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► **To cite this version:**

Susana Rocha, Elísio Costa, Petronila Rocha-Pereira, Fátima Ferreira, Esmeralda Cleto, et al.. Erythrocyte membrane protein destabilization versus clinical outcome in 160 Portuguese Hereditary Spherocytosis patients. *British Journal of Haematology*, 2010, 149 (5), pp.785. 10.1111/j.1365-2141.2010.08166.x . hal-00552588

HAL Id: hal-00552588

<https://hal.science/hal-00552588>

Submitted on 6 Jan 2011

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**Erythrocyte membrane protein destabilization versus
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|-------------------------------|---|
| Journal: | <i>British Journal of Haematology</i> |
| Manuscript ID: | BJH-2009-01964.R1 |
| Manuscript Type: | Ordinary Papers |
| Date Submitted by the Author: | 11-Feb-2010 |
| Complete List of Authors: | <p>Rocha, Susana; Faculdade de Farmácia, Universidade do Porto; Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto</p> <p>Costa, Elísio; Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto; Instituto de Ciências da Saúde, Universidade Católica Portuguesa</p> <p>Rocha-Pereira, Petronila; Centro de Investigação em Ciências da Saúde (CICS), Universidade da Beira Interior; Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto</p> <p>Ferreira, Fátima; Serviço de Hematologia Clínica, Hospital S. João Cleto, Esmeralda; Sector de Hematologia Pediátrica, Centro Hospitalar do Porto – Hospital Santo António</p> <p>Barbot, José; Serviço de Hematologia, Centro Hospitalar do Porto – Hospital Maria Pia</p> <p>Quintanilha, Alexandre; Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto; Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto</p> <p>Belo, Luís; Faculdade de Farmácia, Universidade do Porto; Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto</p> <p>Santos-Silva, Alice; Faculdade de Farmácia, Universidade do Porto; Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto</p> |
| Key Words: | HEREDITARY ANAEMIAS, MEBRANE, CELL MEMBRANE |

Title: Erythrocyte membrane protein destabilization *versus* clinical outcome in 160 Portuguese Hereditary Spherocytosis patients

Running title: Erythrocyte membrane proteins in HS

Authors: Susana Rocha^{1,2}, Elísio Costa^{2,3}, Petronila Rocha-Pereira^{2,4}, Fátima Ferreira⁵, Esmeralda Cleto⁶, José Barbot⁷, Alexandre Quintanilha^{2,8}, Luís Belo^{1,2}, Alice Santos-Silva^{1,2}

Affiliations: ¹Faculdade de Farmácia, Universidade do Porto, Porto, Portugal; ²Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Porto, Portugal; ³Instituto de Ciências da Saúde, Universidade Católica Portuguesa, Porto, Portugal; ⁴ Centro de Investigação em Ciências da Saúde (CICS), Universidade da Beira Interior, Covilhã, Portugal; ⁵ Serviço de Hematologia Clínica, Hospital S. João, Porto, Portugal; ⁶ Sector de Hematologia Pediátrica, Centro Hospitalar do Porto – Hospital Santo António, Porto, Portugal; ⁷ Serviço de Hematologia, Centro Hospitalar do Porto – Hospital Maria Pia, Porto, Portugal; ⁸ Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Porto, Portugal

Corresponding author: Alice Santos Silva/Susana Rocha
Serviço de Bioquímica,
Faculdade de Farmácia da Universidade do Porto
Rua Aníbal Cunha, 164
4050-047, Porto, Portugal
Phone: +351 222 078 906/7
Fax: +351 222 003 977
Email: assilva@ff.up.pt; srocha@ff.up.pt

Summary

Hereditary Spherocytosis (HS) is a haemolytic anaemia caused by erythrocyte protein membrane defects – spectrin, ankyrin, band 3 or protein 4.2 – that lead to membrane destabilization. Ours aims were to evaluate the prevalence of protein deficiencies and the role of membrane proteins or of membrane linked proteins in membrane disturbance and in HS clinical outcome. We studied 215 Portuguese individuals – 203 from 71 families plus 12 individual unrelated subjects, and found that 160 of them were HS patients. They were classified as presenting mild, moderate or severe forms of HS according to the degree of haemolytic anaemia. We performed the standardized electrophoretic erythrocyte membrane protein analysis to identify and quantify protein deficiencies. We found that band 3 and ankyrin account for the majority of the erythrocyte protein defects underlying HS. Increasing isolated protein deficiency or increasing unbalance between combined protein deficiencies seem to underlie HS severity, by increasing membrane destabilization. We found an increased membrane linkage of the cytosolic proteins, glyceraldehyde-3-phosphate dehydrogenase and peroxiredoxin 2, and of denatured haemoglobin, suggesting that this linkage could interfere with membrane structure. Our data suggest that the quantification and the analysis of RBC membrane proteins may be helpful in predicting the clinical outcome of HS.

