-melamide (EMA)-bound red blood cells screening test.

Study design We compared laboratory indices of 151 samples obtained from 139 different individual patients with negative, borderline, or positive EMA-test results. We also compared the clinical data of the patients in each EMA test results group.

Results Borderline EMA-test results were obtained for 13 patients and were associated with more severe anemia, and lower reticulocyte count and reticulocyte production index compared with samples with positive EMA-test results. A receiving operator characteristic analysis identified mean corpuscular hemoglobin concentration of <32.5 g/dL as a cut-off, between positive/borderline and negative test results with 100% sensitivity. A higher prevalence of clinical markers typical of hereditary spherocytosis was found in patients with borderline or positive compared with negative EMA test samples.

Conclusions Based on laboratory data, borderline EMA-test results may be an indication of a more severe form of hereditary spherocytosis. Using mean corpuscular hemoglobin concentration as a cut-off may help predict and reduce negative EMA tests without compromising sensitivity. This finding needs to be further validated in other flow cytometry laboratories with a large EMA test sample pool. *(J Pediatr 2022; ■:1-4)*.

Eosin-5-maleimide (EMA) is a fluorescent dye that binds to the ϵ-NH_2 group of lysine on the anion exchange protein band 3, CD47, and Rh-related proteins on the red blood cell (RBC) membrane.[1] Cytoskeletal defects resulting from deficiency or dysfunction of spectrin, ankyrin, protein 4.2, and band 3 that are associated with hereditary spherocytosis are associated with reduced mean fluorescence intensity of the EMA signal.[2] Patients with hereditary spherocytosis usually show >21% lower EMA signals compared with control specimens. Hence, measurement of the degree of reduced EMA signal in RBCs is the basis of a validated and highly specific and sensitive flow cytometry screening test developed for detection of hereditary spherocytosis.[3,4] However, some patients with hereditary spherocytosis present with borderline staining (16%-21% reduced EMA staining).[5]

The EMA screening test is used by many laboratories as it is more reliable than the osmotic fragility test.[6,7] However, performing this test requires expensive equipment, high skills, and is time- and effort-consuming. The objectives of this retrospective analysis were to investigate differences in routine laboratory indices between samples with negative, borderline, and positive EMA screening test results, and to search for routine laboratory indices that can assist in reducing redundant tests.

Methods

After obtaining approval from the Galilee Medical Center ethics committee according to the declaration of Helsinki (approval #0178-19-NHR), data from medical records and results of all the peripheral blood samples of patients subjected to EMA screening test in the Galilee Medical Center Flow Cytometry Laboratory were collected. The following clinical data were collected: presence of jaundice, history of blood transfusions, splenomegaly, cholecystectomy, and splenectomy.

Laboratory data that were collected in parallel to EMA screening test included the following hemocytometry measures: erythrocyte (Er) count, hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean cell hemoglobin, mean

Er	Erythrocyte
EMA	Eosin-5-maleimide
Hb	Hemoglobin
HCT	Hematocrit
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MFI	Mean fluorescence intensity
PBS	Phosphate buffered saline
RBC	Red blood cell
RPI	Reticulocyte production index

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corpuscular hemoglobin concentration (MCHC), RBC distribution wide, absolute reticulocytes, % reticulocytes, and reticulocyte production index (RPI). Biochemical measures included bilirubin, lactic dehydrogenase, chloride, and vitamin B12. The Sysmex XN-1000 instrument (Sysmex) was used to conduct the study. Some old samples were analyzed by the ADVIA 2120i (Siemens HealthCare Ltd).

