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Osmotic gradient ektacytometry: A valuable screening test for hereditary spherocytosis and other red blood cell membrane disorders

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Abstract

Introduction: New generation osmotic gradient ektacytometry has become a powerful procedure for measuring red blood cell deformability and therefore for the diagnosis of red blood cell membrane disorders. In this study, we aim to provide further support to the usefulness of osmotic gradient ektacytometry for the differential diagnosis of hereditary spherocytosis by measuring the optimal cutoff values of the parameters provided by this technique.

Methods: A total of 65 cases of hereditary spherocytosis, 7 hereditary elliptocytosis, 3 hereditary xerocytosis, and 171 normal controls were analyzed with osmotic gradient ektacytometry in addition to the routine red blood cell laboratory techniques. The most robust osmoscan parameters for hereditary spherocytosis diagnosis were determined using receiver operating characteristic curve analysis.

Results: The best diagnostic criteria for hereditary spherocytosis were the combination of decreased minimal elongation index up to 3% and increased minimal osmolality point up to 5.2% when compared to the mean of controls. Using this established criterion, osmotic gradient ektacytometry reported a sensitivity of 93.85% and a specificity of 98.38% for the diagnosis of hereditary spherocytosis.

Conclusion: Osmotic gradient ektacytometry is an effective diagnostic test for hereditary spherocytosis and enables its differential diagnosis with other red blood cell membrane diseases based on specific pathology profiles.

KEYWORDS
diagnosis, hereditary spherocytosis, membranopathies, osmotic gradient ektacytometry, screening

1 | INTRODUCTION

Red blood cell (RBC) membrane disorders constitute one of the major causes of chronic hereditary hemolytic anemia. Although they are worldwide distributed, their health burden vary among geographical areas depending on population ethnic background, related severity in clinical manifestations, and delay in appropriate diagnosis or misdiagnosis.[1]

RBC membrane is a highly dynamic structure consisting of an outer phospholipid bilayer anchored to a spectrin-based network through two principal linking protein complexes: the ankyrin and the junctional complexes. These vertical linkages assure a strong membrane cohesion preventing membrane vesiculation, while lateral linkages between spectrin dimers and among spectrin-actin-4.1R in the junctional complex are the key regulators of membrane mechanical stability preventing membrane fragmentation[2-4] Moreover, there are various associated ion transporters, co-transporters, and channels embedded in the RBC membrane that mediates the maintenance of the cell volume and hydration status.

Main RBC membrane disorders, namely hereditary spherocytosis (HS), hereditary elliptocytosis (HE), and hereditary stomatocytosis (HSt), alter membrane cohesion, membrane mechanical stability, and RBC volume, respectively. As a consequence, RBC deformability is compromised leading to their premature removal from circulation by the spleen, manifested as hemolytic anemia.[5-8]

HS and HE are RBC membrane disorders characterized by mutations in genes encoding membrane or skeletal proteins respectively, and its mutations alter the membrane complex structure. HS is the most common inherited RBC membrane disorder with one case of 2000 individuals in the Northern European countries, and probably even higher prevalence due to under diagnosis of minor or even moderate forms. The inheritance pattern is dominant in 75% of cases, and it is caused by defects in the vertical interactions that confer cohesion to the RBC membrane.[9-12] HE is a common RBC hemolytic anemia, with an estimated prevalence of 3 to 5 of 10 000 individuals, although real frequency is likely to be higher due to a significant number of asymptomatic patients. HE presents an autosomal dominant inheritance, exception made for the most severe forms, named hereditary pyropoikilocytosis (HPP), which inheritance is autosomal recessive.[6,13] HS and HE patients phenotype varies from very mild or symptomless defect to moderate or severe neonatal hemolytic anemia. RBC life span is shortened in both diseases mainly due to RBC trapping in the billroth cords of the spleen, and their phagocytosis by the macrophages. As a consequence, a regenerative hemolytic anemia is associated with splenomegaly and icterus due to increased free plasma bilirubin level results.[14,15] In HSt syndromes, the ion homeostasis is compromised due to alterations in membrane channels leading to a leak of the univalent cations (Na^+ and K^+) and an altered RBC water content. Xerocytosis or dehydrated hereditary stomatocytosis (dHSt) is the most prevalent HSt syndrome, and its main characteristic is RBC dehydration. The inheritance is autosomal dominant even though the molecular bases are still unclear. dHSt patients might also present heterogeneous clinical manifestations.[7,13,16-18]