Keywords: Hereditary Spherocytosis, erythrocyte membrane proteins, protein defects, glyceraldehyde-3-phosphate dehydrogenase, clinical severity

Introduction

Hereditary Spherocytosis (HS) is a common red blood cell (RBC) membrane disorder affecting about one in 2000 individuals from northern European ancestry (Delaunay 2007, Perrotta, *et al* 2008). Clinically, HS can range from asymptomatic to a life threatening anaemia. It is classified as mild, moderate and severe, according to the degree of the haemolytic anaemia and the associated symptoms (Bolton-Maggs, *et al* 2004, Perrotta, *et al* 2008). HS is caused by membrane protein deficiencies in spectrin, ankyrin, band 3 or protein 4.2 that lead to membrane destabilization and vesiculation, changing the RBCs into denser and more rigid cells, the spherocytes, which will be removed in the spleen, leading to the development of a haemolytic anaemia. Familial private mutations in several genes, *SPTA1*, *SPTB*, *ANK1*, *SLC4A1* and *EPB42* cause α -spectrin, β -spectrin, ankyrin, band 3 or protein 4.2 protein deficiencies, respectively. Although the majority of HS cases present an autosomal dominant inheritance pattern (75%), about one quarter of the patients arise from autosomal recessive or *de novo* mutations (Bolton-Maggs, *et al* 2004, Delaunay 2007, Perrotta, *et al* 2008).

The molecular defect in one of the proteins underlying HS causes a primary deficiency in that protein; however, in some cases more than one protein is deficient (although only one protein molecular defect exists), as the primary protein deficiency triggers secondary proteins deficiencies in proteins with which that protein interacts (An and Mohandas 2008, Delaunay 2007, Perrotta, *et al* 2008). Molecular defects in the *SLC4A1* gene (that cause band 3 protein deficiency) have been associated with a secondary protein deficiency in protein 4.2 (Delaunay 2007, Inoue, *et al* 1994, Mariani, *et al* 2008); mutations in the gene encoding ankyrin were associated with secondary protein deficiencies in spectrin and/or protein 4.2. Actually, reticulocytosis can mask the primary protein deficiency in ankyrin, and only the secondary protein deficiencies in spectrin and/or in protein 4.2 reflect this primary deficiency (Delaunay 2007, Hanspal, *et al* 1991, Lanciotti, *et al* 1997, Miraglia del Giudice, *et al* 1997).

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3 Peroxiredoxin 2 (Prx2) is one of the most abundant cytoplasmatic proteins of the
4 erythrocyte (Moore, *et al* 1991, Rabilloud, *et al* 1995, Schroder, *et al* 2000) and is a
5 typical 2-cys peroxiredoxin, with a thiol-dependent H₂O₂ scavenger activity that plays a
6 role in the antioxidant systems within the RBC (Low, *et al* 2007, Low, *et al* 2008, Wood,
7 *et al* 2003). The association of Prx2 with the erythrocyte membrane (Cha, *et al* 2000,
8 Moore, *et al* 1997, Murphy, *et al* 2004, Rocha, *et al* 2008) has been described and it is
9 not the only cytosolic protein interacting with the membrane; It is known that when
10 haemoglobin (Hb) is denatured, under oxidative stress conditions, it binds to the
11 cytoplasmatic domain of band 3 (Murthy, *et al* 1984, Premachandra 1986, Walder, *et al*
12 1984). Furthermore, though glyceraldehyde-3-phosphate dehydrogenase (G3PD)
13 exerts its activity in the cytoplasm, the majority (90%) of erythrocyte G3PD is bound to
14 the RBC membrane in its inactive form, becoming active when released into the
15 cytoplasm after band 3 phosphorylation (Mallozzi, *et al* 1995, Rogalski, *et al* 1989,
16 Tsai, *et al* 1982).

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33 In a previous study involving 51 HS patients (Rocha, *et al* 2005), we reported that an
34 unbalance between primary and secondary protein deficiencies may account for the
35 enhancement in membrane destabilization and that the value of the unbalance
36 appeared to be related to the clinical outcome of HS. Recently, we reported the
37 detection of Prx2 in the RBC membrane of some HS patients, as well as, the linkage of
38 increased amounts of Hb and G3PD (Rocha, *et al* 2008).

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Our aims were to evaluate, in a large Portuguese HS population, the prevalence of the
membrane primary and secondary protein deficiencies and to study the role of
membrane proteins or of membrane linked proteins, in membrane RBC destabilization
and in the clinical outcome of HS.

Materials and Methods

Subjects

This study was approved by the Ethics Committees of the participant Hospitals (Hospital Maria Pia and Hospital Santo António from Centro Hospitalar do Porto and Hospital S. João). All subjects gave their informed consent to participate in the study.

We studied 215 individuals from the north region of Portugal with a suggestive family and/or clinical history of HS – 203 individuals from 71 families and 12 unrelated subjects, as only the *propositus* was available for study. Out of the 215 studied individuals, 160 were diagnosed with HS, according to haematological and biochemical data and to clinical/family history. The patients were classified as presenting mild, moderate or severe HS according to the *Guidelines for the diagnosis and clinical management of Hereditary Spherocytosis* (Bolton-Maggs, *et al* 2004). For splenectomised patients we used the clinical classification defined before splenectomy and only those who were submitted to surgery more than one year before this study were included. Blood collection was only performed when the last transfusion took place at least 4 months before.

Some adult HS mild patients had been splenectomised, as they developed a symptomatic vesicular lithiasis not predictable by clinical criteria; the severe HS children had not undergone splenectomy, as their age was still not compatible with that procedure.

The control samples used for the electrophoretic analysis of RBC membrane proteins were from healthy blood donors.

Preparation of erythrocyte membrane suspensions for electrophoretic analysis:

Blood samples (EDTA as anticoagulant) were collected and, after the basic haematological and reticulocyte count analysis, they were processed to further studies.

