Clinical and hematologic features of 300 patients affected by hereditary spherocytosis grouped according to the type of the membrane protein defect

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ABSTRACT

Background
Hereditary spherocytosis is a very heterogeneous form of hemolytic anemia. The aim of this study was to relate the type of molecular defect with clinical and hematologic features and response to splenectomy using information from a large database of patients.

Design and Methods
Data from 300 consecutive patients with hereditary spherocytosis, grouped according to the results of sodium dodecyl sulphate-polyacrylamide gel electrophoresis, were analyzed and the sensitivity of red cell osmotic fragility tests was compared in various subsets of patients.

Results
Band 3 and spectrin deficiencies were the most common protein abnormalities (54% and 31%, respectively); 11% of cases were not classified by the electrophoretic analysis. Spectrin deficiency was more frequently diagnosed in childhood and band 3 deficiency in adulthood. Hemoglobin concentration was slightly lower, spherocyte number and hemolysis markers higher in spectrin deficiency than in band 3 deficiency. The sensitivity of the osmotic fragility tests ranged from 48% to 95%, and was independent of the type and amount of the membrane defect. The association of the acidified glycerol lysis test and the NaCl test on incubated blood reached a sensitivity of 99%. Splenectomy corrected the anemia in patients with all subtypes of hereditary spherocytosis although spectrin-deficient patients still showed increased reticulocyte numbers and levels of unconjugated bilirubin. Splenectomy allowed the identification of the membrane defect in all the previously unclassified patients, most of whom had spectrin and/or ankyrin deficiency.

Conclusions
The definition of the red cell membrane defect in hereditary spherocytosis has no major clinical implications, but may be useful for a differential diagnosis from other hematologic disorders that mimic this hemolytic anemia.

Key words: hereditary spherocytosis, erythrocyte membrane, SDS-PAGE, protein deficiencies, splenectomy.

Citation: Mariani M, Barcellini W, Vercellati C, Marcello AP, Fermo E, Pedotti P, Boschetti C, and Zanella A. Clinical and hematologic features of 300 patients affected by hereditary spherocytosis grouped according to the type of the membrane protein defect. Haematologica 2008; 93:1310-1317. doi: 10.3324/haematol.12546

Funding: this work was supported by a research grant from IRCCS OMPM-RE Foundation, n. RC2007, 160/04.

Manuscript November 23, 2007. Revised version arrived on April 23, 2008. Manuscript accepted May 26, 2008.

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Introduction

Hereditary spherocytosis (HS) is the most common congenital hemolytic anemia in Caucasians, with an estimated prevalence ranging from 1:2000 to 1:5000.[1-5] Approximately 75% of cases display an autosomal dominant pattern of inheritance, the remaining comprising recessive forms and *de novo* mutations.[3,6,7] The main clinical features of HS are hemolytic anemia, which can be from compensated to severe, sometimes requiring exchange transfusion at birth and/or repeated blood transfusions, variable jaundice, splenomegaly and gallstones.[8,9]

The molecular defect is highly heterogeneous, involving the genes encoding for spectrin, ankyrin, band 3 and protein 4.2.[10-12] The deficiency or dysfunction of any of these proteins, which are involved in the attachment of the cytoskeleton to the membrane integral domain, results in a loss of surface area and leads to spheroidal, osmotically fragile cells that are selectively trapped in the spleen.[13,14] The defective protein can be detected by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which allows the identification of different subsets of patients;[3,15,16] although some HS subjects remain unclassified by this technique;[16,17-19] efforts to identify the protein defect by genetic analysis in unclassified cases have been unsuccessful.[20]

It is usually thought that the clinical phenotype of spectrin deficiency is more severe than that of band 3 deficiency, and that the level of residual spectrin inversely correlates with the severity of anemia.[16,21,22] Consistently, the observation that spectrin/ankyrin-deficient red blood cells display lower maximal deformability than band 3 deficient ones, both before and after splenectomy, suggests more severe disease in the former.[23] Since information on the clinical and laboratory features of the most common HS defects has so far been referred to a small number of subjects, we analyzed data from 300 HS patients grouped according to the results of SDS-PAGE, to ascertain whether the clinical and hematologic features and the response to splenectomy are related to the type of molecular defect. We also compared the sensitivity of the most common laboratory screening tests for HS in various subsets of patients.

Design and Methods

Patients

Three hundred consecutive HS patients (141 males and 159 females, median age 20 years, range 1-80 years) belonging to 212 unrelated families from different Italian regions (109 from the north, 29 from the center, 62 from the south) and 12 from foreign countries were investigated. 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.

At the time of the study 121 patients were aged <18 years (40% of cases) and 179 were adults. Forty-one patients had been splenectomized before the time of the study, and 21 underwent splenectomy thereafter. Indications for splenectomy in the latter group were symptomatic anemia, or cholelithiasis and age <39 years.[24] In non-splenectomized cases the age at the time of the study corresponded to the age at diagnosis. As regards splenectomized patients, clinical and laboratory data referred to the time of the study.