Abnormal RBC morphology is often the first clue for RBC membrane disorders diagnosis. According to guidelines,[9] patients with a family history of HS, typical clinical features, and laboratory investigations (hemolytic anemia with raised mean corpuscular hemoglobin concentration, high percentage of hyperdense cells, and spherocytes in the blood smear) do not require any additional test to be diagnosed as HS. However, several common circumstances may hamper the diagnosis, for example, family history of HS is not present in many severe cases, commonly inherited in a recessive pattern or resulting from a significant rate of the novo mutations, or laboratory investigations may be altered by intense reticulocytosis or in neonatal period. Therefore, advanced and specific hematological tests, available only in specialized laboratories, will be required for the diagnosis of unclear HS cases, severe forms of HE and dHSt. In addition, dHSt patients can be misdiagnosed as HS due to shared alterations in hematological parameters and disease unawareness. Differential diagnosis between HS and dHSt is critical as splenectomy is an indicated treatment for HS patients but not in dHSt patients as, for still unclear reasons, it may lead to severe thrombosis.[19,20]

Specific hematological tests for HS diagnosis are only available in specialized laboratories and include osmotic fragility test (OFT), acidified glycerol lysis test (AGLT), hypertonic cryohemolysis test, eosin-5-maleimide (EMA)-binding test, RBC membrane thermostability, and osmotic gradient ektacytometry.[21-26]

Osmotic gradient ektacytometry, originally designed in the seventies, has been for many years the reference method for RBC deformability measurement but, due to its technical complexity and lack of implementation, was not used for clinical purposes. However, the new generation ektacytometer LoRRca MaxSis (Mechatronics Instruments BV®, Zwaag, The Netherlands) has become a more robust and user friendly equipment allowing the transference from research to clinical laboratory requiring harmonization for its inclusion in the diagnostic algorithm for HS and other RBC membrane disorders.[25,27,28]

In this study, we analyzed the differences of the several parameters obtained after performing the osmoscan module of LoRRca MaxSis among healthy controls and patients affected by HS, HE, and dHSt and determined the optimal cutoffs for HS diagnosis. The aim of this study was to evaluate the present technique as an adequate assay to perform screening of membranopathies, focusing on the differential diagnosis between HS and nonspherocytic membrane defects such as HE and dHSt.

2 | MATERIALS AND METHODS

2.1 | Patients and inclusion protocol

A total of 75 patients with chronic hemolytic anemia oriented as hereditary RBC membrane disorders (hemoglobin disorders discarded and negative Coombs test) were included during a period comprised between January 2015 and August 2016 (20 months). The age of the patients varied from 3 months to 71 years and both sexes were represented (32 females and 43 males). Transfused patients were excluded except if blood transfusion was not required within the 3 months before

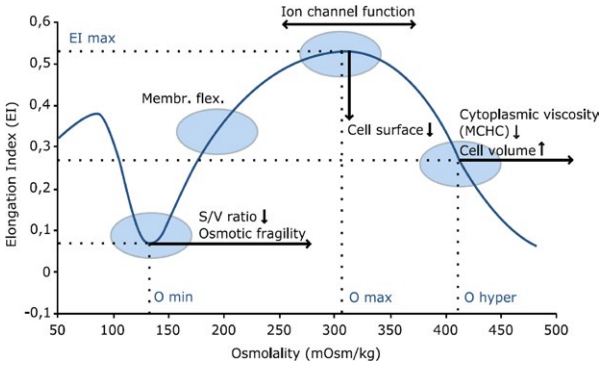

FIGURE 1 Osmoscan curve relevant parameters: Omin, EImin, Omax, EImax, Ohyper, and EIhyper. AUC is not represented. Adapted from RR Mechatronics, brochure/website *Lorrca® ektacytometer*, Figure 5; with permission [Colour figure can be viewed at wileyonlinelibrary.com]

the blood draw. Normal controls were obtained from blood donors. The study was approved by the Ethical Committee for Clinical Investigations of the Hospital Clínic de Barcelona (reference number 2013/8436), and informed consents were obtained according to the current laws regulating personal data protection (LIB 14/2007 and LOPD 15/1999).

