Diagnostic power of laboratory tests for hereditary spherocytosis: a comparison study in 150 patients grouped according to molecular and clinical characteristics

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ABSTRACT

Background
The laboratory diagnosis of hereditary spherocytosis commonly relies on NaCl-based or glycerol-based red cell osmotic fragility tests; more recently, an assay directly targeting the hereditary spherocytosis molecular defect (eosin-5'-maleimide-binding test) has been proposed. None of the available tests identifies all cases of hereditary spherocytosis.

Design and Methods
We compared the performances of the eosin-5'-maleimide-binding test, NaCl-osmotic fragility studies on fresh and incubated blood, the glycerol lysis test, the acidified glycerol lysis test, and the Pink test on a series of 150 patients with hereditary spherocytosis grouped according to clinical phenotype and the defective protein, with the final aim of finding the combination of tests associated with the highest diagnostic power, even in the mildest cases of hereditary spherocytosis.

Results
The eosin-5'-maleimide-binding test had a sensitivity of 93% and a specificity of 98% for detecting hereditary spherocytosis: the sensitivity was independent of the type and amount of molecular defect and of the clinical phenotype. The acidified glycerol lysis test and Pink test showed comparable sensitivity (95% and 91%) . The sensitivity of NaCl osmotic fragility tests, commonly considered the gold standard for the diagnosis of hereditary spherocytosis, was 68% on fresh blood and 81% on incubated blood, and further decreased in compensated cases (53% and 64%, respectively). The combination of the eosin-5'-maleimide-binding test and acidified glycerol lysis test enabled all patients with hereditary spherocytosis to be identified. The eosin-5'-maleimide-binding test showed the greatest disease specificity.

Conclusions
Each type of test fails to diagnose some cases of hereditary spherocytosis. The association of an eosin-5'-maleimide-binding test and an acidified glycerol lysis test enabled identification of all patients with hereditary spherocytosis in this series and, therefore, represents a currently effective diagnostic strategy for hereditary spherocytosis including mild/compensated cases.

Key words: hereditary spherocytosis, laboratory tests, specificity, sensitivity.

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Introduction

Hereditary spherocytosis is the most common congenital hemolytic anemia in Caucasians, affecting approximately 1 in 1000-2000 individuals.[1,2] The molecular defect is highly heterogeneous involving the genes encoding for spectrin, ankyrin, band 3 and protein 4.2,[3] and the degree of hemolysis varies widely, from fully compensated to transfusion-dependent anemia.

The typical laboratory hallmark of hereditary spherocytosis, although not specific, is the presence of spherocytes on a peripheral blood smear, which are detectable in 97% of patients.[4] However, there may be very few spherocytes in some patients[4,5] and skilled operators are, therefore, necessary to detect them; moreover, microscopic examination of red cells is increasingly omitted in the era of laboratory automation. The laboratory diagnosis of hereditary spherocytosis does, therefore, commonly rely on tests that exploit the surface area-to-volume ratio, typically reduced in spherical erythrocytes, in particular red cell osmotic fragility tests at various sodium chloride (NaCl) concentrations on fresh and incubated blood,[6] and assays that measure the extent or the rate of lysis of red cells suspended in buffered glycerol solutions, i.e. the glycerol lysis test (GLT),[7] the acidified glycerol lysis test (AGLT)[8] and the Pink test.[9] However, these tests do not pick up a variable proportion of cases of hereditary spherocytosis, particularly the mildest ones,[6,10-12] and do not differentiate hereditary spherocytosis from secondary spherocytosis associated with other conditions, mainly autoimmune hemolytic anemias.[13-15]

More recently, the cryohemolysis test,[16,17] based on the observation that red cells from patients with hereditary spherocytosis are particularly susceptible to cooling at 0° C in hypertonic conditions, and the flow cytometric analysis of eosin-5'-maleimide-labeled intact red blood cells (EMA-binding test)[18] have been proposed as new methods for identifying hereditary spherocytosis.[19] The latter in particular has been proven to be a sensitive and specific diagnostic test for hereditary spherocytosis[12,18,20-29] directly targeting the structural lesion of this disease, since the fluorescent probe, eosin-5'-maleimide, interacts with the protein band 3 complex.[30]

The performance of the available direct or indirect diagnostic tests has been mostly evaluated individually and on limited number of cases. The sensitivity of the test varies greatly and each method fails to identify several patients with hereditary spherocytosis.[4,6,12] In this study, we compared the performances of EMA-binding, NaCl-induced osmotic fragility of fresh and incubated blood, GLT, AGLT and Pink tests on a series of 150 patients with hereditary spherocytosis grouped according to the clinical phenotype and molecular lesion, with the final aim of finding the combination of tests associated with the highest diagnostic power for hereditary spherocytosis, including the mildest cases which are usually difficult to diagnose.