Hereditary spherocytosis was diagnosed on the basis of the clinical history, physical examination and the results of laboratory tests: complete blood count, blood smear examination, reticulocyte count, bilirubin concentration, red blood cell osmotic fragility tests, and negative direct antiglobulin test.[25] Autohemolysis, screening for abnormal or unstable hemoglobins, Ham and sucrose hemolysis test and, in some cases, assay of the most important erythrocyte enzyme activities of the glycolytic and pentose phosphate pathways were also investigated. All patients underwent SDS-PAGE analysis of the red cell membrane proteins and were divided into different groups according to whether they had deficiency of band 3, spectrin, ankyrin, both spectrin and ankyrin, or band 4.2,[3] or no detectable defect. Patients splenectomized during the follow-up (21 cases) were re-evaluated after splenectomy. Anemia was defined as severe (Hb < 8 g/dL), moderate (Hb 8-10 g/dL) and mild (Hb > 10 and <11.5 g/dL for females and Hb > 10 and <13.5 g/dL for males).

Hematologic assays and iron status assessment

The great majority of samples were collected in our institute. The few samples collected from different centers in Italy were transported at a temperature of 4°C and always processed within 24 hours. Tests were performed in a single site. None of the patients had had transfusions within the 3 months preceding the study. Hematologic parameters were determined on an automated hematology analyzer (Automatic Beckman Coulter LH-750, CA, USA). The reference values for adults in our hospital were[26]: Hb 12.2-15.1 g/dL for females, 13.6-16.0 g/dL for males; mean corpuscular volume (MCV) 81-98 fL; mean cell hemoglobin content (MCHC) 31-36 g/dL; reticulocyte count 24-84×10^9/L; reference values for children were obtained from Nathan *et al.*[27] Bilirubin, serum iron, total iron binding capacity, and ferritin were determined using Integra 800 (Roche, Mannheim, Germany) (reference values: unconjugated bilirubin <12.8 IU, transferrin saturation 16-54%, serum ferritin 15-355 ng/mL).The number of spherocytes in peripheral blood was assessed indepently by two experts. For each patient, red cell osmotic fragility was evaluated by performing the following tests: NaCl osmotic fragility test on both fresh and incubated blood,[28] the standard glycerol lysis test,[29] the acidified glycerol lysis test[30] and the pink test.[31] The reference group consisted of 274 healthy blood donors giving blood for the first time;[30] moreover, since one normal sample (from a healthy donor) was always run together with samples from patients, the baseline reference values were periodically updated.

The cryohemolysis test[32] and the flow cytometric eosin-5-maleimide binding test,[33] recently recommended as screening tests for HS,[25] were performed in the last 33 consecutive patients. Activities of enzymes in the glycolytic and pentose phosphate pathways were assayed as reported elsewhere,[34] using the methods described by Beutler.[35]

Analysis of red cell membrane proteins

Red cell ghosts were prepared within 24 hours of blood collection using the method of Dodge *et al.*[36] with the following modifications: whole blood was collected in EDTA and centrifuged at 1,000 g for 10 min, plasma and buffy-coat were removed and red cells were filtered through a cellulose mixture made of α-cellulose and microcrystalline cellulose[37] equilibrated with phosphate-buffered saline [154 mM NaCl, 10 mM phosphate buffer, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), pH 7.4]. The lysis buffer was made of 5 mM phosphate buffer, 1 mM EDTA, 0.5 mM PMSF, pH 7.4. Free hemoglobin ghosts were stored frozen in aliquots after addition of 1% SDS and 5mM N-ethylmaleimide.[38] Red cell membrane proteins were analyzed, within 15 days of preparation of the ghosts, by SDS-PAGE using a 4% to 12% gradient of acrylamide according to Fairbanks *et al.*[39] and the discontinuous buffer system of Laemmli with an acrylamide linear gradient from 6% to 14%.[40] Each patient and control sample was loaded at least four times on each gel and the average densitometric reading from Coomassie blue stained gels was considered (GS-800 Calibrated Densitometer, Bio-Rad). Quantitation of bands was expressed as ratios to band 3. Spectrin and ankyrin deficiencies were defined as a ratio to band 3 below the lower limit of the reference range calculated from 100 normal subjects (reference ranges: spectrin/band 3: 0.95-1.21, ankyrin/band 3: 0.13-0.23). Band 3 deficiency was defined as a spectrin/band 3 ratio higher than the upper limit of the reference range (see above). Protein 4.2 deficiency was defined as an isolated, substantial decrease of the protein 4.2/band 3 ratio (<50% of the lower limit of the reference range: 0.12-0.18).

Statistical analysis

Continuous variables were analyzed as means ± standard deviation or as medians and range, whereas discrete variables were analyzed as percentages. χ^2 tests were performed on discrete variables, in order to study whether there were significant differences in clinical data between different groups of patients. When a large number of cells contained fewer than five subjects, a Monte Carlo χ^2 test[41] was performed, based on 1,000 simulations. Significant differences in mean values of continuous variables were assessed using a one-way ANOVA with protein defect as a predictor. A contrast test was used to evaluate whether significant differences in mean values were attributable to the two prevalent protein defects (band 3 or spectrin). T-tests were performed to analyze differences between splenectomized and non-splenectomized subjects. For all statistical tests, a p value <0.05 was considered statistically significant. Statistical analyses were performed using SAS version 8e.

