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

by Paola Bianchi, Elisa Fermo, Cristina Vercellati, Anna P. Marcello, Laura Porretti, Agostino Cortelezzi, Wilma Barcellini, and Alberto Zanella

Haematologica 2011 [Epub ahead of print]

Citation: Bianchi P, Fermo E, Vercellati C, Marcello AP, Porretti L, Cortelezzi A, Barcellini W, and Zanella A. Diagnostic power of laboratory tests for hereditary spherocytosis: a comparison study on 150 patients grouped according to the molecular and clinical characteristics. Haematologica. 2011; 96:xxx

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.

Haematologica (pISSN: 0390-6078, eISSN: 1592-8721, NLM ID: 0417435, www.haematologica.org) publishes peer-reviewed papers across all areas of experimental and clinical hematology. The journal is owned by the Ferrata Storti Foundation, a non-profit organization, and serves the scientific community with strict adherence to the principles of open access publishing (www.doaj.org). In addition, the journal makes every paper published immediately available in PubMed Central (PMC), the US National Institutes of Health (NIH) free digital archive of biomedical and life sciences journal literature.

Support Haematologica and Open Access Publishing by becoming a member of the European Hematology Association (EHA) and enjoying the benefits of this membership, which include participation in the online CME program.
Diagnostic power of laboratory tests for hereditary spherocytosis: a comparison study on 150 patients grouped according to the molecular and clinical characteristics

Running title: Diagnostic power of laboratory tests for HS

Paola Bianchi,1 Elisa Fermo, 1 Cristina Vercellati,1 Anna P. Marcello, 1 Laura Porretti, 2 Agostino Cortezezzi, 1,3 Wilma Barcellini1, and Alberto Zanella1

1U.O. Ematologia 2, Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico, Milan, Italy; 2Centro di Medicina Trasfusionale, Terapia Cellulare e Criobiologia, Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico, Milano, Italy, and 3U.O. Ematologia 1 e Centro Trapianti di Midollo, Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico and Università degli Studi di Milano, Milan, Italy

Correspondence
Paola Bianchi, Hematology 2 Unit, Fondation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Via Francesco Sforza, 35, 20122 Milan, Italy. E-mail:paola.bianchi@policlinico.mi.it

Funding
This work was supported by a grant from Foundation IRCCS Ca’ Granda Ospedale Maggiore Policlinico of Milan, RC2009 160/01.
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 (EMA-binding test) has been proposed. None of the available tests identify all hereditary spherocytosis cases.

**Design and methods:** We compared the performances of EMA-binding, NaCl-osmotic fragility on fresh and incubated blood, glycerol lysis test, acidified glycerol lysis test (AGLT), and Pink test on a series of 150 patients with hereditary spherocytosis grouped according to the clinical phenotype and the defective protein, with the final aim to find the combination of tests associated with the highest diagnostic power, even in the mildest cases.

**Results:** EMA-binding displayed 93% sensitivity and 98% specificity: sensitivity was independent of the type and amount of molecular defect and of clinical phenotype. A comparable sensitivity was shown by AGLT (95%) and Pink test (91%). The sensitivity of NaCl osmotic fragility tests, commonly considered the gold standard for diagnosis of hereditary spherocytosis, was 68% on fresh and 81% on incubated blood, and further decreased in compensated cases (53% and 64%). The combination of EMA and AGLT enabled to identify the totality of patients. EMA-binding test shows the greater disease specificity.

**Conclusions:** Each individual test fails to diagnose a portion of cases. The association of EMA-binding and AGLT allowed to identify all the hereditary spherocytosis patients of this series and therefore represents at present an effective diagnostic tool for hereditary spherocytosis also in mild/compensated cases.
INTRODUCTION

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

The typical laboratory hallmark of HS, although not specific, is the presence of spherocytes on peripheral blood smear, which are detectable in 97\% of patients.\textsuperscript{4} However, spherocytes may be very few in several patients\textsuperscript{4,5} requiring skilled operators to be detected; moreover, the microscopic examination of red cells is increasingly omitted in an era of laboratory automation. The laboratory diagnosis of HS therefore commonly relies on tests that exploit the surface area-to-volume ratio, typically reduced in spherical-shaped erythrocytes, in particular the red cell osmotic fragility (OF) tests at various sodium chloride (NaCl) concentrations on fresh and incubated blood,\textsuperscript{6} and assays that measure the extent or the rate of lysis of red cells suspended in buffered glycerol solutions, i.e. Glycerol Lysis (GLT),\textsuperscript{7} Acidified Glycerol Lysis (AGLT)\textsuperscript{8} and Pink test.\textsuperscript{9} However, these tests miss a variable portion of HS cases, particularly the mildest ones,\textsuperscript{6,10-12} and do not differentiate HS from secondary spherocytosis associated with other conditions, mainly autoimmune hemolytic anemias.\textsuperscript{13-15}