Design and Methods

Subjects

One hundred and fifty consecutive patients with hereditary spherocytosis (79 males and 71 females, median age 26 years, range 0-79 years) belonging to 128 unrelated families were investigated. At the time of the study, 22 patients were splenectomized and 128 not splenectomized.

All patients underwent clinical and physical evaluation and the following laboratory tests: complete blood counts, blood smear examination, reticulocyte count, assays of bilirubin and haptoglobin concentration, assessment of iron status, direct antiglobulin test (DAT), screening for abnormal or unstable hemoglobins, NaCl osmotic fragility test on fresh and incubated blood, GLT, AGLT, Pink test and EMA-binding test. In some cases, the activity of the most important glycolytic and pentose phosphate pathway enzymes was investigated. In a few patients, the mitogen-stimulated DAT[31] was also performed to rule out the diagnosis of DAT-negative hemolytic anemia.

Hereditary spherocytosis was diagnosed on the basis of clinical and laboratory signs of chronic hemolysis, presence of spherocytes at peripheral blood smear examination, positivity of at least one red cell fragility test, family history of hereditary spherocytosis if any, and exclusion of other causes of secondary spherocytosis.[19] Anemia was defined as severe (Hb<8 g/dL), moderate (Hb 8-10 g/dL), mild (Hb>10 and <11.5 g/dL for females and Hb>10 and <13.5 g/dL for males) and compensated (Hb>11.5 g/dL for females and >13.5 g/dL for males).

Samples from all patients were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) for analysis of the red cell membrane proteins and divided according to whether there was deficiency of band 3, spectrin or ankyrin, combined spectrin/ankyrin deficiency, and no detectable defect.[4]

Five hundred and seventy-five healthy blood donors and 84 cases with hemolytic anemia other than hereditary spherocytosis (17 with autoimmune hemolytic anemia, 10 with red cell enzyme disorders, 10 with hereditary elliptocytosis, 15 with congenital dyserythropoietic anemia, 9 with paroxysmal nocturnal hemoglobinuria, 3 with stomatocytosis, 3 with mechanical anemia, and 17 with anemia of unknown cause) were also studied.

Hematologic assays

Peripheral blood was collected from patients and controls during diagnostic procedures after obtaining informed consent and approval from the Institutional Human Research Committee. The procedures followed were in accordance with the Helsinki international ethical standards on human experimentation. The great majority of samples were collected in our institute; samples collected from other centers were shipped maintaining a temperature of 4°C and always processed within 24 h. All tests were performed in a single site. None of the patients had been transfused within the 3 months preceding the study. Hematologic parameters were determined on an automated hematology analyzer (Automatic Beckman Coulter LH-750, CA, USA). Routine hematologic investigations were carried out according to Dacie & Lewis.[32] Bilirubin, haptoglobin, and ferritin levels were determined using Integra 800 (Roche, Mannheim, Germany). The number of spherocytes in peripheral blood was assessed by two independent and expert operators. Red cell osmotic fragility was evaluated by performing the NaCl osmotic fragility test on both fresh and incubated blood,[6] the standard GLT,[7] the AGLT[8] and the Pink test[9] on samples from each patient.

The EMA-binding test was performed as described by King *et al.*[18] with minor modifications. In particular, fluorescence intensity, expressed as median channel fluorescence, was determined for 10,000 events in the FL-1 channel, using a Becton Dickinson FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA, USA). The EMA dye stock solution was stored in small aliquots at -80° C over a period of 6 months. The test was performed once a week grouping all cases in the same day[25] after confirming the reproducibility of results on blood samples stored up to 6 days at 4°C.

In order to decrease intra-assay variation, patients' samples

were compared with those of six normal controls. Results were expressed as the percentage of fluorescence reduction of the patient's sample compared to the mean fluorescence of the six normal controls as proposed by Girodon *et al.*[25] A receiver operating characteristic (ROC) curve based on logistic regression analysis was used to determine the optimum cut-off between values from patients with hereditary spherocytosis and those from normal individuals. According to the ROC curve analysis, the optimum decrease in fluorescence to separate normal subjects from patients with hereditary spherocytosis was 11%. In these conditions test specificity computed on 575 healthy subjects was 98%.