Results

Clinical, hematologic and biochemical features of splenectomized and non-splenectomized hereditary spherocytosis patients

The inheritance pattern of HS was dominant in 210/300 patients (70%) and 116/212 families (55%). Separate analysis of cases diagnosed in childhood and in adulthood (Figure 1A) showed that anemia, neonatal jaundice and transfusion requirement were more frequent in subjects diagnosed during childhood. Neonatal jaundice was rather common (occurring in one-third of all cases), but exchange transfusion was required in only 14/82 cases. When clinical features were divided according to the membrane defect (Figure 1B), we found that splenomegaly and gallstones were more frequent in band 3 deficient patients, whereas anemia, neonatal jaundice and transfusion requirement were more common in those with spectrin/ankyrin deficiency. As regards exchange transfusion, no difference was found between the two groups. Interestingly, isolated or combined spectrin

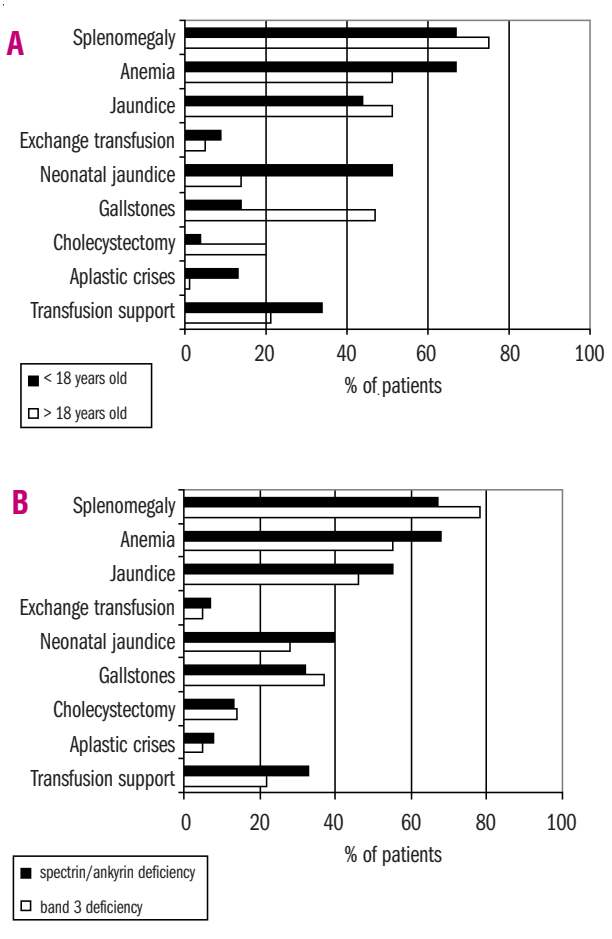

Figure 1. Main clinical data of non-splenectomized HS patients divided according to age at diagnosis (A): ■<18 years old (n=121), and □>18 years old (n=138), and to the type of membrane defect (B): ■spectrin/ankyrin deficiency (n=90), and □ band 3 deficiency (n=139).

and ankyrin deficiency was more frequently diagnosed in childhood than in adulthood (55% *vs.* 45%), unlike band 3 deficiency (60% in adults *vs.* 40% in children).

Table 1 shows the hematologic and biochemical data of HS patients grouped according to whether they had not been splenectomized or had been splenectomised before the time of the study. In the former group MCV was decreased in 8% of adults and 16% of children, and MCHC increased in 14% of adults and 16% of children (considering the age-related reference values); 22 patients (8%) had less than 2% spherocytes, and eight of them (five with band 3 deficiency and three with spectrin deficiency) had no detectable spherocytes. Hemolysis markers were increased in all patients but four (two with band 3 deficiency and two with spectrin deficiency). Red cell osmotic fragility was assessed by a battery of tests, with sensitivities ranging from 48% to 95%. The association of the acidified glycerol lysis and NaCl tests on incubated blood reached a sensitivity of 99%, even including atypical patients, i.e. those with normal reticulocyte counts (n=16) and/or no spherocytes in the peripheral blood (n=8). The sensitivity of the cryohemolysis and the eosin-5-maleimide binding test, performed in the last 33 consecutive patients only, was 53% and 84%, respectively (*data not shown*). As regards serum iron parameters, a serum ferritin concentration >500 ng/mL was detected in eight out of 189 non-splenectomized and never transfused patients; three of these eight patients had both increased serum ferritin concentration and transferrin saturation, and were heterozygous for the HFE mutation His63>Asp.

Considering the splenectomized patients, the hemoglobin levels were normal in all cases but one (who had severe spectrin deficiency), and markers of hemolysis were significantly reduced as compared to those in non-splenectomized subjects. Interestingly, among splenectomized patients the percentage of positive cases was increased for all the osmotic fragility tests, as compared with non-splenectomized cases; in particular, both the acidified glycerol lysis and NaCl tests on incubated blood reached 100% sensitivity. The most common protein abnormalities were band 3 and spectrin deficiencies in both non-splenectomized and splenectomized patients. This also held true when one individual from each kindred was considered (104 with band 3 deficiency and 64 with spectrin deficiency). Substantial isolated protein 4.2 deficiency was found in two patients belonging to the same family. A small decrease of protein 4.2 was found in association with band 3 or spectrin deficiency in 7% and 16% of cases, respectively. The membrane protein defect was undetectable in 3% of splenectomized patients and in 11% of non-splenectomized patients.