More recently, the cryohemolysis test,\textsuperscript{16,17} based on the observation that HS red cells are particularly susceptible to cooling at 0\degree C in hypertonic conditions, and the flow cytometric analysis of eosin-5\'-maleimide-labelled intact red blood cells (EMA-binding test)\textsuperscript{18} have been proposed as new methods of identifying HS.\textsuperscript{19} The latter in particular has been proven to be a sensitive and specific diagnostic tool for HS\textsuperscript{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.\textsuperscript{30}

The performance of the available direct or indirect diagnostic tests has been mostly evaluated individually and on limited number of cases: in particular, sensitivity is greatly variable, and each method fails to identify several HS patients.\textsuperscript{4,6,12} In this study, we compared the performances of EMA-binding, NaCl OF on fresh and incubated blood, GLT, AGLT and Pink tests on a series of 150 HS patients grouped according to the clinical phenotype and the molecular lesion, with the final aim to find the combination of tests associated with the highest diagnostic power towards HS, included the mildest cases which are of more difficult diagnosis.
DESIGN AND METHODS

Subjects.
150 consecutive HS patients (79 males and 71 females, median age 26 yrs, range 0-79 yrs) 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, bilirubin and haptoglobin concentration, iron status, direct antiglobulin test (DAT), screening for abnormal or unstable hemoglobins, NaCl OF 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-direct antiglobulin test (MS-DAT)\(^3\) was also performed to rule out the diagnosis of DAT-negative hemolytic anemia.

HS 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 tests, family history of HS if any, and exclusion of other causes of secondary spherocytosis.\(^1\) 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).

All patients underwent SDS-PAGE analysis of the red cell membrane proteins and were divided into the following groups: deficiency of band 3, spectrin, ankyrin or combined spectrin/ankyrin, and no detectable defect.\(^4\)

Five-hundred seventy five healthy blood donors and 84 hemolytic anaemias other than HS (17 autoimmune hemolytic anemia (AIHA), 10 erythroenzymopaties, 10 hereditary elliptocytosis (HE), 15 congenital dyserythropoietic anemia (CDA), 9 paroxysmal nocturnal hemoglobinuria (PNH), 3 stomatocytosis, 3 mechanical, 17 unknown) 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 hrs. All tests were performed in a single site. None of the patients had been transfused within the three months preceding the study. Hematologic parameters were determined on an automated hematology analyzer (Automatic Beckman Coulter LH-750, CA, USA). Routine
hematological investigations were carried out according to Dacie & Lewis. Bilirubin, aptoglobin, and ferritin 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 performing on each patient the following tests: NaCl OF test on both fresh and incubated blood, standard glycerol lysis test (GLT), acidified glycerol lysis test (AGLT) and Pink test.

EMA-binding test was performed as described by King et al. with minor modifications. In particular, fluorescence intensity, expressed as median channel fluorescence (MCF), 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). 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 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 compared to the mean fluorescence of the six normal controls as proposed by Girodon et al. Receiver Operating Characteristic (ROC) curve based on logistic regression analysis was used to determine the optimum cut-off between HS and normal individuals. According to the ROC curve analysis, the optimum decrease in fluorescence to separate normal subjects from HS patients was 11%. In these conditions test specificity computed on 575 healthy subjects was 98%.

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

Table 1 shows the hematologic and biochemical data of HS patients 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 only). The majority of non-splenectomized subjects displayed 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 9 (7%) non-splenectomized subjects, seven of whom had a positive family history of HS; 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 HS. EMA-binding failed to identify 10/150 (7%) cases (4 band 3 deficiency, 5 spectrin deficiency and 1 “undetected”). The mean decrease in fluorescence was 27% ± 10% in HS vs 0 % ± 8% in reference subjects (P<0.001). The percent fluorescence reduction was directly related to the spherocyte number and indirectly with MCV; no correlation was found with either hemoglobin, reticulocyte absolute number and red cell distribution width (RDW). The test’s sensitivity was independent of the type and amount of molecular defect, although it was slightly lower in “undetected” patients that displayed the mildest median decrease in fluorescence (15% vs 30% and 28% in band 3 and spectrin deficiency respectively). It is worth noting that the EMA-binding test sensitivity was slightly higher in splenectomized than in non-splenectomized patients (Figure 1).