Red cell ghosts were prepared within 24 h of blood collection using the method described by Dodge *et al.*[33] with slight modifications.[4] Red cell membrane proteins were analyzed within 15 days of ghost preparation by SDS-PAGE using a 4% to 12% gradient of acrylamide according to Fairbanks *et al.*[34] and the discontinuous buffer system of Laemmli[35] with an acrylamide linear gradient from 6% to 14%, as previously described.[4] Activities of enzymes of the glycolytic and pentose phosphate pathways were assayed using the methods described by Beutler.[36]

Results

Table 1 shows the hematologic and biochemical data of the patients with hereditary spherocytosis grouped according to whether they had not been splenectomized or had been splenectomized before the time of the study, and according to the clinical phenotype (in non-splenectomized patients only). The majority of non-splenectomized subjects had mild to compensated hemolysis; 18% had very few spherocytes (≤3%). The most common protein abnormalities were spectrin and band 3 deficiencies in both non-splenectomized and splenectomized patients. The membrane protein defect was undetectable in nine (7%) non-splenectomized subjects, seven of whom had a positive family history of hereditary spherocytosis; the remaining two displayed mild anemia, reticulocytosis and 5% spherocytes, in the absence of other causes of chronic hemolytic anemia.

Table 2 compares the sensitivity of the diagnostic laboratory tests for hereditary spherocytosis. EMA-binding failed to identify 10/150 (7%) cases (4 with band 3 deficiency, 5 with spectrin deficiency and 1 with an undetected defect). The mean decrease in fluorescence was 27% ± 10% in patients with hereditary spherocytosis *versus* 0 % ± 8% in reference subjects ($P<0.001$). The percent fluorescence reduction was directly related to the number of spherocytes and indirectly with the mean corpuscular volume; no correlation was found with hemoglobin concentration, absolute number of reticulocytes or red cell distribution width. The test's sensitivity was independent of the type and amount of molecular defect, although it was slightly lower in patients with undetected defects who had the smallest median decrease in fluorescence (15% *versus* 30% and 28% in band 3 and spectrin deficiency, respectively). It is worth noting that the sensitivity of the EMA-binding test was slightly higher in splenectomized than in non-splenectomized patients (Figure 1).

The sensitivity of the various red cell fragility assays investigated was very variable and generally higher in splenectomized than in non-splenectomized hereditary spherocytosis, and lower (with the exception of AGLT) in the patients with undetected defects than in those with detectable defects (Table 2A). As regards the NaCl osmotic fragility test, the sensitivity was greater when performed on incubated blood than on fresh blood. Overall, NaCl osmotic fragility tests (both on fresh and incubated blood) had a lower sensitivity than the more commonly used glycerol-based tests. Among these latter assays, AGLT had the highest sensitivity, also in the cases with undetected defects, comparable to that of the EMA-binding test.

All patients with hereditary spherocytosis were positive to at least two different tests with the exception of two patients (1 with band 3 deficiency and 1 with spectrin

Table 1. Hematologic and biochemical data of patients with hereditary spherocytosis grouped according to whether they had or had not been splenectomized.

	Not splenectomized (n=128)				Splenectomized (n=22)
	Severe to moderate anemia (n= 32)	Mild anemia (n=48)	Compensated anemia (n=48)	All (n=128)	
Hematologic parameters[a]					
Hemoglobin (g/dL)	9 (5.5-10)	11.3 (10.2-13)	13.8 (11.7-16.2)	11.4 (5.5-16.2)	14.9 (11.2-18.8)
Mean cell volume (fL)	77 (65.6-105)	84 (62.5-105)	88 (83-104)	85.5 (62.5-123)	87 (82.1-96.8)
MCHC (g/dL)	33.25 (31.4-37.7)	35.25 (30.4-38.3)	35.3 (27.9-37.7)	34.8 (27.9-38.4)	35.5 (31.5-37.1)
Spherocytes (%)	8 (1-28)	7 (1-23)	9 (1-43)	8 (1-43)	8 (2-60)
Reticulocytes ($\times 10^9$/L)	40 (52-463)	252 (19-579)	180 (34-512)	222 (19-579)	116.5 (41-415)
Unc. bilirubin (mg/dL)	2.3 (0.1-22.2)	1.75 (0.4-9.23)	2.6 (0.5-9.9)	2.5 (0.1-22.2)	0.71 (0.24-4.7)
Defective protein[b]					
Band 3	11	23	17	51	8
Spectrin	16	21	25	62	11
Combined spectrin-ankyrin	4	0	2	6	3
Undetected	1	4	4	9	0