Table 1. Hematologic and biochemical data of non-splenectomized and splenectomized HS patients.

	Non-splenectomized (n=259)			*Splenectomized (n=41)*	
Hematologic parameters[a]					
Hemoglobin (g/dL)	12	(6-18.9)	$p<0.001$	14.8	(9.2-18.4)
MCV (fL)	86	(68-112)	$p<0.03$	90	(76-112)
MCHC (g/dL)	35.5	(27.6-39.9)		35.6	(29.4-38.8)
Spherocytes (%)	7	(0-56)		10	(0-44)
Markers of hemolysis[a]					
Reticulocytes ($\times 10^9$/L)	244	(9-909)	$p<0.0001$	78	(37-439)
Unc. bilirubin (I.U.)	30.8	(5.1-215.4)	$p<0.0001$	13.7	(3.4-66.7)
Osmotic fragility tests[b]					
Standard glycerol lysis	124	(48)	$p<0.0001$	39	(95)
Acidified glycerol lysis	246	(95)		41	(100)
Pink	197	(76)		37	(89)
NaCl on fresh blood	174	(67)	$p=0.0001$	40	(97)
NaCl on incubated blood	223	(86)	$p=0.02$	41	(100)
All tests	259	(100)		41	(100)
Iron status parameters[a]					
Transferrin saturation (%)	29	(8-93)		33	(0.3-85)
Serum ferritin (ng/mL)	115	(3-1403)		133	(12-1617)
Defective protein[b]					
Band 3	139	(54)		19	(46)
Spectrin	81	(31)		17	(41)
Ankyrin or combined spectrin/ankyrin	9	(3)		4	(10)
Band 4.2	2	(1)		0	(0)
Undetected	28	(11)		1	(3)

[a]Median values (ranges); the t-test was used to compare splenectomized and non-splenectomized patients; [b]Number of positive cases (percentage); the χ^2 test was used to compare frequencies in splenectomized and non-splenectomized patients.

Hematologic features of hereditary spherocytosis patients grouped according to the type of membrane defect

Figure 2 shows the hematologic data of non-splenectomized HS patients grouped according to the type of the membrane defect. No significant differences were observed among the various groups, although hemoglobin levels were slightly lower, and median spherocyte number and hemolysis markers slightly higher in spectrin-deficient patients than in those with band 3 or protein 4.2 deficiency. This was also true considering the different forms of HS according to the clinical classification proposed by Bolton-Maggs *et al.*:[25] HS presented as trait, mild, moderate, and severe in 42%, 38%, 11%, and 9% of spectrin/ankyrin-deficient patients, and in 50%, 30%, 16%, and 4% of band 3-deficient cases, respectively. Table 2 shows the hematologic features of patients with band 3 deficiency and isolated spectrin deficiency divided according to whether they had been splenectomized or not. In both defects, splenectomized patients had normal hemoglobin values, and significantly reduced reticulocyte numbers and unconjugated bilirubin levels compared to non-splenectomized cases; however, spectrin-deficient splenectomized patients still had increased reticulocyte counts. Consistently, considering only splenectomized patients, spectrin-deficient cases had significantly higher reticulocyte counts ($p=0.05$) compared with band 3-deficient cases. The sensitivity of osmotic fragility tests was similar in patients with band 3 and spectrin deficiency irrespective of the amount of protein deficiency, and also in patients without a detectable membrane defect (*data not shown*). The HS patients with unclassified membrane defect were comparable to the other groups in terms of hemoglobin