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3 To ensure the minimal RBC membrane preparation variability, the protocol was
4 performed by the same individual during the course of this study.
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7 To prepare the erythrocyte membranes, plasma and leukocytes were isolated from
8 RBCs and discarded after centrifugation on a double density gradient (Histopaque
9 1.077 and 1.119, Sigma). Erythrocytes were washed in saline solution and, afterwards,
10 submitted to hypotonic lyses, according to Dodge *et al* (1983) (Dodge, *et al* 1963). The
11 obtained membranes were washed in Dodge buffer, adding in the first two washes
12 phenylmethylsulphonyl fluoride 0.1 mM, a protease inhibitor. The protein concentration
13 of the RBC membrane suspensions was determined by the Bradford's method
14 (Bradford 1976). The membrane suspensions were treated with an equal volume of a
15 solubilisation buffer (0.125M Tris-HCl pH 6.8, 4% sodium dodecyl sulfate (SDS), 20%
16 glycerol, 10% 2-mercaptoethanol) and heat denatured.
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31 *Identification and quantification of erythrocyte membrane proteins:*

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33 The electrophoretic analysis of the RBC proteins was carried out on a discontinuous
34 system of polyacrylamide gels in the presence of SDS (SDS-PAGE), using a 5-15%
35 linear acrylamide gradient gel (8 µg protein/lane) and a 3.5-17% exponential
36 acrylamide gradient gel (6 µg protein/lane), according to Laemmli (Laemmli 1970) and
37 Fairbanks *et al.* (Fairbanks, *et al* 1971), respectively. The proteins were stained with
38 Coomassie brilliant blue, and scanned (Darkroom CN UV/wl, *BioCaptMW version 99*,
39 Vilber Lourmat). The relative amount of each major protein (as a percentage of the
40 total), was quantified by densitometry (*Bio1D++ version 99*, Vilber Lourmat). This
41 technique allowed the identification and quantification of the major protein constituents
42 of erythrocyte membrane (α -spectrin, β -spectrin, ankyrin, band 3 protein 4.1, protein
43 4.2, actin, G3PD and stomatin/tropomyosin) and, thus, the identification of the protein
44 membrane deficiency that underlies HS.
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59 The electrophoretic analysis for each RBC membrane sample was performed in
60 duplicate gels, and, in each gel, duplicates of each sample were loaded alongside with

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3 four to six control samples. The mean values presented by the studied subjects were
4 compared with those of the controls. A protein deficiency was considered as significant
5 when its mean value was lower than the mean value minus 2 times the standard
6 deviation presented by the controls for the same protein. The protein deficiency value
7 was calculated in relation to controls (protein deficit (%) = [(control's mean value
8 protein - patient's mean value protein)/control's mean value protein] x 100).

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10 We also analyzed the balance/ratio between protein deficiency values whenever the
11 patients presented combined protein deficiencies: for band 3 primary deficiency, we
12 determined the ratio between the deficiency values of band 3 and protein 4.2 and for
13 ankyrin primary deficiency, the ratio between spectrin and protein 4.2; afterwards, we
14 calculated the difference of these ratios to one, in absolute numbers (balance between
15 the protein deficiency values = |1- ratio bd3/pt4.2| or |1- ratio sp/pt4.2|, respectively). It
16 should be noted that in relation to our prior work (Rocha, *et al* 2005), we altered the
17 way we determined this balance, as we found that the relationship for
18 primary/secondary protein deficiencies was sometimes inverse; therefore, we revised
19 that formula in order to represent this protein balance for all the ratios.

20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 *Immunoblot analysis for Prx2:*

42 **The positive or negative detection of membrane linked Prx2 in the Coomassie**
43 **blue stained gels after SDS-PAGE, was confirmed by immunoblotting in all**
44 **samples (control and HS).**

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49 Erythrocyte membrane protein suspensions were submitted to SDS-PAGE in a 12%
50 acrylamide gel (30 µg protein/lane), using the discontinuous Laemmli system (Laemmli
51 1970). Proteins were electrophoretically transferred from SDS gels to a nitrocellulose
52 sheet with a porosity of 0.2 µm (Towbin, *et al* 1979). Additional reactive sites on the
53 nitrocellulose were blocked by incubation in low fat dry milk (5% w/v) and 0.1% Triton-X
54 100 in PBS pH 7.0, for 1h at room temperature, under gentle rotation. Prx2 immunoblot
55 was then carried out adding monoclonal antibodies anti-human Prx2 (dilution 1:3000),
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3 produced in mouse (LabFrontier) and incubating for 5 h; washing of the nitrocellulose
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5 was followed by the incubation with anti-mouse IgG peroxidase linked (Sigma-Aldrich)
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7 for 1h (dilution 1:3000). An enhanced chemiluminescence method (SuperSignal West
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9 Pico Chemiluminescent Substrate, Pierce) and a X-ray film (Kodak) were used to
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11 develop the immunoblot. The immunoblots were scanned (Darkroom CN UV/wl,
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13 BioCaptMW version 99, Vilber Lourmat).
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18 *Membrane bound haemoglobin measurement:*

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20 The membrane bound haemoglobin (MBH) was measured by spectrophotometry, as
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22 described elsewhere (Santos-Silva, *et al* 1995).
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27 *Statistical analysis*

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29 For statistical analysis we used the Statistical Package for Social Sciences (SPSS),
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31 version 16.0, for Windows. We present our data as mean \pm SD or as median values
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33 (inter-quartile range). To evaluate the differences between groups, we used the
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35 OneWay Anova associated to Bonferroni's multiple comparisons test whenever the
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37 parameters presented a Gaussian distribution. The non-parametric Kruskal-Wallis H
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39 test was used in the case of a non-Gaussian distribution and, when statistical
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41 significance was achieved single comparisons (two groups) were made by using the
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43 Mann-Whitney U test. Pearson's correlation coefficient was used to evaluate
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45 relationships between sets of parametric data and the Spearman's rank correlation
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47 coefficient between sets of non-parametric data. A *p* value lower than 0.05 was
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49 considered as statistically significant.
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Results

We found that 160 out of 215 studied individuals from the north region of Portugal were HS patients (108 unsplenectomised (unspl) and 52 splenectomised (spl) patients) and the identification and quantification of protein deficiencies was determined in all of them.