[a]*median values (range);* [b]*number of cases; MCHC: mean cell hemoglobin concentration; Unc.: unconjugated.*

deficiency) who were positive only in the EMA-binding test. We found that the combination of EMA and AGLT enabled identification of all the patients with hereditary spherocytosis (Table 2B). In particular, 133/150 (88%) of hereditary spherocytosis cases were EMA-positive, AGLT-positive, 7/150 (5%) were EMA-positive, AGLT-negative and 10/150 (7%) were EMA-negative, AGLT-positive.

When the performance of the various tests was analyzed in non-splenectomized patients as a function of the clinical phenotype, EMA-binding, the AGLT and the Pink test maintained high sensitivity in the different clinical subsets, whereas the sensitivity of the NaCl osmotic fragility test on fresh blood and after incubation dropped markedly in compensated cases (Figure 1).

The results of various tests in a series of 84 patients with hemolytic conditions other than hereditary spherocytosis are depicted in Figure 2. EMA-binding showed the greater disease specificity being negative in all patients with autoimmune hemolytic anemia, even in the presence of marked spherocytosis.

Discussion

This is the first extensive comparison study of the most currently used laboratory methods for diagnosing hereditary spherocytosis, carried out on a large number of patients grouped according to the molecular defect, the degree of hemolysis and the presence or absence of the spleen. The finding that half of the examined patients had mild/compensated anemia and were, therefore, more difficult to diagnose, and that 18% had very few spherocytes on peripheral blood smear, makes the population examined particularly suitable for sensitivity studies. We did not include the cryohemolysis test[17] in this study since the basis of the susceptibility of hereditary spherocytosis red blood cells to cooling has not been elucidated so far, and opinions on its routine utilization for the diagnosis of hereditary spherocytosis are controversial.[4,5,16,17,19,37,38]

Among the diagnostic methods considered, the recently proposed EMA-binding test is certainly the most interesting one, and it is being increasingly used by specialized laboratories because of its high sensitivity and specificity.[12,18,23-29] This method directly targets the structural lesion of the disease, since the fluorescent probe eosin-5'-maleimide interacts with transmembrane proteins band 3, Rh protein, Rh glycoprotein and CD47 which are reduced in red cells from patients with hereditary spherocytosis;[30] defects of other cytoskeletal proteins, such as spectrin and protein 4.2, also induce a decrease in fluorescence intensity, likely because they create a long-range modulation effect on the dye binding site in band 3 protein.[39] The sensitivity of the EMA-binding test in this series is higher than that recently reported by Crisp *et al.*,[12] and similar to

Table 2. (A) Sensitivity of single tests in patients with hereditary spherocytosis (HS) grouped according to the biochemical defect. (B) The sensitivity of combined tests in total HS cases. The number represents the ratio of positive cases/total cases with percent values in brackets.

A

	EMA-binding	GLT	AGLT	Pink	OF (NaCl fresh)	OF (NaCl inc.)
Total HS patients	140/150 (93%)	92/150 (61%)	143/150 (95%)	136/150 (91%)	102/150 (68%)	122/150 (81%)
HS with biochemical defect	132/141 (94%)	90/141 (64%)	135/141 (96%)	131/141 (93%)	100/141 (71%)	119/141 (84%)
Spectrin	68/73 (93%)	45/73 (61%)	70/73 (96%)	67/73 (92%)	51/73 (70%)	62/73 (85%)
Band 3	55/59 (93%)	38/59 (64%)	56/59 (93%)	55/59 (93%)	43/59 (73%)	49/59 (83%)
Combined spectrin/ankyrin	9/9 (100%)	7/9 (78%)	9/9 (100%)	9/9 (100%)	6/9 (67%)	8/9 (89%)
HS with undetectable defect	8/9 (88%)	2/9 (22%)	8/9 (88%)	2/9 (22%)	3/9 (33%)	4/9 (44%)

B

	EMA + AGLT	EMA + OF (NaCl fresh)	EMA + OF (NaCl inc.)	EMA + Pink	OF (NaCl inc.) + AGLT
Total HS patients	150/150 (100%)	143/150 (95%)	143/150 (95%)	149/150 (99%)	146/150 (97%)

OF: osmotic fragility; Inc.: incubated.