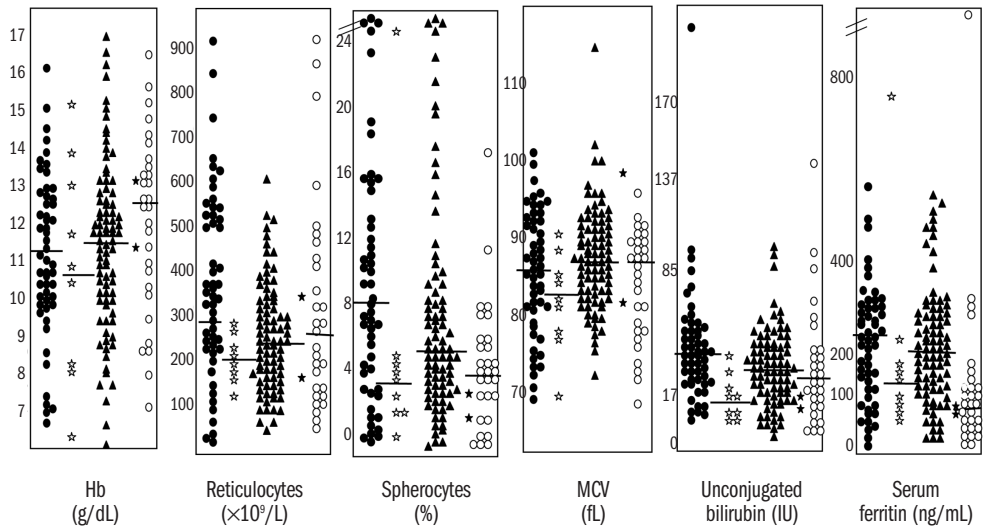

Figure 2. Hematologic parameters of 259 non-splenectomized HS patients grouped according to the results of SDS-PAGE analysis. ▲ band 3 deficiency, ● spectrin deficiency, ☆ ankyrin and combined ankyrin and spectrin deficiency, ★ band 4.2 deficiency, ◦ unclassified membrane defect. Horizontal bars indicate median values.

Table 2. Hematologic data of band 3- or spectrin- deficient HS patients grouped according to whether they had or had not been splenectomized.

	Band 3 deficiency					*Spectrin deficiency*				
	Non-splenectomized (n=139)			*Splenectomized[a] (n=27)*		*Non-splenectomized (n=81)*			*Splenectomized[a] (n=26)*	
Hematologic parameters[b]										
Hemoglobin (g/dL)	12.1	(6-18.9)	$p<0.0001$	15.4	(11.8-18.8)	11.4	(12.2-16.2)	$p<0.0001$	14.3	(9.2-18.4)
MCV (fL)	88	(71-112)		91	(74-112)	86	(68-100)		88	(74-110)
MCHC (g/dL)	36	(28-40)		36	(29-39)	35.4	(28-40)		35	(30-37)
Spherocytes (%)	7	(0-45)		10	(3-21)	8.5	(0-56)		7	(0-44)
Markers of hemolysis[b]										
Reticulocytes (x10⁹/L)	244	(9-700)	$p<0.0001$	64	(11-153)	263	(38-909)	$p<0.0001$	90	(22-439)
Unc. bilirubin (I.U.)	36.9	(5.1-152)	$p<0.0001$	16.9	(8.2-45.1)	38.9	(7.2-202)	$p=0.0004$	14.7	(3.4-121.4)
Osmotic fragility tests[c]										
Standard glycerol lysis	80	(58)	$p<0.01$	23	(85)	31	(39)	$p<0.0001$	21	(81)
Acidified glycerol lysis	133	(95)		25	(93)	76	(95)		26	(100)
Pink	107	(78)		21	(78)	64	(80)		21	(81)
NaCl on fresh blood	80	(58)	$p<0.03$	22	(81)	61	(76)	$p<0.01$	26	(100)
NaCl on incubated blood	116	(83)		26	(96)	71	(89)		26	(100)
All tests	139	(100)		27	(100)	81	(100)		26	(100)
Iron status parameters[b]										
Transferrin saturation (%)	29	(10-79)		34	(1-61)	34	(12-93)		39	(10-85)
Serum ferritin (ng/mL)	145	(7-1296)		107	(11-844)	177	(3-1403)	$p=0.03$	143	(13-1617)

[a]Patients splenectomized before the study and during follow-up; [b]Median values (ranges); the t-test was used to compare splenectomized and non-splenectomized patients; [c]Number of positive cases (percentage); the χ^2 test was used to compare frequencies in splenectomized and non-splenectomized patients.

levels, hemolysis markers, osmotic resistance and prevalence of splenomegaly.

Effect of splenectomy on hematologic and biochemical features

The analysis of 21 patients (seven with band 3 deficiency, five with spectrin deficiency, one with band 4.2 deficiency and eight with an undetected abnormality) evaluated before and after surgery showed, in all cases, an increase of median hemoglobin levels (from 10.8 g/dL to 13.9 g/dL), and a decrease of reticulocyte count (from 337×10^9/L to 51×10^9/L) and unconjugated bilirubin (from 32.5 IU to 12 IU). When the effect of splenectomy was related to the membrane defect, the mean hemoglobin increase was higher in band 3-deficient patients than in spectrin-deficient ones (4.25 g/dL; range, 0.8-6.1, versus 3.6 g/dL; range, 0.8-5.2), although the difference was not statistically significant. Furthermore, the reduction of reticulocytes and unconjugated bilirubin was more evident in patients with band 3 deficiency. The effect of splenectomy was comparable in young (n=6) and adult patients. Interestingly, splenectomy allowed the identification of the membrane defect in all the previously unclassified cases (four with spectrin deficiency, three with spectrin/ankyrin deficiency and one with band 3 deficiency).