The protocol for the diagnosis of RBC membrane disorders included CBC and reticulocyte count, blood smear morphology, OFT,[29] AGLT,[30] EMA-binding test,[31] and RBC membrane heat stability [32,33] (Supplemental methods). Based on the results, 65 patients from 47 families were classified as HS, accomplishing at least three of the following criteria: hyperdense RBC (CHCM > 350 g/L and/or % > 4), presence of spherocytes in peripheral blood smear, increased OFT, decreased AGLT, and decreased fluorescence in EMA-binding test (decreased fluorescence > 11% "gray zone," > 21% HS). Seven patients from 6 families were classified as HE by the presence of elliptocytes in blood smear and absence of HS criteria. Finally, 3 patients of the same family were classified as dHSt, accomplishing at least two of the following criteria: CBC parameters (increased CHCM > 350 g/L, reticulocytes > 2%) and presence of stomatocytes in blood smear and increased RBC membrane heat stability.

2.2 | Osmotic gradient ektacytometry

Osmotic gradient ektacytometry was performed using the osmoscan module of the Laser-assisted Optical Rotational Deformability Cell Analyzer: LoRRca MaxSis (RR Mechatronics, The Netherlands).[34] There are several parameters defined in the osmoscan curve (Figure 1): (i) EImin: minimal elongation index, (ii) Omin: osmolality at EImin, directly correlates with the osmolality of 50% lysis point in OFT and represents the area/volume ratio, (iii) EImax: maximal RBC deformability, (iv) Omax: osmolality at EImax, represents the ion channel function, (v) Ohyper: osmolality at 50% of EImax in the hypertonic region, reflects the hydration state (vi) EIhyper (varies according to EImax): elongation index at Ohyper, (vii) AUC: the area under curve is directly calculated by the software from Omin to 450 mOsm/kg.[35]

The osmoscan module of LoRRca MaxSis is performed by adding 250 uL of whole blood to 5 mL of isosmolar polyvinylpyrrolidone (iso PVP). The diluted RBC suspension is submitted to an increasing osmotic gradient (from 80 mOsmol/L to 500 mOsmol/L) under a constant shear stress of 30 mPa.

2.3 | Statistical analysis

Osmoscan parameters' statistical differences among controls and patients groups were analyzed with unpaired (two samples) *t*-test as Gaussian distribution was confirmed with D'Agostino and Pearson test.

Evaluation of osmoscan parameters robustness for HS diagnosis was performed using the receiver operating characteristic (ROC) curve analysis. The analysis compared a single HS group with a unique non-HS group that included normal controls, HE, and dHSt patients as well as with each one of these groups separately. The optimal cutoff was determined as the one with the highest likelihood ratio. Statistical analysis was operated with GraphPad Prism (GraphPad Software, La Jolla, California, USA).

3 | RESULTS

3.1 | Hematological parameters and HS patient's classification

Patient's general hematological data (CBC and reticulocyte count) are summarized in Table 1.

3.1.1 | Hereditary spherocytosis (HS)

A total of 65 patients with HS were classified into two groups: nonsplectomized (46 patients) and splenectomized (19 patients). The splenectomy corrected the anemia and reticulocytosis in 17 of the 19 patients. In the remaining 2, a high reticulocytosis (retics > $450x10^9$/L, > 9%) without anemia (164 g/L) persisted in a woman and with a slight anemia (Hb 122 g/L) in a man. According to HS guideline criteria,[9] the 46 nonsplenectomized HS patients were clinically classified into four main groups: I) severe (Hb < 80 g/L, retics > 10%): 3 patients, II) moderate (Hb 80-120 g/L, retics > 6%): 29 patients, III) mild (Hb 100-150 g/L, retics 3%-6%): 13 patients, and IV) trait (Hb normal, retics < 3%): 1 patient.