EMA Screening Test by Flow Cytometry

Flow cytometry EMA tests were performed on fresh peripheral blood samples that were collected in EDTA tubes (Ref#454217, Greiner Bio-One GmbH). Briefly, 50 μL peripheral blood samples collected from patients and from 6 individuals, were placed in 4-mL glass tubes and washed 3 times with 0.9% sodium chloride solution (Ref# IE1324, Teva Medical Ltd). The control samples used to perform the EMA test were collected from healthy individuals or outpatients who were selected from the hematology laboratory on the same day of analysis of the test samples. Controls were all age-matched and verified to have normal hemoglobin and RBC measures. Then, 5 μL of each sample were transferred into a polypropylene flow cytometry and fluorescence-activated cell sorting tube (Ref# 400800 Deltalab SL) and stained with 25 μL 500 μg/mL EMA reagent (Ref# 63184 Sigma-Aldrich Ltd) for 1 hour, at room temperature, in the dark. The cells were then washed twice with 4 mL phosphate buffered saline (PBS) (Ref# 02-023-1A, Biological Industries) and resuspended in 500 μL PBS. Then, 100 μL of each sample were transferred into a new flow cytometry and fluorescence-activated cell sorting tube and diluted in 500 μL PBS. Flow-Check Pro Fluorescence beads (Ref#A63493, Beckman Coulter Inc) were used for daily quality control to ensure stability of instrument fluidics and optical alignment as well as standardization of fluorescence intensity and light scatter. RBCs were gated using forward vs side scatter and 20 000 events were read by the FL1 channel of a Beckman Coulter Navios flow cytometer instrument. The mean fluorescence intensity (MFI) of the peaks was recorded by the cytometry list mode acquisition and Analysis Software Navious Cytometer 1.3 (Beckman Coulter Inc). The MFI of the patient peak was subtracted from the mean MFI of the 6 controls. The result was multiplied by 100 to calculate the percentages of decreased EMA staining in the patient samples, according to the formula:

$$\frac{(MFI\ of\ patient - mean\ MFI\ of\ 6\ controls)}{mean\ MFI\ of\ 6\ controls} x100$$

Patients with <16% lower EMA staining compared with controls, were classified as negative. Patients with 16%-21% lower EMA staining were classified as borderline. Patients with >21% lower EMA staining were categorized as positive.[5]

Data Analyses

Pearson χ^2 analysis was used to compare nonparametric variables between negative, borderline, and positive samples. Two-sided t tests were performed to compare continuous variables between samples with negative vs positive EMA results and between samples with borderline vs positive EMA results. A receiver operating characteristic curve was generated and the area under the curve was calculated to evaluate

Table I. Patients and samples characterization

Total patients	139 (100)
Sex male (%):female (%)	70(50.3):69(49.7)
Age (y) (median + range)	0 (0-75)
Age (d) (median + range)	79.5 (0-27 333)
Total sample N (%)	151 (100)
EMA negative	115 (76.16)
EMA borderline	13 (8.6)
EMA positive	23 (15.2)

Table II. Comparison of laboratory indices between samples with negative borderline and positive EMA test

Indices	EMA Negative	EMA Borderline	EMA Positive	*P* value EMA Negative vs Positive	*P* value EMA Borderline vs Positive
Age (years) (Average ± SD)	10.25 ± 18.91 (0-74)	8.09 ± 8.89 (0-26)	7.05 ± 10.7 (0-30)	0.5	0.7
Age (years) (Median)	0	4	0		
RBCs X103/μL (range)	3.27 ± 1.13 (0.9-5.99)	3.14 ± 0.69 (2.09-3.68)	3.99 ± 0.92 (2.18-5.47)	0.0051	0.0072
Hb g/dL	9.9 ± 3.4 (3.1-20.8)	9.2 ± 2.2 (6.3-12)	12.2 ± 3.9 (6.6-20.7)	0.0057	0.015
HCT %	29.7 ± 10.2 (9.2-61.1)	26.5 ± 6.0 (18.5-34.9)	34.6 ± 11.1 (19.1-59)	0.04	0.02
Mean cell volume fL	91.8 ± 12.6 (56.2-119.6)	84.3 ± 5.7 (75.3-97.2)	83.3 ± 17.2 (28.6-109.3)	0.0064	0.84
Mean cell Hb pg	30.8 ± 4.5 (15.6-41.1)	29.2 ± 2.1 (25.1-33.4)	30.3 ± 4.4 (22.2-39.2)	0.62	0.40
MCHC g/dL	33.5 ± 1.8 (27.8-42)	34.7 ± 1.5 (32.9-37.9)	35.4 ± 1.4 (32.6-38.6)	<0.0001	0.16
Red cell distribution Width %	17.0 ± 3.2 (11.9-28.2)	19.5 ± 3.8 (11.7-25)	19.9 ± 3.5 (13.3-26.9)	0.0001	0.70
Absolute reticulocytes X109/L	164.2 ± 132.4 (1.6-713.2)	160.7 ± 147.9 (0.1-426.9)	293.7 ± 168.1 (0.2-539)	0.0002	0.03
Reticulocytes %	6.3 ± 6.1 (0.16-35.8)	5.8 ± 5.3 (0.3-16.6)	7.4 ± 3.5 (1.9-14.4)	0.43	0.30
Reticulocyte Index	2.0 ± 2.4 (0-13.3)	1.1 ± 1.3 (0-3.5)	4.1 ± 3.6 (0-11.4)	0.0013	0.01
Bilirubin total mg/dL	5.9 ± 5.0 (0-19.3)	1.5 ± 1.1 (0.5-3.6)	6.4 ± 4.6 (1.2-15.1)	0.73	0.003
Bilirubin direct mg/dL	0.9 ± 1.1 (0.2-5.7)	0.5 ± 0.2 (0.4-0.6)	0.7 ± 0.4 (0.3-1.7)	0.56	0.20
Lactate Dehydrogenase IU/L	583.2 ± 623.9 (107-3201.8)	579.6 ± 305.1 (189-623)	361.7 ± 161.8 (239-598)	0.32	0.34
Chloride mmol/L	104.3 ± 2.9 (96.6-116.4)	103.2 ± 2.2 (99.5-106)	106.8 ± 2.1 (103-110)	0.0063	0.002
B12 pg/ml	598.4 ± 406.7 (195-1846)	457.7 ± 77.1 (400.4-444.5)	777.2 ± 844.9 (180-1744)	0.54	0.47