Discussion

This is the largest study correlating the clinical and hematologic features of patients with HS with the defective cytoskeletal protein, as assessed by SDS-PAGE analysis which is currently the reference test for the identification of the molecular lesion in this disease.[42] Band 3 deficiency was the most frequent red cell membrane abnormality in our patients, in line with data reported by Cynober *et al.*[17] and Rocha *et al.*,[43] but at variance with others,[15,44-47] who found that isolated spectrin and combined ankyrin/spectrin deficiencies were the more frequent defects in smaller HS series. The proportion of unclassified patients was lower than that found by Saad *et al.*[48] and Cynober *et al.*,[17] and similar to that in more recent reports.[16,18,47] The lack of substantial clinical and hematologic differences between classified and unclassified HS subjects rules out the hypothesis[25] that the failure of SDS-PAGE to show the defective protein was related to a milder clinical expression of the disease. It is interesting that splenectomy revealed the underlying cytoskeletal abnormality in all eight previously unclassified cases, of whom seven were found to be spectrin and/or ankyrin-deficient. One case of spectrin deficiency disclosed by splenectomy was reported by Saad *et al.*,[48] who hypothesized that spleen conditioning could peel out some band 3 molecules resulting in overestimation of the spectrin to band 3 ratio; moreover, spectrin deficiency was reported to be quantitatively more pronounced in splenectomized than in non-splenectomized patients.[49] In line with these observations, our data indicate that almost all unclassified HS patients are likely to be spectrin and/or ankyrin deficient.

The correlation between the type of defective protein and the clinical features of the disease has never been extensively investigated, the few large studies on HS being focused on either clinical[50,51] or biochemical aspects only.[16,18] In small series spectrin was reported to be associated with a more severe clinical phenotype than band 3 deficiency.[16,21,22] We did not detect significant differences in the clinical or hematologic features among the various HS subsets, although spectrin-deficient patients had slightly lower hemoglobin levels and higher hemolytic markers than those with band 3 deficiency. Splenectomy corrected the anemia in all cases but one, who had severe spectrin deficiency, in line with previous reports.[4,21] After splenectomy, spectrin-deficient patients had a slightly lower median increase in hemoglobin concentration, and a higher reticulocyte count than band 3-deficient patients, suggesting that the former is a more severe clinical condition. These *in vivo* findings are in agreement with the observation of Reliene *et al.*[23] that red cell deformability, measured as the elongation index by an ectacytometer, is lower in spectrin/ankyrin-deficient erythrocytes than in band 3-deficient ones, both before and after splenectomy.

HS may present at any age, although typically in childhood and adolescence. We showed that splenomegaly was the most frequent sign in adults, followed by jaundice, anemia and gallstones, and confirmed that anemia and splenomegaly were the most frequent complaints in children.[50,52] The observation that spectrin deficiency was more frequently diagnosed in childhood and that anemia, jaundice and transfusion requirement were more frequent in spectrin-deficient than in band 3-deficient patients is in favor of an earlier and more overt clinical presentation of the former defect.

The laboratory hallmarks of HS are the presence of spherocytes in a blood smear and/or the demonstration of increased red cell fragility. We showed that the number of spherocytes was not related to either the type or the severity of the membrane defect. It is worth noting that about 10% of HS cases had very few or no detectable spherocytes, and this may account for HS being misdiagnosed in some patients. Red cell fragility tests are, therefore, an important diagnostic investigation in less typical cases. These tests, which are known to have different sensitivities,[25] have been mostly used individually,[6,15,43] and their performance has never been related to the defective protein. Our results confirmed that the sensitivity of these tests varies greatly[30] and showed that it was independent of the cytoskeletal abnormality and of the extent of the protein deficiency. We also demonstrated that, among the traditional screening tests for HS, the acidified glycerol lysis test had the highest sensitivity, even higher than that reported for the flow cytometric eosin-5-maleimide binding test,[33] recently recommended by the General Haematology Task Force of the British Committee for Standards in Haematology.[25]

In conclusion, the data presented in this paper show that the definition of the red cell membrane defect in HS, whatever the clinical form of the disease (trait, mild, moderate and severe),[25] has no clear clinical implications. Nevertheless, the finding that spectrin/ankyrin deficiency was more frequently diagnosed in childhood, and that splenectomy was more frequently performed in patients with spectrin/ankyrin deficiency, may suggest that spectrin/ankyrin-deficiency tends to produce a more severe clinical phenotype than band 3 deficiency. Our findings are in line with the guidelines for the diagnosis of HS, which recommend that SDS-PAGE analysis be performed only in selected cases. However, it is worth mentioning that SDS-PAGE may be an effective investigation for differentiating HS from cases of congenital dyserythropoietic anemia type II, which mimics HS due to the presence of mild chronic hemolytic anemia, splenomegaly and microspherocytes in a peripheral blood smear.[53]

Authorship and Disclosures

MM: biochemical characterizartion of red cell membranes, preparation of the draft of the article; WB: clinical follow-up; CV: laboratory diagnosis and biochemical characterization; APM: laboratory diagnosis; EF: laboratory diagnosis; PP: statistical analysis; CB: clinical follow-up; AZ: director, critical revision of the manuscript.

The authors reported no potential conflicts of interest.