A dominant inheritance pattern for HS was found in 53 (75%) out of the 71 studied families. In the other 18 families only the *propositus* presented the disease, therefore, only molecular studies could establish whether a recessive or non-dominant pattern was present, such as a *de novo* mutation. We could not ascertain about the inheritance pattern of the 12 unrelated patients, as the family members were not available to participate in this study.

We found that seventy-two (45.0%) patients presented mild HS (6 were spl), sixty (37.5%) presented moderate HS (29 were spl) and twenty-eight (17.5%) presented severe HS (17 were spl).

The analysis of the erythrocyte membrane protein profile showed that 109 patients presented a primary protein deficiency in band 3 [isolated band 3 deficiency ($n=20$) or band 3 deficiency combined with protein 4.2 ($n=89$)], 35 patients a primary ankyrin deficiency [ankyrin deficiency associated to both spectrin and protein 4.2 deficiencies ($n=14$), ankyrin deficiency combined with spectrin ($n=1$), ankyrin deficiency combined with protein 4.2 ($n=1$) or combined deficiencies in spectrin and protein 4.2 in patients presenting reticulocytosis ($n=19$)], 14 patients an isolated deficiency in spectrin and 2 patients an isolated deficiency in protein 4.2 (Table I). These protein defects presumably result from private family mutations; hence, band 3, ankyrin, spectrin and protein 4.2 protein defects account for 65.1% (54 families), 24.1% (20 families), 9.6% (8 families) and 1.2% (1 family) of HS families, respectively.

In Table I, we present the relative amount of each of the major erythrocyte membrane proteins and the MBH value for control and HS patients (unspl and spl), according to

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3 the most likely primary protein deficiency underlying the disease. For all types of
4 protein defects, except for protein 4.2, G3PD was statistically and significantly higher in
5 HS than in control samples and MBH was increased for all types of protein defects.
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7 Actually, MBH was increased in 112 out of 160 patients (70%) and G3PD in 109 (68%)
8 of the HS patients. MBH was higher in spl patients, as compared to unspl with the
9 same protein defect, though only for band 3 and ankyrin defects this difference was
10 statistically significant (Table I).
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18 Concerning unspl *versus* spl patients we found that no statistically significant
19 differences were found between them (Table I) (except for MBH); therefore, in the
20 subsequent studies we analyzed them together, considering only the most likely
21 molecular/protein defect.
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27 Concerning clinical classification, the patients showing band 3 primary protein
28 deficiency, presented mild HS in 52 (48%) patients, while moderate HS was observed
29 in 42 (38%) cases and severe HS in 15 (14%) patients. The group presenting an
30 ankyrin protein defect included 14 (40%) mild HS patients, 14 (40%) moderate HS and
31 7 (20%) severe HS patients. In the case of isolated spectrin deficiency, 5 (36%)
32 patients presented mild HS, 3 (21%) moderate HS and 6 (43%) severe HS. For protein
33 4.2 defect one patient showed mild HS and the other a moderate form of the disease.
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42 Regarding the study of the relation between the protein deficiencies and the clinical
43 outcome of HS, we found that, for patients showing isolated protein deficiencies in
44 band 3, spectrin or protein 4.2, the severity of HS was associated with an increase in
45 the value of protein deficiency (Table II). In the case of primary band 3 deficiency
46 combined with protein 4.2 deficiency, we found that band 3 deficiency value presented
47 a trend to decrease with the worsening of the disease and that this reduction was
48 associated to a significant increase in protein 4.2 deficiency value (Table II); thus, there
49 is an increasing unbalance between primary and secondary protein deficiencies from
50 mild to severe HS (Table II). For primary ankyrin deficiency, we found a trend to an
51 increasing ankyrin deficiency value with worsening of HS; concerning the secondary
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3 deficiencies, we found that spectrin presented an increasing deficiency value, whereas
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5 the opposite was observed for protein 4.2, thus, there was an unbalance between the
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7 secondary protein deficiencies from mild to severe HS (Table II).
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10 In a previous work (Rocha, *et al* 2008), we identified as Prx2 (Fig 1, band i), an unusual
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12 protein band of about 22 kDa in SDS-PAGE that was observed in some HS samples.