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

All HS patients were positive to at least two different tests with the exception of two (one band 3 and one spectrin deficient cases) who were EMA-binding positive only. We found that the combination of EMA and AGLT enabled to identify the totality of HS patients (Table 2B). In particular, 133/150 (88%) of HS cases tested EMA+ AGLT+, 7/150 (5%) EMA+ AGLT- and 10/150 (7%) EMA- AGLT+. 

DOI: 10.3324/haematol.2011.052845
When the performance of various tests in not splenectomized patients was analyzed as a function of the clinical phenotype, EMA binding, AGLT and Pink test maintained high sensitivity in the different clinical subsets, whereas the sensitivity of NaCl OF test on fresh blood and after incubation markedly dropped in compensated cases (Figure 1).

The results of various tests on a series of 84 patients with hemolytic conditions other than HS are depicted in Figure 2. EMA-binding shows the greater disease specificity being negative in all patients with AIHA, even in the presence of marked spherocytosis.
DISCUSSION

This is the first extensive comparison study of the most currently used laboratory methods for diagnosing HS, 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 displayed 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 didn’t include in this study the cryohemolysis test, since the basis of HS red cells susceptibility to cooling has not been so far elucidated, and opinions on its routine utilization for diagnosis of HS are controversial. Among the diagnostic methods considered, the recently proposed EMA-binding test is certainly the most interesting one, and it is increasingly used by specialized laboratories for its high sensitivity and specificity. 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 HS red cells; defects of other cytoskeletal proteins, such as spectrin and protein 4.2, induce as well a decrease in fluorescence intensity likely because they create a long-range modulation effect on the dye binding site in band 3 protein. The sensitivity of EMA-binding test found in this series is higher than recently reported by Crisp et al., and similar to that found by others; moreover, the test’s performance appears to be independent of the type of red cell membrane defective protein, and decreases only slightly in HS patients with undetectable defect, in line with what observed by King et al. and Girodon et al. Interestingly, sensitivity is independent of clinical phenotype, being high also in patients with compensated anemia. The separate analysis of non-splenectomized and splenectomized HS patients showed that sensitivity increased in the latter group, a finding generally common to all the tests investigated; this observation pinpoints to the need to precisely define the clinical characteristics of patients when testing the performance of diagnostic methods for HS, and possibly to limit the analysis to non-splenectomized subjects. A further advantage of EMA-binding test is that results are not influenced by shipping or storage up to 6 days as shown in Figure 3, thus enabling shipping of samples as reported by Girodon et al. Moreover, results are not affected by recent transfusions since the method discriminates different red cell populations. As regards the NaCl OF tests, we found that they miss nearly a quarter of HS patients, in agreement with what observed by others, and that their sensitivity is generally lower than that of the other diagnostic laboratory tests evaluated in this series. In spite of this, incubated NaCl OF is still
commonly considered the gold standard in diagnosing HS in patients with Coombs-negative hemolytic anemias.\textsuperscript{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 tool for the diagnosis of HS is based on studies carried out on limited number of patients with not clearly defined clinical characteristics; moreover, the interpretation of the NaCl OF curves may be difficult in less typical cases.\textsuperscript{8} The high number of patients considered in this series enabled us to correlate the performance of NaCl OF test with the clinical expression of the disease: the observation that in compensated HS cases the sensitivity of OF tests decreased to nearly 60\%, much more than reported by Korones & Pearson,\textsuperscript{13} further limits the utility of this method in mildest and less typical cases. This is also confirmed by the finding that the sensitivity drops to 30\% in HS patients with undetectable biochemical defect.

The glycerol-based red cell fragility tests, with the exception of the original version GLT, are more sensitive than NaCl OF; in particular AGLT displayed in this series 95\% sensitivity, similarly to what found by others\textsuperscript{1,15,43,44} and higher than reported by Cynober et al.\textsuperscript{10} and Bucx et al.\textsuperscript{45} The AGLT’s sensitivity was high also in compensated and biochemically undetected cases. Moreover, it is worth noting that AGLT allowed to identify the 10 EMA-negative HS.