3.1.2 | Hereditary elliptocytosis (HE)

Four of the seven patients were asymptomatic or presented very mild anemia and three showed moderate anemia. In addition, the patient with the lowest Hb concentration (86 g/L) also presented splenomegaly (echography 21 cm), reticulocytosis (7.2%). and hyperdense cells (6.1%). No HE patient underwent splenectomy.

3.1.3 | Dehydrated hereditary stomatocytosis (dHSt)

The 3 related patients, propositus, mother, and grandfather presented compensated chronic hemolysis associated with increased MCHC and reticulocytosis. It is worth mentioning the significant differences in

TABLE 1 Sex, age, and hematological parameters of patients included in the study grouped according to the RBC membrane disorder

	Sex (female: male)	Age (years)	Hematological parameters						
			Hb (120-170 g/L)	RBC (3.90-5.50 10^{12}/L)	MCV (80-100 fl)	MCHC (310-350 g/L)	Hyper (0.1-2.8%)	Retics (25-100 10^{9}/L)	Retics (0.5-2%)
Hereditary Spherocytosis (n = 65)									
Nonsplenectomized (n = 46)	17: 31	8 (1-69)	112.5 (54-171)	3.95 (2.42-6.07)	80.95 (67-98.1)	352.5 (311-400)	11.1 (0.8-32.5)	307.75 (47-783.5)	8.6 (0.96-20.1)
Severe (n = 3)	0: 3	4 (4-4)	66 (54-78)	2.57 (2.42-2.9)	80.7 (70.3-84.7)	317 (317-319)	3.7 (1.2-4.5)	259.5 (210.5-307.4)	10.6 (8.2-10.72)
Moderate (n = 29)	12: 17	6 (1-54)	104 (84-129)	3.69 (2.99-4.68)	78.90 (67-98.10)	355 (311-400)	12.7 (0.8-32.5)	371.2 (144.3-783.5)	10.34 (3.9-20.1)
Mild (n = 13)	3: 10	12 (1-69)	132 (112-142)	4.28 (3.97-4.77)	82.4 (73.9-95.2)	353 (338-383)	12.1 (2.2-29)	197.7 (152.1-426.2)	4.64 (3.53-10.03)
Trait (n = 1)	1: 0	2	134	4.88	80.3	343	2.1	47	0.96
Splenectomized (n = 19)	9: 10	37 (10-48)	142 (122-172)	4.87 (4.17-6.07)	85.6 (77.7-99.7)	348 (324-370)	3.7 (0.6-30.4)	132.7 (58.1-471.9)	2.75 (1.09-10.47)
Hereditary Elliptocytosis (n = 7)	5: 2	26 (2-39)	120 (86-155)	4.5 (2.8-5.9)	85.6 (60.2-98.6)	314 (287-341)	1.3 (0.3-6.1)	102.6 (61.8-202)	2.18 (1.4-7.2)
Dehydrated Hereditary Stomatocytosis (n = 3)	2: 1	44 (12-71)	147 (137-165)	4.19 (4.2-4.7)	98 (97-100.6)	348 (340-358)	2.1 (0.9-2.3)	321.2 (201.1-631.1)	7.66 (4.3-15.2)

Patient data for age and hematological parameters are expressed as median (minimum value- maximal value).
Hb, hemoglobin; RBC, red blood cell count; MCV, mean cell volume; MCHC, mean corpuscular hemoglobin concentration; Hyper, hyperdense cells; Retics, reticulocytes.

clinical expression between the child, presenting with severe anemia, high reticulocytosis (15.22%), jaundice, gallstones, and early cholecystectomy (before puberty), and her relatives, who underwent cholecystectomy in adulthood. None was splenectomized.