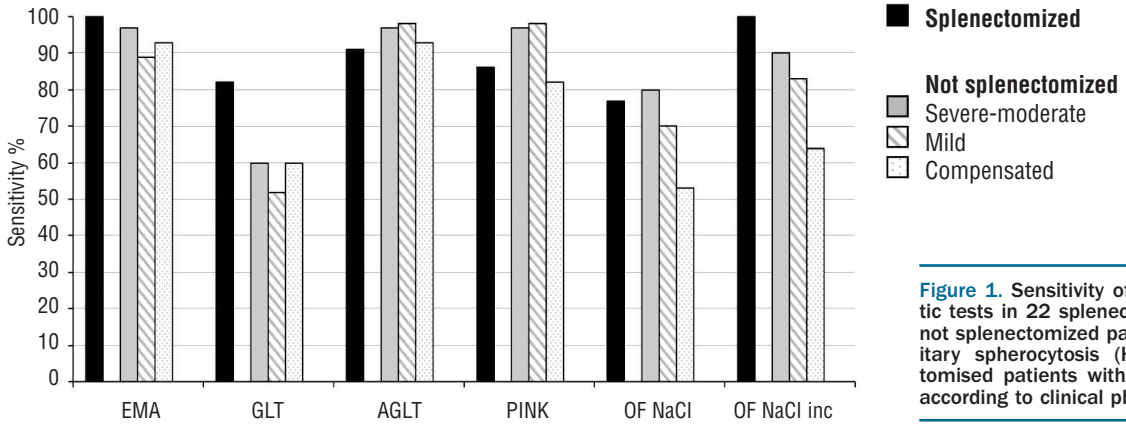

Figure 1. Sensitivity of various diagnostic tests in 22 splenectomized and 128 not splenectomized patients with hereditary spherocytosis (HS). Non-splenectomised patients with HS were divided according to clinical phenotype.