13 **In the present work, Prx2 was observed in about one third of the studied patients**
14 **(57 out of 160) and it was never present in the control samples. We analyzed the**
15 **linkage of Prx2 to the membrane according to RBC membrane defect and to HS**
16 **clinical outcome in terms of percentage of detectable cases, instead of**
17 **quantifying its amount, since, unlike G3PD and MBH, Prx2 was not detected in**
18 **the membrane of all samples; moreover, as Prx2 was analysed separately by**
19 **immunoblotting, we were not able to evaluate the relative proportion of this**
20 **protein in relation to the other membrane proteins.** We found that the percentage of
21 patients with detectable Prx2 presenting band 3 and ankyrin primary deficiencies was
22 similar (35% and 34%, respectively) and, that this percentage in HS patients with
23 spectrin deficiency was slightly higher (43%); concerning MBH, a trend to higher values
24 of MBH, which reached statistical significance for ankyrin deficiency, was observed in
25 the patients with membrane linked Prx2 (Table III). The percentage of cases with
26 detectable Prx2 was similar for all the different clinical forms of HS; MBH also
27 presented a trend (severe HS) or higher (mild HS) values in the patients with Prx2
28 linked to the membrane, with the exception of moderate HS cases, where an inverse
29 trend was observed (Table III).
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50 We analyzed the value of MBH and of membrane linked G3PD, according to Hb
51 concentration and clinical outcome, for spl and unspl patients, separately. We found
52 that in spl patients MBH and Hb concentration presented a positively and statistically
53 significant correlation (Fig 2A) and that membrane G3PD was correlated negatively in a
54 statistically significant manner with Hb concentration in the unspl patients (Fig 2B).
55 Also, membrane linked G3PD was statistically and significantly correlated with
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3 reticulocyte count in the unspl patients ($r=0.500$; $p<0.001$) and that, this correlation was
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5 not observed in the spl patients ($r=0.069$; $p=0.628$). In addition, unspl patients
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7 presented statistically and significantly higher reticulocyte count than spl patients
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9 (reticulocytes = 217.6×10^{-9} versus 95.7×10^{-9} ; $p<0.001$). In both spl and unspl patients,
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11 no changes were observed in MBH with worsening of HS, though the MBH values were
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13 significantly higher in the spl patients (Table IV). The value of G3PD linked to the
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15 membrane was statistically and significantly higher in moderate and severe unspl
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17 patients, as compared to the spl cases. However, in unspl patients, membrane linked
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19 G3PD increased with the severity of HS in a statistically significant manner (Table IV).
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Discussion

HS has very heterogeneous clinical/biochemical/molecular patterns. The present work, intended to characterize a large HS population from the north region of Portugal concerning HS RBC membrane protein deficiencies. As far as we know, this is the largest study of this type in Portugal (Granjo, *et al* 2003) and the third largest in Europe (Iolascon and Avvisati 2008, Mariani, *et al* 2008).

A dominant autosomal inheritance pattern was observed in 75% (53/71) of our HS families, which is the same frequency described elsewhere (Bolton-Maggs, *et al* 2004, Delaunay 2007, Perrotta, *et al* 2008). This value is slightly higher than that previously reported by us (67%) in a smaller HS population (Rocha, *et al* 2005).

HS is clinically classified according to the severity of the anaemia and symptoms (Bolton-Maggs, *et al* 2004). After splenectomy, anaemia is corrected since the cause for premature RBC destruction is removed; thus, we assessed if the membrane protein profile of unsplenectomised *versus* splenectomised patients could also differ. We observed no statistically significant differences in the relative amounts of the major erythrocyte membrane proteins between unspl and spl patients, with the exception of MBH (Table I). As HS is a hereditary disorder, it makes sense that splenectomy is not a factor that would significantly alter membrane protein composition, since the molecular defect is the same before and after removal of the spleen, independently of RBC survival in circulation.

We found that 45%, 38% and 18% of the patients presented mild, moderate or severe HS, respectively. All clinical outcomes were observed in all types of protein deficiencies, with the exception of protein 4.2, as only two patients were found with this protein deficiency. This is in accordance with the literature (Bolton-Maggs, *et al* 2004, Delaunay 2007, Perrotta, *et al* 2008) however, the prevalence of mild, moderate and severe HS in our studied population differs from others, which present moderate HS as the most common presentation (Perrotta, *et al* 2008).