References

1. Morton NE, MacKinney AA, Kosower N. Genetics of spherocytosis. Am J Hum Gen 1962;14:170-8.
2. Godal HC, Heisto H. High prevalence of increased osmotic fragility of red blood cells among Norwegian blood donors. Scand J Haematol 1981;27:30-4.
3. Tse WT, Lux SE. Red blood cell membrane disorders. Br J Haematol 1999;104:2-13.
4. Eber SW, Pekrun A, Neufeldt A, Schroter W. Prevalence of increased osmotic fragility of erythrocytes in German blood donors: screening using a modified glycerol lysis test. Ann Hematol 1992;64:88-92.
5. Delaunay J. The molecular basis of hereditary red cell membrane disorders. Blood Rev 2007;21:1-20.
6. Eber SW, Ambrust R, Schroeter W. Variable clinical severity of hereditary spherocytosis: relation to erythrocytic spectrin concentration, osmotic fragility, and autohemolysis. J Pediatr 1990;117:409-16.
7. Miraglia del Giudice E, Nobili B, Francese M, D'Urso L, Iolascon A, Eber S, et al. Clinical and molecular evaluation of non-dominant hereditary spherocytosis. Br J Haematol 2001;112:42-7.
8. Hassoun H, Palek J. Hereditary spherocytosis: a review of the clinical and molecular aspects of the disease. Blood Rev 1996;10:129-47.
9. Gallagher PG. Red cell membrane disorders. Hematology. American Society of Hematology, Educational Program 2005.
10. Maillet P, Alloisio N, Morlè L, Delaunay J. Spectrin mutation in hereditary elliptocytosis and hereditary spherocytosis. Hum Mutat 1996;8:97-100.
11. Gallagher PG, Forget BG. Hematologically important mutations: band 3 and protein 4.2 variants in hereditary spherocytosis. Blood Cell Mol Dis 1997;23:417-21.
12. Gallagher PG, Sabatino DE, Basseres DS, Nilson DM, Wong C, Cline AP, et al. Erythrocyte ankyrin promoter mutations associated with recessive hereditary spherocytosis cause significant abnormalities in ankyrin expression. J Biol Chem 2001;276: 41683-9.
13. Cooper RA, Jandl JH. The role of membrane lipids in the survival of red cells in hereditary spherocytosis. J Clin Invest 1969;48:736-44.
14. Da Costa L, Mohandas N, Sorette M, Grange MJ, Tchernia G, Cynober T. Temporal differences in membrane loss lead to distinct reticulocyte features in hereditary spherocytosis and in immune hemolytic anemia. Blood 2001;98: 2894-9.
15. Miraglia del Giudice E, Iolascon A, Pinto L, Nobili B, Perrotta S. Erythrocyte membrane protein alterations underlying clinical heterogeneity in hereditary spherocytosis. Br J Haematol 1994;88:52-5.
16. Jarolim P, Murray JL, Rubin HL, Taylor WM, Prchal JT, Ballas SK, et al. Characterization of 13 novel band 3 gene defects in hereditary spherocytosis with band 3 deficiency. Blood 1996;88:4366-74.
17. Cynober T, Mohandas N, Tchernia G. Red cell abnormalities in hereditary spherocytosis: relevance to diagnosis and understanding of the variable expression of clinical severity. J Lab Clin Med 1996;12: 259-69.
18. Ricard MP, Gilsanz F, Millan I. Erythroid membrane protein defects in hereditary spherocytosis. A study of 62 Spanish cases. Haematologica 2000;85:994-5.
19. Lee YK, Cho HI, Park SS, Lee YJ, Ra E, Chang YH, et al. Abnormalities of erythrocyte membrane proteins in Korean patients with hereditary spherocytosis. J Kor Med Sci 2000; 15:284-8.
20. Yawata Y, Kanzaki A, Yawata A, Doerfler W, Ozcan R, Eber S. Characteristic features of the genotype and phenotype of hereditary spherocytosis in the Japanese population. Int J Hematol 2000;71:118-35.
21. Agre P, Asimos A, Casella JF, McMillan C. Inheritance pattern and clinical response to splenectomy as a reflection of erythrocyte spectrin deficiency in hereditary spherocytosis. N Engl J Med 1986; 315:1579-83.
22. Nakanishi H, Kanzaki A, Yawata A, Yamada O, Yawata Y. Ankyrin gene mutations in Japanese patients with hereditary spherocytosis. Int J Hematol 2001;73:54-63.
23. Reliene R, Mariani M, Zanella A, Reinhart WH, Ribeiro L, Miraglia del Giudice E, et al. Splenectomy prolongs in vivo survival of erythrocytes differently in spectrin/ankyrin- and band 3- deficient hereditary spherocytosis. Blood 2002;100:2208-15.
24. Marchetti M, Quaglini S, Barosi G. Prophylactic splenectomy and cholecystectomy in mild hereditary spherocytosis: analyzing the decision in different clinical scenarios. J Int Med 1998;244:217-26.
25. Bolton-Maggs PHB, Stevens RF, Dodd NJ, Lamont G, Tittensor P, King MJ. Guidelines for the diagnosis and management of hereditary spherocytosis. Br J Haematol 2004; 126:455-74.
26. Sirchia G. Il sistema sangue. Centro Trasfusionale Ospedale Maggiore Policlinico di Milano, 1983.
27. Nathan DG, Orkin SH Nathan & Oski's Hematology of Infancy and Childhood. 5th edition. W.B: Saunders Company 1998.
28. Parpart AK, Lorenz PB, Parpart ER, Gregg JR, Chase AN. The osmotic resistance (fragilities) of human red cells. J Clin Invest 1947;26:636-45.
29. Gottfried EL, Robertson MA. Glycerol lysis time as a screening test for erythrocyte disorders. J Lab Clin Med 1974;83:323-33.
30. Zanella A, Izzo C, Rebulla P, Zanuso F, Perroni L, Sirchia G. Acidified glycerol lysis test (AGLT): a screening test for spherocytosis. Br J Haematol 1980;45: 481-6.
31. Vettore L, Zanella A, Molaro GL, De Matteis MC, Pavesi M, Mariani M. A new test for the laboratory diagnosis of spherocytosis. Acta Haematologica 1984;72:258-63.
32. Streichman S, Gesheidt Y, Tatarski I. Hypertonic cryohemolysis: a diagnostic test for hereditary spherocytosis. Am J Hematol 1990;35: 104-9.
33. King MJ, Behrens J, Rogers C, Flynn C, Greenwood D, Chambers K. Rapid flow cytometric test for the diagnosis of membrane cytoskeleton-associated haemolytic anaemia. Br J Haematol 2000;111: 924-33.
34. Zanella A, Fermo E, Bianchi P, Valentini G. Red cell pyruvate kinase deficiency: molecular and clinical aspects. Br J Haematol 2005; 130:11-25.
35. Beutler E. Red Cell Metabolism: A Manual of Biochemical Methods. New York, NY: Grune and Stratton, Inc. 1984.
36. Dodge JT, Mitchell C, Hanahan DJ. The preparation and chemical characteristics of hemoglobin free ghosts of human erythrocytes. Arch Biochem Biophys 1963;100: 119-30.
37. Beutler E, West C, Blume KG. The removal of leukocytes and platelets from whole blood. J Lab Clin Med 1976;88:328-33.
38. Lutz UH, Wipf G. Naturally occurring autoantibodies to skeletal proteins from human red blood cells. J Immunol 1982;128:1695-9.
39. Fairbanks G, Steck TL, Wallach DFH. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 1971;10:2606-17.
40. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
41. Roff DA, Bentzen P. The statistical analysis of mitochondrial DNA polymorphisms: chi^2 and the problem of small samples. Mol Biol Evol 1989;6:539-45.
42. Walensky LD, Mohandas N, Lux SE. Disorders of the red blood cell membrane. In: Handin RI, Lux SE, Stossel TP, eds. Blood Principles and Practice in Hematology. 2nd edition, Lippincott Williams and Wilkins, 2003.
43. Rocha S, Rebelo I, Costa E, Catarino C, Belo L, Castro EBM, et al. Protein deficiency balance as a predictor of clinical outcome in hereditary spherocytosis. Eur J Haematol 2005;74:374-80.
44. Lanciotti M, Perutelli P, Valetto A, Di Martino D, Mori PG. Ankyrin deficiency is the most common defect in dominant and non dominant hereditary spherocytosis.