3.2 | Osmoscan LoRRca MaxSis profiles

Specific patterns of osmoscan LoRRca MaxSis were observed for HS, HE, and dHSt (Figure 2). HS curves were bell shaped, but two different profiles were identified: HS1 curves present a classical bell shape explained by an increased Omin and decreased Ohyper (moreover they present decreased EImax and AUC) and HS2 curves are moved to the right, explained by an increased Omin and also increased Ohyper (moreover they present increased Omax and decreased EImax and AUC). HE curves showed a characteristic trapezoidal shape with a decreased EImax, Omax, and AUC, with several heterogeneous profiles. dHSt curves showed a specific decrease in Ohyper and a slight increase in EImin.

The values of each osmoscan parameter obtained from the three different membrane disorders (HS, HE, and dHSt) were compared with the reference ranges established with 171 normal controls, and the observed differences were statistically analyzed (Table S1). The only parameter that appeared to be significantly different between normal and all membrane defects profiles was AUC. However, HS and HE profiles differed in the osmolality-related parameters (Omin, Omax, and Ohyper). Meanwhile, dHSt profile differed from HS and HE in EImax parameter.

3.3 | Osmoscan LoRRca MaxSis for HS diagnosis

To identify the most useful parameters for HS diagnosis, ROC curve analysis was performed to all the parameters that showed statistical significant differences between HS (HS1 and HS2) and non-HS (controls, HE, and dHSt): Omin, EImax, Omax, and AUC. The results showed that the best parameters were as follows: AUC (AUC 0.9903; $P < .0001$) and Omin (AUC 0.9642, $P < .0001$) and its optimal cutoff: AUC < 145.1 (sensitivity 86.15%, specificity 99.45%) and Omin > 170.5 (sensitivity 67.69%, specificity 99.45%). If the results are expressed as % of variation from the mean of normal controls, the cutoffs are less than −14.9% for AUC and more than +12.8% for Omin.

In addition, ROC curve analysis was also performed for HS and each one of the non-HS groups separately. The results determined that EImax was the parameter that better differentiated HS from normal controls (AUC 0.9998, $P < .0001$) and from dHSt, while the Omin was the best parameter to separate HS from HE (AUC 0.959, $P < .0001$). The optimal EImax cutoff to differentiate HS from normal controls and dHSt was <0.5975 (sensitivity 98.46%, specificity 99.42%), while the optimal Omin cutoff to differentiate HS from HE was >159.0 (sensitivity 95.38%, specificity 85.71%). If the results are expressed as % of variation from the mean of normal controls, the best combination of parameters for HS diagnosis is less than −3% for EImax and more than +5.2% for Omin. When the combination of EImax/Omin was applied in the 171 normal controls and the 75 patients, 62 samples were identified as HS and 184 as non-HS (Figure 3). The coincidence between the EImax/Omin combination results and the clinical diagnosis of HS was of 61 of 62 cases, resulting in a specificity of 98.38%. On the other hand, the correlation with the non-HS clinical diagnosis was coincident in 180 cases of 184, resulting in a sensitivity of 93.85%.

Osmoscan LoRRca parameters were analyzed in combination with the other laboratory tests currently used for HS diagnosis (CBC parameters, morphology, OFT, and EMA-binding test). No correlations were identified among the routine laboratory techniques and LoRRca MaxSis Osmoscan. Regarding HS diagnosis, the combination of results for each one of the tests was analyzed (Table 2). In 53 HS patients, all the diagnosis tests had been performed, and in 32 of them, all the tests suggested HS. The results showed that morphology was the most sensitive test (100%) followed by LoRRca (92.5%). EMA-binding test sensitivity varied from 83.0% to 96.22% when using the threshold >−21% or >−11%, respectively.

4 | DISCUSSION

Osmotic gradient ektacytometry has risen during the last years, as a potential powerful technique for measuring RBC deformability [5,6] and therefore for HS diagnosis, the most relevant RBC membrane disorder in clinical practice due to its prevalence and clinical manifestations.[6,28]

A total of 75 patients with hereditary membranopathies were included in this study: 65 HS (86.7%), 7 HE (9.3%), and 3 dHSt (4%). Despite the low number of HE and dHSt patients, the osmotic gradient ektacytometry profiles obtained were similar to those reported so far.[6] Therefore, the results here obtained were considered as illustrative for the statistical analysis when compared to HS.