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3 Studies involving individuals of northern European ancestry have reported ankyrin
4 followed by band 3 deficiencies (isolated or combined) as the most common molecular
5 defects underlying HS and, less frequently, spectrin and protein 4.2 deficiencies
6 (Bolton-Maggs, *et al* 2004, Delaunay 2007, Perrotta, *et al* 2008). In our population, the
7 majority of the HS cases were due to band 3 primary protein deficiency (65%) followed
8 by ankyrin (24%), spectrin (10%) and protein 4.2 (1%) molecular defects. This
9 prevalence, reflecting the distribution of the ethnic groups living in the north of Portugal,
10 is similar to the reported for the southern region of Europe, in a study of 300 Italian HS
11 patients (Mariani, *et al* 2008). Possibly, there are different patterns in clinical outcome
12 and in molecular defects underlying HS, between northern and southern Europe.
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16 We confirmed the hypothesis proposed in our previous work (Rocha, *et al* 2005) that
17 an unbalance between protein deficiency values (observed in combined protein
18 deficiencies) was likely to be responsible for an increased membrane destabilization
19 and, therefore, could explain a more severe clinical pattern (Table II). Actually, in both
20 studies, we found that similar values of protein combined deficiencies, accounting for a
21 more stable membrane structure, were observed in mild HS cases; when those values
22 were increasingly different, the patients presented moderate to severe HS. Moreover,
23 in the present work, we also demonstrated that for isolated protein deficiencies the
24 value of protein deficiency increased with HS severity (Table II). Interestingly, protein
25 4.2, which is believed to play a crucial role in membrane stabilization (Satchwell, *et al*
26 2009), presented a deficiency value that increased with HS severity in almost all
27 molecular defects (Table II).
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51 It is known that methaemoglobin (metHb), resulting from a progressive oxidation of
52 haemoglobin, links to the cytoplasmatic domain of band 3 (Kay 1978, Kay 1984, Lutz,
53 *et al* 1987). In the spleen, the erythrocytes are submitted to mechanical stress. This,
54 favours membrane vesiculation of erythrocytes and, eventually, its sequestration;
55 moreover, this will further stress the metabolism of the HS cells (Caprari, *et al* 1992,
56 Kiefer and Snyder 2000, Mansouri and Perry 1987). In agreement, we found that MBH
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3 was increased in all HS patients when compared to controls (Tables I and IV),
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5 especially in the spl patients. In these patients, the HS RBCs have a longer lifespan, as
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7 the spleen as been removed, and, therefore, to maintain membrane integrity, are
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9 metabolically stressed cells, accumulating oxidative changes, as occurs along RBC
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11 aging or under oxidative stress conditions (Santos-Silva, *et al* 1998). Moreover, we
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13 found that a statistically significant positive correlation between oxidized haemoglobin
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15 (MBH) and Hb concentration (Fig 2A) only occurred in spl patients. This suggests that
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17 Hb oxidation is dependent not only in the amount of Hb in the cell but also on the
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19 erythrocyte lifespan, along which the oxidized Hb accumulates bound to the
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21 membrane.
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25 The increase of erythrocyte membrane G3PD that we found in our patients (Table I),
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27 has been reported in HS and in other pathologies (Antonelou, *et al* 2003, Margetis, *et*
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29 *al* 2007, Orntoft and Clausen 1994, Rocha, *et al* 2008). Considering that membrane
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31 structure is altered in HS, it may interfere with the release of G3PD into the cytoplasm,
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33 thus, inactivating and/or hindering the enzyme's activity in the cytoplasm, where it is
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35 believed to be important against free radical haemoglobin attack, and, therefore, it may
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37 contribute to the accumulation of oxidized Hb (Mallozzi, *et al* 1995, Schrier 1966).
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39 Furthermore, it was reported that G3PD activity is stimulated by oxyhaemoglobin
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41 (Brookes, *et al* 1997). Actually, we found that Hb concentration and the G3PD linked to
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43 the membrane were inversely correlated in a statistically significant manner in unspl
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45 patients (Fig 2B), suggesting that the reduction of oxyhaemoglobin in the cell may
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47 contribute to the non release of G3PD from the membrane. Interestingly, the value of
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49 membrane linked G3PD in unspl HS patients seems to increase with HS clinical
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51 severity (Table IV), suggesting that this value or the lack of activity of this enzyme, are
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53 potential markers of HS clinical outcome. It has been reported that erythrocytes from
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55 HS patients contain more G3PD in the membrane and that this could be related to the
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57 age of the RBCs, presenting younger erythrocytes higher membrane content of G3PD
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59 (Orntoft and Clausen 1994). In our study, we found a statistical significant positive
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3 correlation between membrane linked G3PD and reticulocyte count ($r=0.500$; $p<0.001$)
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5 in the unspl patients that was not observed in spl patients. This, further suggests a
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7 disturbance in G3PD release from the membrane; indeed, both unspl and spl patients
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9 presented similar values of membrane G3PD (Table I) but, in the spl patients this value
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11 did not correlate with the number of reticulocytes and in these patients reticulocyte
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13 count was significantly lower than in unspl patients.
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16 The increase in bound Hb, G3PD and band 8 in RBC membranes of HS patients was
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18 reported by Margetis *et al.* (2007) (Margetis, *et al* 2007). We believe that band 8 is
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20 Prx2, as in a previous study from our group, we were able to identify a protein with the
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22 same molecular weight, as Prx2 (Fig 1) (Rocha, *et al* 2008). Recently, we reported that
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24 this linkage of Prx2 to the membrane seems to occur with oxidative stress development
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26 within the HS erythrocytes (Rocha, *et al* 2009). This is in accordance with the findings
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28 of the present study, as the linkage of Prx2 to RBC membrane appeared to be also
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30 associated to oxidative stress. Indeed, we observed that most of the HS patients with
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32 detectable Prx2 in the membrane, also presented higher MBH values (Table III), which
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34 has been reported as a good marker of Hb oxidation (Coimbra, *et al* 2006, Costa, *et al*
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36 2008, Santos-Silva, *et al* 1995, Santos-Silva, *et al* 1998).
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40 In conclusion, we report that band 3 and ankyrin account for the majority of erythrocyte
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42 protein membrane defects underlying HS in a large population from the north region of
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44 Portugal. An increasing isolated protein deficiency or increasing unbalance between
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46 combined protein deficiencies seem to underlie HS severity, by increasing membrane
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48 destabilization. We propose that further and more extensive studies about the
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50 cytoplasmatic proteins that are able to link to the RBC membrane, such as G3PD,
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52 metHb and Prx2, should be performed to clarify their contribution to membrane
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54 destabilization. **Finally, it is noteworthy that, as HS is a genetic disease, in the**
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56 **future, molecular analysis will facilitate the elucidation of the genotype-**
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58 **phenotype interactions, as well as, the influence of genetic modifiers in HS**
59
60 **clinical outcome.**

Acknowledgments

This study was supported by a PhD grant (SFRH/BD/22442/2005) attributed to S. Rocha by Fundação para a Ciência e Tecnologia (FCT) and Fundo Social Europeu (FSE). For blood collection we thank nurses team from Consulta de Pediatria do Hospital S. João, Serviço de Hematologia Laboratorial do Hospital Geral Santo António and laboratory technician Elisabete Machado from Serviço de Hematologia do Hospital de Crianças Maria Pia, Centro Hospitalar do Porto.