Haematologica 1997;82:460-2.
45. Dhermy D, Bournier O, Bourgeois M, Grandchamp B. The red blood cell band 3 variant (band 3 Bicetrel: R 490C) association with dominant hereditary spherocytosis causes defective membrane targeting of the molecule and a dominant negative effect. Mol Membr Biol 1999; 16:305-12.
46. Premetis E, Stamoulakatou A, Loukopoulos D. Hereditary spherocytosis in Greece: collective data on a large number of patients. Hematology 1999;4:361-6.
47. Sanchez-Lopez JY, Camacho AL, Magana MT, Ibarra B, Perea FJ. Red cell membrane protein deficiencies in Mexican patients with hereditary spherocytosis. Blood Cells Mol Dis 2003;31:357-9.
48. Saad STO, Costa FF, Vicentim DL, Salles TSI, Pranke PHL. Red cell membrane protein abnormalities in hereditary spherocytosis in Brazil. Br J Haematol 1994;88:295-9.
49. Jarolim P, Brabec V, Ballas SK, Prchal JT, Poon MC, Castleberry R, et al. Biochemical heterogeneity of the hereditary spherocytosis syndrome (abstract). Paper presented at the 24th Congress of the International Society of Haematology, London, 1992.
50. Pinto L, Iolascon A, Miraglia del Giudice E, Matarese S, Nobili B, Perrotta S. The Italian pediatric survey on hereditary spherocytosis. Int J Pediatr Hematol/Oncol 1995; 2:43-7.
51. Michaels LA, Cohen AR, Zhao H, Raphael RI, Manno CS. Screening for hereditary spherocytosis by use of automated erythrocyte indexes. J Pediatr 1997;130:957-60.
52. Krueger HC, Burgert EO Jr. Hereditary spherocytosis in 100 children. Mayo Clin Proc 1966;41: 821-30.
53. Mariani M, Vercellati C, Bianchi P, Marzorati S, Caneva L, Soligo C, et al. Congenital dyserythropoietic anemia type II (CDA II) mimicking hereditary spherocytosis (HS): report of 12 cases detected by SDS-PAGE. Hematol J 2002;3:353. (abstract).