The majority (46 patients) of total HS patients (65 patients) were nonsplenectomized (70.8%), most of them (45; 97.8%) presenting anemia: severe (6.5%), moderate (63.0%), and mild (28.3%). The 3 patients with severe hemolytic anemia were less than 5-year-old nonsplenectomized children. Following the HS guidelines,[9] only 4 of the 46 patients fulfilled the first-line criteria for diagnosis (family history, circulating spherocytes, CHCM >360 g/L and hyper >4%), and, accordingly, further laboratory analyses were required for final diagnosis confirmation in 42 patients (91.3%). The 19 splenectomized patients (29.2%) showed a rapid improvement of hemoglobin concentration in all cases exception made of two cases that maintained a slight anemia associated with a high reticulocyte count suggesting persistence of higher degree of hemolysis than usually expected in HS patients after splenectomy.

LoRRca MaxSis osmoscan analysis of the 75 patients with RBC membrane defects revealed 4 different profiles: two for HS, one for HE, and one for dHSt with a common characteristic decrease in AUC in all the cases. Despite the HS1 and HS2 profiles presented increased Omin and decreased EImax and AUC, they significantly differed in the values of Omax and Ohyper parameters, both related with channel function and RBC hydration, respectively.[35] Despite all the patients

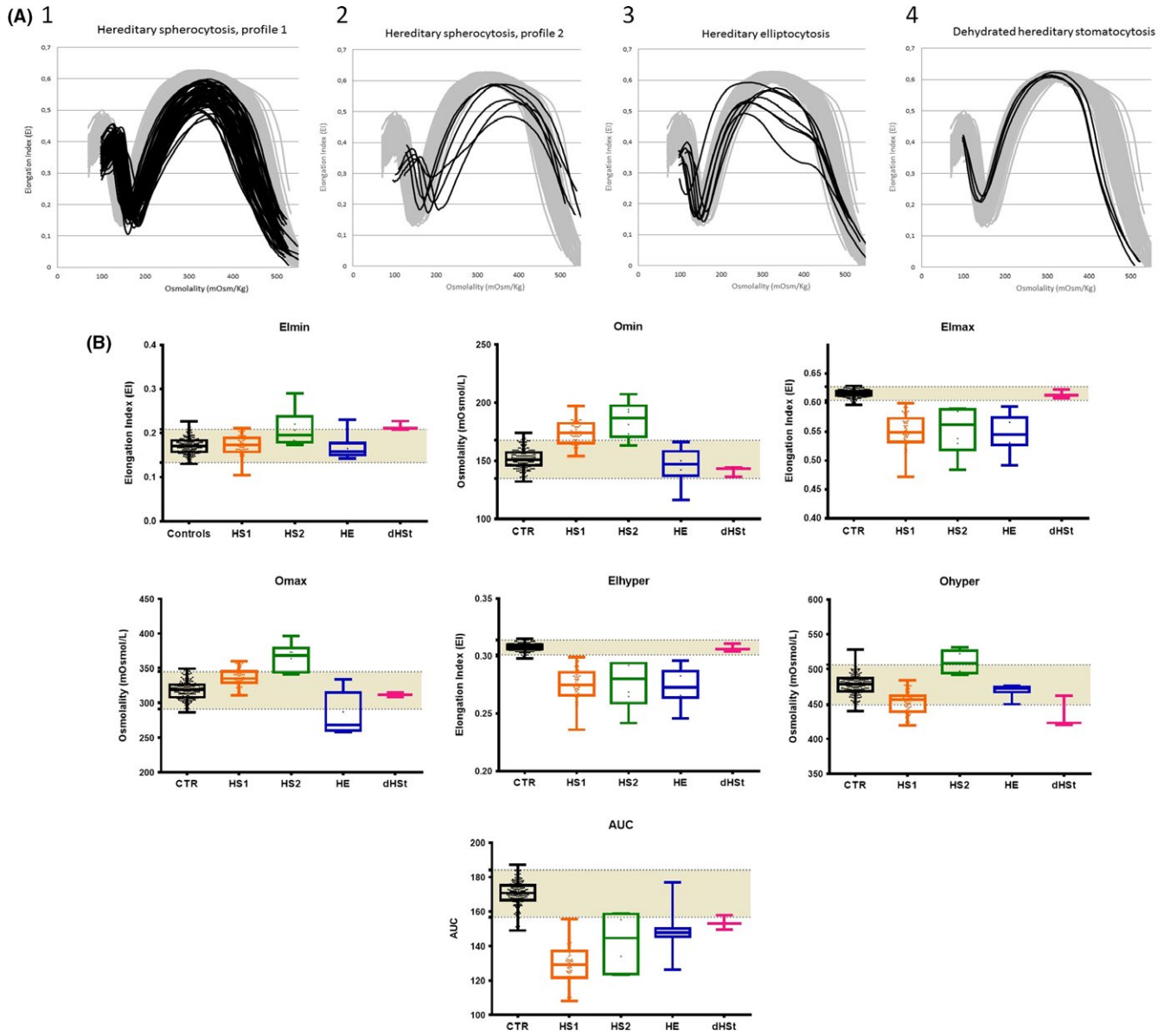

FIGURE 2 LoRRca MaxSis osmoscan profiles and parameters for controls and RBC membrane disorders. A) HS, HE, and dHSt osmoscan profiles (controls colored in gray): A1. Osmoscan profiles for HS profile 1, A2. Osmoscan profiles for HS profile 2, A3. Osmoscan profiles for HE; A4. Osmoscan profiles for dHSt. B) Osmoscan parameters (EImin, Omin, EImax, Omax, EIhyper, Ohyper, and AUC) represented in boxes and whiskers for the different control and