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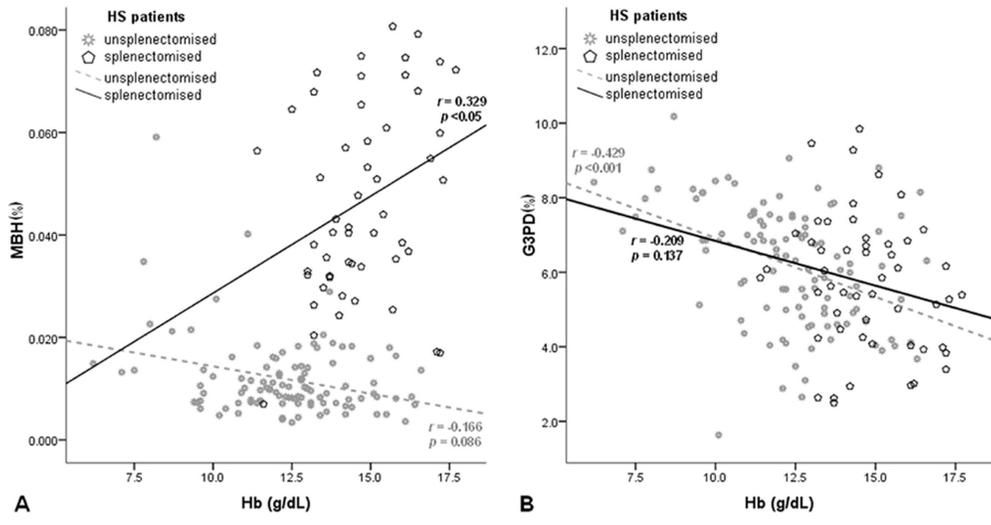
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Figure Captions

Figure 1 – Electrophoretic profile of the major erythrocyte membrane proteins for one HS patient (P) **presenting band 3 and protein 4.2 combined protein deficiencies** , in duplicate, and for two control samples (C1 and C2), in a linear gradient (5-15%) gel stained with Coomassie blue. **a** – α -spectrin; **b** – β -spectrin and ankyrin; **c** – band 3; **d** – protein 4.1; **e** – protein 4.2; **f** – actin; **g** – glyceraldehyde-3-phosphate dehydrogenase; **h** – stomatin/tropomyosin; **i** – peroxiredoxin 2 (22kDa); **j** – globin chains.

Figure 2 – Haemoglobin (Hb) concentration *versus* **(A)** membrane bound haemoglobin (MBH) and **(B)** membrane linked glyceraldehyde-3-phosphate dehydrogenase (G3PD).

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Table 1 – Major erythrocyte membrane protein profile (%) and membrane bound haemoglobin for control and HS samples, according to the most likely protein defect underlying HS.

| | α -Spectrin | β -Spectrin | Total Spectrin | Ankyrin | Band 3 | Protein 4.1 | Protein 4.2 | Actin | G3PD | Stomatin Tropomyosin | MBH $\times 10^{-2}$ (%) |
|--------------------------|--------------------------|--------------------------|--------------------------|-----------------------|--------------------------|----------------------|-----------------------|----------------------|-----------------------|--------------------------|-----------------------------|
| Control (n=24) | 14.2 (13.8 – 14.9) | 14.0 (13.4 – 14.8) | 28.3 (27.3 – 29.5) | 7.0 (6.3 – 7.5) | 39.2 (38.1 – 40.9) | 7.8 (7.5 – 8.3) | 5.4 (5.2 – 5.7) | 6.4 (6.2 – 6.7) | 3.7 (3.3 – 4.0) | 1.7 (1.4 – 1.9) | 0.58 (0.47 – 0.69) |
| Protein defect | | | | | | | | | | | |
| Band 3 | | | | | | | | | | | |
| unspl (n=73) | 15.4*** (14.8 – 16.1) | 15.6*** (14.9 – 16.4) | 31.0*** (29.6 – 32.4) | 7.9*** (7.3 – 8.6) | 34.4*** (33.3 – 35.9) | 8.03* (7.6 – 8.5) | 4.6*** (4.3 – 4.9) | 6.5 (6.0 – 7.1) | 6.0*** (4.6 – 7.2) | 1.5* (1.4 – 1.7) | 1.00*** (0.72 – 1.43) |
| spl (n=36) | 15.6*** (14.4 – 16.8) | 16.1*** (14.4 – 16.8) | 31.9*** (30.0 – 33.3) | 8.1*** (7.2 – 8.8) | 33.0*** (31.3 – 35.5) | 8.6** (7.6 – 8.8) | 4.5*** (4.2 – 4.7) | 7.0** (6.2 – 7.5) | 5.5*** (4.3 – 7.0) | 2.1***§§§ (1.7 – 2.5) | 4.23***§§§ (3.45 – 5.92) |
| Ankyrin | | | | | | | | | | | |
| unspl (n=25) | 12.4*** (11.9 – 13.7) | 12.3*** (12.1 – 12.9) | 24.9*** (24.1 – 26.3) | 6.6 (6.2 – 7.4) | 40.5 (39.1 – 41.1) | 8.0 (7.4 – 8.7) | 4.8*** (4.4 – 5.2) | 6.2 (6.1 – 6.5) | 6.9*** (5.7 – 7.8) | 1.9 (1.5 – 2.3) | 0.91*** (0.77 – 1.37) |
| spl (n=10) | 12.8** (12.2 – 14.4) | 12.7** (11.9 – 14.3) | 25.2** (24.2 – 28.8) | 6.2* (5.8 – 6.9) | 41.5* (38.7 – 43.2) | 8.5 (7.5 – 8.7) | 4.8*** (4.5 – 5.1) | 6.1 (5.7 – 6.5) | 5.8*** (4.2 – 6.6) | 1.7 (1.5 – 1.9) | 4.94***§§§ (2.58 – 7.10) |
| Spectrin | | | | | | | | | | | |
| unspl (n=9) | 13.2*** (11.9 – 13.6) | 12.8** (11.8 – 13.6) | 26.0*** (23.5 – 27.1) | 7.4 (6.7 – 7.8) | 39.0 (36.9 – 40.4) | 8.1 (7.6 – 8.2) | 5.3 (4.9 – 5.4) | 6.2 (5.4 – 6.7) | 7.5*** (5.8 – 8.4) | 1.8 (1.5 – 2.6) | 1.24*** (0.83 – 1.46) |
| spl (n=5) | 12.6** (10.7 – 13.3) | 12.6** (10.8 – 13.1) | 25.1** (21.5 – 26.4) | 7.5 (6.8 – 8.0) | 38.9 (37.2 – 29.9) | 9.5** (8.0 – 9.9) | 5.7 (4.8 – 6.3) | 6.9 (6.1 – 7.8) | 6.1** (5.0 – 7.0) | 2.1* (1.9 – 2.5) | 5.12*** (1.83 – 7.31) |
| Protein 4.2 | | | | | | | | | | | |
| unspl (n=1) | 13.5 | 13.4 | 26.9 | 6.6 | 41.3 | 8.1 | 5.0 | 6.3 | 3.9 | 1.9 | 0.50 |
| spl (n=1) | 17.4* | 14.4 | 31.2 | 6.9 | 38.3 | 7.2 | 5.0 | 4.8* | 4.0 | 2.2 | 1.72* |