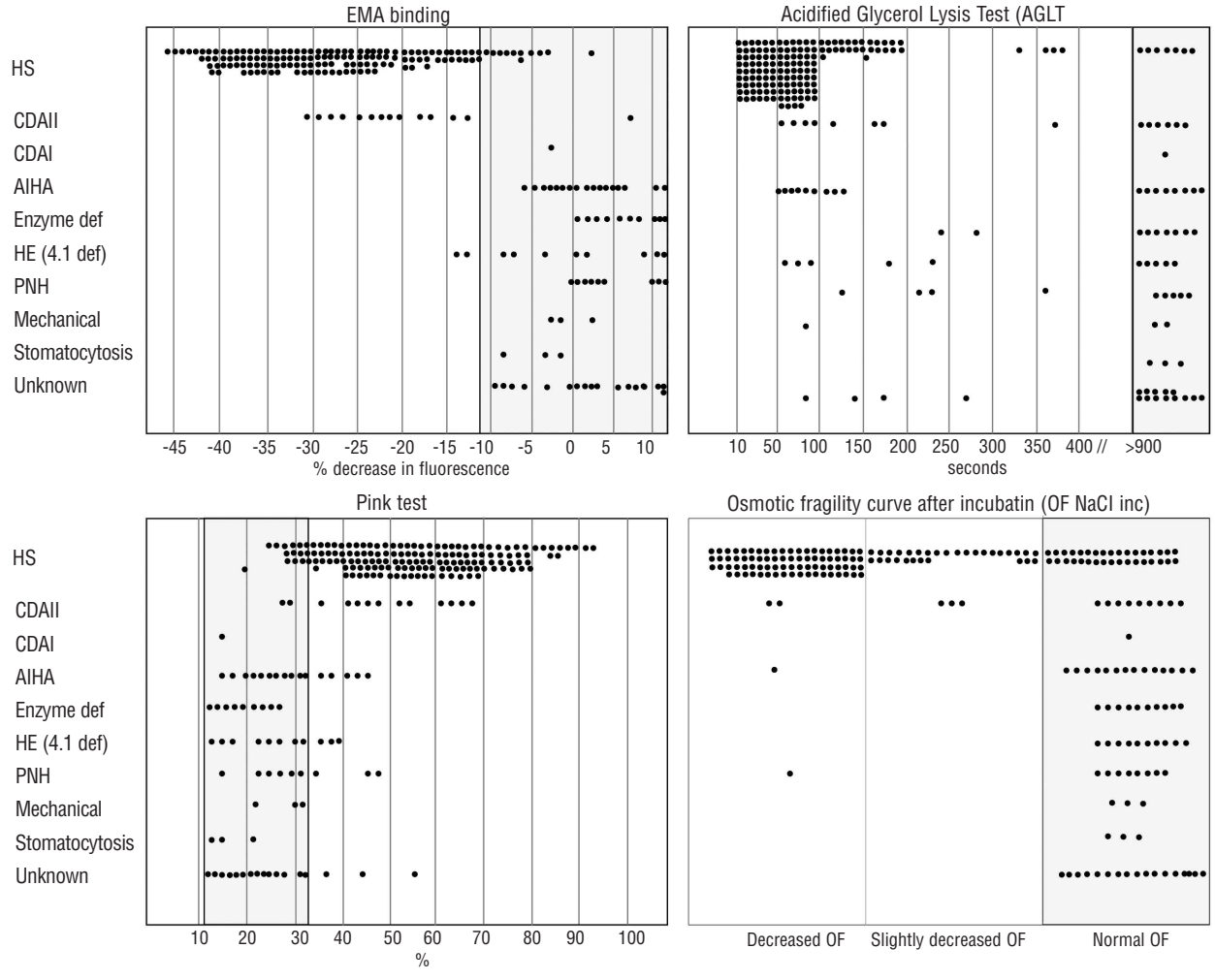

Figure 2. Results of individual diagnostic tests in patients with hemolytic anemias other than hereditary spherocytosis (HS), compared with those with HS. The shaded area represents normal reference intervals. CDA: congenital dyserythropoietic anemia; AIHA: autoimmune hemolytic anemia; HE: hereditary elliptocytosis; PNH: paroxysmal noctural hemoglobinuria

that found by others;[18,23-29] moreover, the test's performance appears to be independent of the type of red cell membrane protein deficiency, and decreases only slightly in hereditary spherocytosis patients with an undetectable defect, in line with what was observed by King *et al.*,[18] and Girodon *et al.*[25] Interestingly, sensitivity is independent of clinical phenotype, being high also in patients with compensated anemia. The separate analysis of non-splenectomized and splenectomized patients with hereditary spherocytosis showed that sensitivity increased in the latter group, a finding generally common to all the tests investigated; this observation pinpoints the need to define the clinical characteristics of patients precisely when testing the performance of diagnostic methods for hereditary spherocytosis, and possibly to limit the analysis to non-splenectomized subjects. A further advantage of the EMA-binding test is that results are not influenced by shipping or storage for up to 6 days, as shown in Figure 3, thus enabling shipping of samples as reported by Girodon *et al.*[25] Moreover, results are not affected by recent transfusions since the method discriminates different red cell populations.[20]

As regards the NaCl osmotic fragility tests, we found that these failed to identify nearly a quarter of patients with hereditary spherocytosis, confirming results from other researchers,[12,13,19,40,41] and that their sensitivity was generally lower than that of the other diagnostic laboratory tests evaluated in this series. In spite of this, incubated NaCl osmotic fragility is still commonly considered the gold standard for diagnosing hereditary spherocytosis in patients with Coombs'-negative hemolytic anemias.[5,11,42] Indeed, the analysis of the literature reveals that no systematic studies on sensitivity of this test have been performed in the past, and that the assertion that it is the best method for the diagnosis of hereditary spherocytosis is based on studies carried out on limited numbers of patients with not clearly defined clinical characteristics; moreover, the interpretation of the NaCl osmotic fragility curves may be difficult in less typical cases.[8] The high number of patients considered in this series enabled us to correlate the performance of the NaCl osmotic fragility test with the clinical expression of the disease: the observation that in cases of compensated hereditary spherocytosis the sensitivity of osmotic fragility tests decreased to nearly 60%, much more than reported by Korones & Pearson,[13] further limits the utility of this method in the mildest and less typical cases. This is also confirmed by the finding that the sensitivity drops to 30% in patients

with hereditary spherocytosis with an undetectable biochemical defect.

The glycerol-based red cell fragility tests, with the exception of the original version GLT, are more sensitive than the NaCl osmotic fragility test; in particular, in this series, the AGLT had a sensitivity of 95%, similar to that found by others[1,15,43,44] and higher than that reported by Cynober *et al.*[10] and Bucx *et al.*[45] The sensitivity of the AGLT was also high in compensated cases and in those with an undetected biochemical defect. Moreover, it is worth noting that the AGLT identified the ten EMA-negative cases of hereditary spherocytosis.