The association of EMA-binding and AGLT enabled to identify all HS cases in this series; since flow cytometer is not available in all diagnostic laboratories, it is worth mentioning that AGLT plus incubated NaCl OF raises the sensitivity to 97\%, similarly to what previously reported in a greater series of patients\textsuperscript{4}: this value is in any case higher than that obtained by combining EMA-binding and cryohemolysis test, as recently reported by Crisp et al.\textsuperscript{12}

The disease specificity of the diagnostic tests for HS has been evaluated by including in the study a large group of various hemolytic anemias that may show morphological and laboratory features similar to HS. As expected, the results of EMA-binding, in terms of percent fluorescence reduction, were directly related with the number of spherocytes in HS patients only, but not in AIHA, even in those with marked spherocytosis: this observation is in line with the high disease specificity of this test reported by others.\textsuperscript{19-21,25} We found that the other assays, in particular the glycerol-based ones, are less specific than EMA-binding, being often positive also in acquired hemolytic anemias, as previously reported.\textsuperscript{8,9,14,15,46} It is worth mentioning that none of the available diagnostic tests for HS, neither direct or indirect, differentiates HS from CDAII. This latter, although less prevalent than HS, may mimics HS both in terms of clinical presentation, red cell morphology and increased red cell osmotic fragility, 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 HS were found to be CDAII when examined by SDS-PAGE.\textsuperscript{47}
The diagnostic guidelines for HS of the British Committee for Standards in Hematology, the only so far available, recommend as screening method either the EMA binding or the criohemolysis test, the deciding factor for choice being the availability of a flow cytometer. The guidelines do not specify if, in 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 EMA-binding/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 permits the exclusion of HS. The positivity of EMA (either with positive or negative AGLT) leads to the diagnosis of HS with the exception of those non dominant cases with inadequate reticulocytosis that needs SDS-PAGE analysis to exclude CDAII. SDS-PAGE analysis may also be required in the rare EMA-AGLT+ cases with negative family history, due to the less disease specificity of AGLT.

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

Authorship and Disclosures

PB and EF performed the study and wrote the paper. CV, APM and LP performed the laboratory tests for this study; WB recruited, followed-up the patients and revised the paper. AZ and AC revised the paper and co-ordinated the research.

The authors reported no potential conflicts of interest.
REFERENCES


<table>
<thead>
<tr>
<th></th>
<th>NON SPLENECTOMIZED (n=128)</th>
<th>SPLENECTOMIZED (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Severe to moderate anemia (n=32)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>9 (5,5-10)</td>
<td>11,3 (10,2-13)</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>77 (65,6-105)</td>
<td>84 (62,5-105)</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>33,25 (31,4-37,7)</td>
<td>35,25 (30,4-38,3)</td>
</tr>
<tr>
<td>Spherocytes (%)</td>
<td>8 (1-28)</td>
<td>7 (1-23)</td>
</tr>
<tr>
<td>Reticulocytes (x10^9/L)</td>
<td>240 (52-463)</td>
<td>252 (19-579)</td>
</tr>
<tr>
<td>Unc. bilirubin (mg/dL)</td>
<td>2,3 (0,1-22,2)</td>
<td>1,75 (0,4-9,23)</td>
</tr>
<tr>
<td><strong>Mild anemia (n=48)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>13,8 (11,7-16,2)</td>
<td>11,4 (5,5-16,2)</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>88 (83-104)</td>
<td>85,5 (62,5-123)</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>35,3 (27,9-37,7)</td>
<td>34,8 (27,9-38,4)</td>
</tr>
<tr>
<td>Spherocytes (%)</td>
<td>9 (1-43)</td>
<td>8 (1-43)</td>
</tr>
<tr>
<td>Reticulocytes (x10^9/L)</td>
<td>180 (34-512)</td>
<td>222 (19-579)</td>
</tr>
<tr>
<td>Unc. bilirubin (mg/dL)</td>
<td>2,6 (0,5-9,9)</td>
<td>2,5 (0,1-22,2)</td>
</tr>
<tr>
<td><strong>Compensated anemia (n=48)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>11,4 (5,5-16,2)</td>
<td>14,9 (11,2-18,8)</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>85,5 (62,5-123)</td>
<td>87 (82,1-96,8)</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>34,8 (27,9-38,4)</td>
<td>35,5 (31,5-37,1)</td>
</tr>
<tr>
<td>Spherocytes (%)</td>
<td>8 (1-43)</td>
<td>8 (2-60)</td>
</tr>
<tr>
<td>Reticulocytes (x10^9/L)</td>
<td>222 (19-579)</td>
<td>116,5 (41-415)</td>
</tr>
<tr>
<td>Unc. bilirubin (mg/dL)</td>
<td>2,5 (0,1-22,2)</td>
<td>0,71 (0,24-4,7)</td>
</tr>
<tr>
<td><strong>All (n=128)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>11,4 (5,5-16,2)</td>
<td>14,9 (11,2-18,8)</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>85,5 (62,5-123)</td>
<td>87 (82,1-96,8)</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>34,8 (27,9-38,4)</td>
<td>35,5 (31,5-37,1)</td>
</tr>
<tr>
<td>Spherocytes (%)</td>
<td>8 (1-43)</td>
<td>8 (2-60)</td>
</tr>
<tr>
<td>Reticulocytes (x10^9/L)</td>
<td>222 (19-579)</td>
<td>116,5 (41-415)</td>
</tr>
<tr>
<td>Unc. bilirubin (mg/dL)</td>
<td>2,5 (0,1-22,2)</td>
<td>0,71 (0,24-4,7)</td>
</tr>
</tbody>
</table>