Table III. Clinical markers of hereditary spherocytosis

EMA status	Jaundice	Transfusions	Cholecystectomy	Splenomegaly	Splenectomy
EMA positive (n = 19)	37% (n = 9)	42% (n = 8)	16% (n = 3)	52.5% (N = 10)	16% (N = 3)
EMA borderline (n = 9)	44% (n = 4)	55% (n = 5)	11% (n = 1)	77% (N = 7)	11% (N = 1)
EMA negative (n = 92)	56.5% (n = 52)	33.5% (n = 31)	4.5% (n = 4)	25% (N = 23)	3% (N = 3)

the optimal cut-off of the MCHC that can differentiate between negative and borderline or positive samples with the highest degree of sensitivity and specificity. All statistical analyses were performed using JMP (SAS Inc) statistical software.

Results

Sample Characterization

A total of 151 samples screened using the EMA test and obtained from 139 different patients (4 patients had 2 tests and 4 patients had 3 tests performed) were included in the study. Negative EMA results were obtained for 115 (76.16%) samples, borderline results were obtained for 13 (8.6%) samples (mean reduction in EMA staining ± SD 19 ± 1.5%), and 23 (15.2%) samples were classified as positive (mean reduction in EMA staining 29 ± 0.5%). Patient and sample characteristics are summarized in Table I.

MCHC is the Most Significant Discriminator Between Samples with Positive vs Negative EMA Test Results

Laboratory indices of positive vs negative EMA samples are summarized in Table II. Samples with a positive EMA test result showed higher Er count ($P = .0051$), higher Hb ($P = .0057$), higher HCT ($P = .04$), lower MCV ($P = .0064$), higher MCHC ($P < .0001$), higher RBC distribution wide ($P = .0001$), higher absolute reticulocytes ($P = .0002$), higher RPI ($P = .0013$), and higher chloride ($P = .0063$) levels compared with samples with negative EMA test results. MCHC values were the most significantly different between the negative and positive or borderline EMA groups (mean ± SD 33.54 ± 1.87 vs 35.12 ± 1.45 in negative vs positive and borderline EMA tests, respectively, $P < .0001$). In addition, patients with positive or borderline EMA samples showed higher MCHC/MCV ratios compared with those with negative EMA samples.

Borderline EMA Samples Show More Profound Anemia and Lower Reticulocyte Count Compared to Positive EMA Samples

Comparisons between laboratory indices of borderline vs positive EMA samples are summarized in Table II. Borderline EMA samples showed lower Er count ($P = .0072$), Hb ($P = .015$), HCT ($P = .02$), absolute reticulocytes ($P = .03$), RPI ($P = .01$), and chloride ($P = .002$) levels compared with positive EMA samples.

High Prevalence of Hereditary Spherocytosis Markers in Patients with Borderline and Positive EMA Test Results

Medical history data were available for 19 out of 23 EMA positive samples, 9 out of 13 borderline EMA samples, and 92 out of 115 negative EMA samples. A comparison between clinical markers of hereditary spherocytosis for patients in each EMA test result subgroups is summarized in Table III. According to our data, 55% of the patients with a borderline EMA sample required blood transfusions vs 42% of the patients with a positive EMA sample. In the borderline EMA sample group, 11% of the patients had undergone cholecystectomy vs 16% in the positive EMA group. Splenomegaly was reported for 77% of the patients with borderline EMA samples vs 52.5% of the patients with a positive EMA sample. Both borderline and positive sample groups showed higher prevalence of certain characteristic clinical markers of hereditary spherocytosis compared with the negative EMA test samples. A family history of hereditary spherocytosis was reported for 11% of patients in the borderline EMA group vs 42% of the patients in the positive EMA group.