The association of EMA-binding and the AGLT enabled identification of all the cases of hereditary spherocytosis in this series; since flow cytometer is not available in all diagnostic laboratories, it is worth mentioning that the AGLT plus the NaCl osmotic fragility test on incubated blood raises the diagnostic sensitivity to 97%, similar to that previously reported in a larger series of patients[4]: in any cases, this value is higher than that obtained by combining EMA-binding and the cryohemolysis test, as recently reported by Crisp *et al.*[12]

The disease specificity of the diagnostic tests for hereditary spherocytosis was evaluated by including a large group of patients with different types of hemolytic anemia that may show morphological and laboratory features similar to those of hereditary spherocytosis. As expected, the results of EMA-binding, in terms of percent fluorescence reduction, were directly related to the number of spherocytes only in patients with hereditary spherocytosis, but not in patients with autoimmune hemolytic anemia, even in those with marked spherocytosis: this observation is in line with the high disease specificity of this test reported by others.[19-21,25] We found that the other assays, in particular the glycerol-based ones, are less spe-

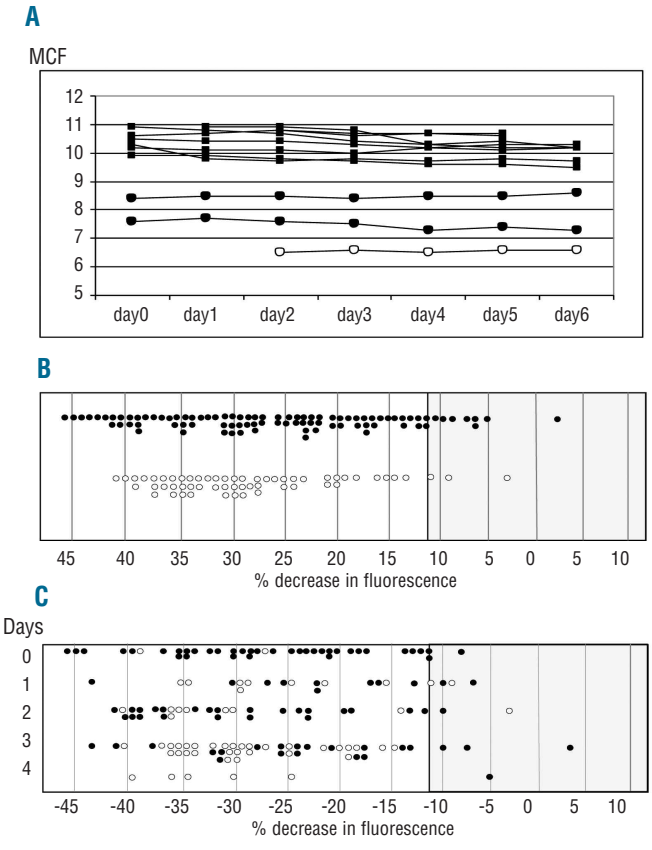

Figure 3. (A) Mean channel fluorescence values in normal controls and patients with hereditary spherocytosis (HS) at different days of storage. (B-C) Results of EMA-binding test in samples from 150 HS patients grouped according to shipping (B) and days of storage before testing (C). ■ Controls, ● HS not shipped, ○ HS shipped.