\*a median values (range)

\*b number of cases
Table 2. A: Sensitivity of single tests in HS patients grouped according to the biochemical defect. B: Combined tests’ sensitivity in total HS cases. Number represents the ratio of positive cases/total cases; in brackets percent values.

### A

<table>
<thead>
<tr>
<th>Test Combination</th>
<th>Total HS patients</th>
<th>HS with biochemical defect</th>
<th>HS with undetectable defect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EMA-binding</td>
<td>GLT</td>
<td>AGLT</td>
</tr>
<tr>
<td>EMA-binding</td>
<td>140/150 (93%)</td>
<td>92/150 (61%)</td>
<td>143/150 (95%)</td>
</tr>
<tr>
<td>GLT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGLT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pink</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OF NaCl fresh</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OF NaCl inc.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>Test Combination</th>
<th>Total HS patients</th>
<th>EMA + OF NaCl fresh</th>
<th>EMA + OF NaCl inc.</th>
<th>EMA + Pink</th>
<th>OF NaCl inc. + AGLT</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMA + AGLT</td>
<td>150/150 (100%)</td>
<td>143/150 (95%)</td>
<td>143/150 (95%)</td>
<td>149/150 (99%)</td>
<td>146/150 (97%)</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Sensitivity of various diagnostic tests in 22 splenectomised and 128 not splenectomised HS patients. Not splenectomised HS were divided according to the clinical phenotype.

Figure 2. Results of individual diagnostic tests in patients with haemolytic anaemias other than HS, compared with HS. The shadowed area represents normal reference intervals.

Figure 3. A) MCF values in normal controls and HS patients 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 HS.
% decrease in fluorescence

EMA binding

Acidified Glycerol Lysis Test (AGLT)

Pink Test

Osmotic Fragility curve after incubation (OF NaCl inc)

-45 -40 -35 -30 -25 -20 -15 -10 -5 0 5 10 seconds

10 50 100 150 200 250 300 350 400 >900

% Decreased OF Slightly decreased OF Normal OF

DOI: 10.3324/haematol.2011.052845
Patients with DAT negative chronic hemolysis and spherocytes

- EMA plus AGLT
  - EMA – AGLT –
    - HS 0/150
      - no HS
      - yes
  - EMA + AGLT+
    - HS 133/150
      - yes
      - Family history of HS?
      - yes
        - yes
          - Band 3 hypoglycosylation by SDS-PAGE?
          - yes
            - CDAII
          - no
        - no
      - no
    - HS 7/150
      - yes
      - “Undetected” HS?
      - yes
  - EMA – AGLT+
    - HS 10/150
      - yes
      - Protein membrane defects by SDS-PAGE?
      - yes
      - no

Exclusion of other causes as enzyme defect, spherostomatocytosis, atypical CDA, Coombs neg AIHA.