membranopathies groups. Boxes show the median and the 25th and 75th percentile, while whiskers determine the minimum and maximal values. Reference ranges for normality established in our laboratory appear as a gray colored area [Colour figure can be viewed at wileyonlinelibrary.com]

included in the HS2 profile exhibited moderate-to-severe anemia, no correlations were found between the two different HS profiles and the clinical classification of HS or their CBC parameters. Accordingly, we hypothesize that these differences may be related with channel polymorphisms that affect ion homeostasis and, in turn, modulate the clinical expression. HE profiles, despite characterized by a typical trapezoid shape, were found to be very heterogeneous showing all of them decreased EImax, Omax, and AUC. HE and HS profiles exhibited similar results for EImax parameter, but not for Omin that was different allowing the discrimination between both membranopathies. Finally, dHSt profiles presented a slight increase in EImin and a decrease in Ohyper and AUC; reflecting the characteristically dehydrated status of the RBCs in this disease. Moreover, normal values for EImax and Omax in dHSt enable its differentiation from HS and HE.

To evaluate osmoscan LoRRca MaxSis for HS diagnosis, we analyzed both the use of single osmoscan parameters and the combination of two of them in scatter plot.

As a single parameter, AUC (cutoff > −14.9%) or Omin (cutoff > 12.8%) was the parameters that better differentiated HS from non-HS, providing further support to previous observations were the use of AUC (cutoff > −18.5%) or Omin (cutoff > 21.5%) have been established for HS diagnosis.[28] However, these parameters are found to be highly specific but less sensitive. In our study, both parameters showed a specificity of 99.45% and a sensitivity of 86.15% for AUC