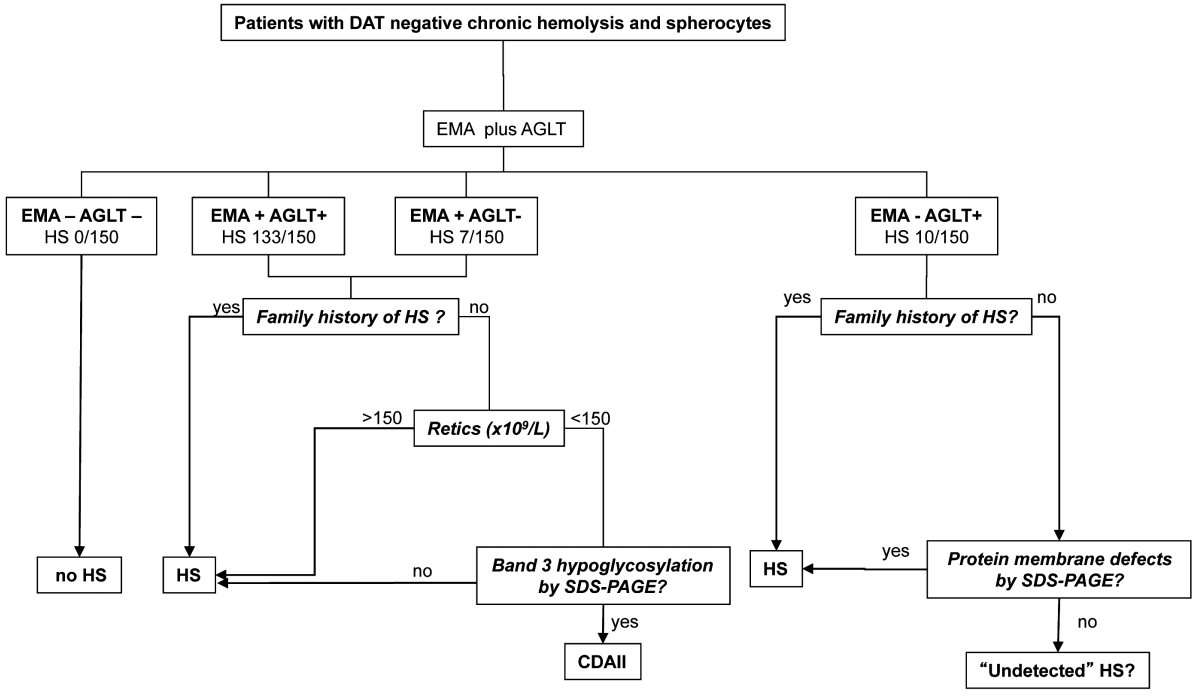

Figure 4. Flow chart for laboratory diagnosis of hereditary spherocytosis (HS).

Exclusion of other causes such as enzyme defects, sphero-stomatocytosis, atypical CDA, Coombs neg AIHA.

cific than EMA-binding, since, as reported previously,[8,9,14,15,46] they are often positive also in acquired hemolytic anemias. It is worth mentioning that none of the available diagnostic tests for hereditary spherocytosis, whether direct or indirect, differentiates hereditary spherocytosis from congenital dyserythropoietic anemia type II. This latter disorder, although less prevalent than hereditary spherocytosis, may mimic the clinical presentation, red cell morphology and increased red cell osmotic fragility of hereditary spherocytosis and may require SDS-PAGE analysis to be identified: Mariani *et al.* reported that 13% of cases referred to a reference laboratory with the provisional label of hereditary spherocytosis were found to be congenital dyserythropoietic anemia type II when examined by SDS-PAGE.[47]

The diagnostic guidelines for hereditary spherocytosis from the British Committee for Standards in Hematology,[19] the only so far available, recommend either EMA-binding or the cryohemolysis test as a screening method,[16] the deciding factor for the choice being the availability of a flow cytometer. The guidelines do not specify whether, in the case of equivocal or borderline results, both tests should be performed. In any case, even the association of these two tests gives a sensitivity of 93%, similar to that of EMA-binding or AGLT used alone, and much lower than that obtained by the combination of EMA-binding plus AGLT.

As seen in Figure 4, in the presence of a patient with DAT-negative chronic hemolysis with spherocytes, the negativity of both EMA and AGLT enables hereditary spherocytosis to be excluded. Positivity of EMA-binding (with either a positive or negative AGLT) leads to the diagnosis of hereditary spherocytosis except in those non-dominant cases with inadequate reticulocytosis[11] which need SDS-PAGE analysis to exclude congenital dyserythropoietic anemia type II. SDS-PAGE analysis may also be required in the rare EMA-negative, AGLT-poistive cases with a negative family history, due to the lesser disease specificity of AGLT.

In conclusion, no single test is able to identify all cases of hereditary spherocytosis. The association of EMA-binding, which directly targets the structural defect of hereditary spherocytosis, and AGLT, which exploits the red cell surface area-to-volume ratio, allowed us to identify all the patients with hereditary spherocytosis in this series and, therefore, represents a very effective diagnostic strategy for hereditary spherocytosis also in mild/compensated cases. However, it must be underlined that the diagnosis of hereditary spherocytosis is the final step of a diagnostic work-up based not only on laboratory tests but also on clinical examination, personal family history, and the exclusion of possible causes of secondary spherocytosis.

Authorship and Disclosures

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