MCHC >32.6 g/dL Identified Borderline and Positive EMA Test with 100% Sensitivity

A receiving operator characteristic analysis identified MCHC of ≥33.81 g/dL as an optimal cut-off value to discriminate between patients with negative EMA test results and patients with positive or borderline EMA test results, with 57.4% specificity and 80.5% sensitivity (area under the curve 0.758, $P < .0001$, false positive 32% and false negative 4.6%). In addition, it identified MCHC of >32.6 g/dL as a cut-off to distinguish between positive/borderline and negative test results with 100% sensitivity (28% specificity and false positive 69%) (Table IV). Using this MCHC cut-off retrospectively identified 30 (20.4%) samples that could have been spared EMA testing.

Discussion

Compared with samples with positive EMA test, borderline EMA test results are associated with more profound anemia and lower reticulocyte count and reticulocyte production index. Borderline EMA test results were shown to align with hereditary spherocytosis diagnosis in high probability and specificity.[7] In our study, patients with borderline EMA samples showed laboratory and clinical markers characteristic of hereditary spherocytosis. Patients with positive or borderline EMA samples showed higher MCHC/MCV ratios compared

Table IV. MCHC receiving operator characteristic table

X-MCHC	Specificity (%)	Sensitivity (%)	True Pos	True Neg	False Pos	False Neg	PPV (%)	NPV (%)
33.81*	57.4	80.5	29	66	49	7	37.2	90.5
32.60†	28	100	36	32	83	0	30.2	100

*MCHC cutoff value to discriminate between patients with negative EMA test results and patients with positive or borderline EMA test results with the highest sensitivity and specificity.
†MCHC cutoff value to distinguish between positive/borderline and negative test results with 100% sensitivity.

with those with negative EMA samples, further suggesting that borderline EMA samples should be referred as hereditary spherocytosis. Hence, the present observations suggest that borderline EMA test results may be indicative of patients with a more severe form of hereditary spherocytosis characterized by uncompensated hemolytic anemia. The increased rate of transfusions and splenomegaly may further indicate a more severe hereditary spherocytosis compared with a positive EMA test result.

The presented findings are in line with a report on milder decrease in EMA staining in patients with moderate or severe hereditary spherocytosis compared with patients with mild hereditary spherocytosis.[8] Hereditary spherocytosis is considered a heterogeneous disease, and considerable differences in disease severity were reported in patients with similar mutations.[9] Hence, borderline EMA results in newly diagnosed patients with hereditary spherocytosis should be taken into consideration as an additional indicator of disease severity, regardless of mutational status. Nonetheless, more research and a larger pool of samples is required to further validate variations of illness severity among patients with positive and borderline EMA samples.

The presented data identified MCHC as a significant discriminator of positive and borderline EMA tests from negative EMA tests. This observation is in line with previous reports showing the association between increased MCHC and hereditary spherocytosis.[10,11] Because of its low specificity, MCHC cut-off cannot be solely used for the diagnosis of hereditary spherocytosis. Nevertheless, the analysis revealed MCHC of ≤32.5 g/dL as a cut-off range beyond which no positive or borderline EMA test results were included. Interestingly, using this MCHC cut-off for determining the need for EMA testing identified 30 (20.4%) samples that could have been spared EMA testing. Hence, MCHC of <32.5 g/dL may be a useful marker for reducing redundant tests. However, as the current sample set included a limited number of samples with positive and borderline EMA tests, this finding needs to be further validated in other flow cytometry laboratories with a large EMA test sample pool. It should also be stated that MCHC is less than specific. The MCHC cut-off may be useful in EMA test referral, however, only in patients with no symptoms of hereditary spherocytosis. Further, final diagnosis cannot be based on one screening test.

In conclusion, these observations imply that a borderline EMA test in a sample suspected of hereditary spherocytosis may be an indication of a more severe form of hereditary spherocytosis. Furthermore, using the measure MCHC as a cut-off may help predict and reduce negative EMA tests without compromising sensitivity. ■

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