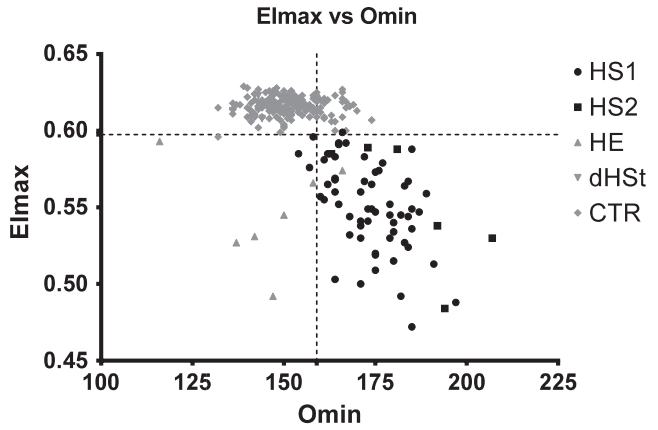

FIGURE 3 Scatter plot representing the distribution of healthy controls, HS, HE, and dHSt patients taking into account Elmax and Omin osmoscan parameters. HS patients (HS1 and HS2) are colored in black and non-HS in gray. The determined cutoffs (Elmax 0.5975 and Omin 159) are indicated by dashed lines

and 67.69% for Omin. Interestingly, the osmoscan parameters that better differentiate HS from controls (and dHSt) could not differentiate HS from HE, and the parameter that better differentiates HS from HE cannot differentiate the HS from controls and dHSt. Therefore, the combination of parameters obtained from our study, Elmax (cutoff < −3%) and Omin (cutoff > 5.2%), has been established as a criteria for HS diagnosis. Regarding the 4 HS patients classified as non-HS by scatter plot parameters combination, one of them was a 2-year-old patient with HS trait, and the other three were two adults in which the splenectomy corrected the anemia and the reticulocytosis, and their son, a nonsplenectomized child that presented moderate anemia (94 g/l) and high reticulocyte counts (6.56%; 236.7 × 10^9/L). On the other hand, this combination of parameters identified one HE patient within the HS group that requires further genetic analysis.

As the diagnostic flow diagram for RBC membrane disorders has not yet been standardized, several HS tests have been proposed as first and second line diagnostic methods, depending on specific laboratories workflows.[5,6,9,25] In this way, EMA-binding test is currently considered the first-line screening test for HS diagnosis with a reported sensitivity of 92.7% and a specificity of 99.1%.[21,22,36,37] However, the cutoff point for HS diagnosis (>−21%, >−16% or >−11%) is still a matter of debate.[21,22,26,36,38] In our study, EMA-binding sensitivity and specificity decreased to 83.0% (using the threshold >−21%) and to 96.22% (using the threshold of >−11%), respectively. However, when using the >−16% threshold, only a 5.6% of HS cases presented EMA values between 11% and 15%, and 3.8% of cases, was below 11%. Accordingly, we consider >16% the optimal EMA cutoff for HS diagnosis.

In conclusion, the combination of EMA-binding test with LoRRca MaxSis osmoscan increases the sensitivity to 100% with an EMA cutoff >−11% and up to 98.1% with the EMA cutoff >−16%. In addition, as LoRRca osmoscan MaxSis profiles clearly distinguish among different RBC membrane defects, the combined analysis of LoRRca osmoscan parameters is, up to now, the most efficient screening procedure for the differential diagnosis of RBC membrane defects, and we strongly recommend its use in the RBC membrane diagnostic workflow. This, together with the complementary use of EMA-binding test will improve the accuracy of HS diagnosis to almost 100% of cases.

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AUTHOR CONTRIBUTION

MMP and JVC designed the research study; JSN, MCS, MGB, ARL, IB, PVP, and JD gathered the clinical data and provided the patient

TABLE 2 Combination of HS diagnostic laboratory techniques results for HS patients

Number of cases (n = 53)	CBC parameters	Morphology	INCUB OFT	EMA	Osmoscan LoRRca
32	✓	✓	✓	✓	✓
5	✗	✓	✓	✓	✓
4	✓	✓	✗	✓	✓
4	✓	✓	✓	○	✓
2	✗	✓	✓	○	✓
1	✓	✓	✓	✗	✓
1	✗	✓	✓	✗	✓
1	✗	✓	✓	○	✗
1	✓	✓	✓	✓	✗
1	✓	✓	✗	✓	✗
1	✗	✓	✗	✓	✗

(✓), Result compatible with HS; (✗), Result no compatible with HS; (○) EMA-binding test >−11% but <−21%.

samples; ELLP, VR, and PGR performed the research; ELLP and MMP analyzed the data; ELLP, JVC, and MMP wrote the paper.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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