Data presented as median (inter-quartil range)

unspl – unsplenectomised HS patients; spl – splenectomised HS patients; G3PD – glyceraldehyde-3-phosphate dehydrogenase; MBH – membrane bound haemoglobin

*p<0.05, **p<0.01, ***p<0.001 HS vs. control; §§§ p<0.001 spl vs. unspl

Table II – Protein deficiency values (%) for patients with isolated protein deficiencies and protein deficiencies (%) and balance between protein deficiencies values for patients with combined protein deficiencies according to HS clinical severity.

| | <i>Isolated Protein Deficiency</i> | | | <i>Combined Protein Deficiencies</i> | | | | | | |
|--------------------|------------------------------------|---------------------|---------------|--------------------------------------|-----------------------------------|----------------------------------|---------------------------------------|----------------------|----------------------|-----------------------|
| | Band 3 | Spectrin | Protein 4.2 | Band 3 + Protein 4.2 | | | Ankyrin and/or Spectrin + Protein 4.2 | | | |
| | | | | band 3 | protein 4.2 | 1- Bd3/pt4.2 | ankyrin | spectrin | protein 4.2 | 1- sp/pt4.2 |
| Mild HS | 11.9 (8.9 – 16.4) (n=16) | 12.2 ± 3.3 (n=5) | 13.8 (n=1) | 14.6 (11.7 – 18.6) (n=36) | 14.0 (12.2 – 17.6) (n=36) | 0.21 (0.11 – 0.45) (n=36) | 15.8 ± 4.9 (n=7) | 11.9 ± 3.6 (n=13) | 13.6 ± 3.2 (n=13) | 0.24 ± 0.14 (n=12) |
| Moderate HS | 14.2 (9.9 – 18.0) (n=4) | 13.7 ± 0.7 (n=3) | 20.8 (n=1) | 13.0 (10.2 – 17.7) (n=38) | 17.2* (13.9 – 21.9) (n=38) | 0.32 (0.17 – 0.53) (n=38) | 13.2 ± 3.5 (n=6) | 9.2 ± 2.2 (n=14) | 14.1 ± 4.1 (n=14) | 0.38 ± 0.20 (n=14) |
| Severe HS | - | 16.0 ± 6.4 (n=6) | - | 11.7 (7.7 – 14.8) (n=15) | 21.1** (18.2 – 24.5) (n=15) | 0.46* (0.26 – 0.56) (n=15) | 9.8 ± 5.6 (n=3) | 8.0 ± 4.2* (n=7) | 15.2 ± 7.6 (n=7) | 0.48 ± 0.23* (n=7) |

Data presented as median (inter-quartile range) or as mean ± SD

|1- Bd3/pt4.2| – unbalance between ratios deficits for band 3 and protein 4.2

|1- sp/pt4.2| – unbalance between ratios deficits for spectrin and protein 4.2

*p<0.05, **p<0.01 HS moderate or severe HS vs. mild HS

Table III – Detection (%) of membrane linked peroxiredoxin 2 (Prx2) and membrane bound haemoglobin (MBH) amount, according to erythrocyte protein membrane defect and to HS clinical severity.

| | Band 3 (n=109) | Ankyrin (n=35) | Spectrin (n=14) | Protein 4.2 (n=2) | Mild HS (n=72) | Moderate HS (n=60) | Severe HS (n=28) | Total HS (n=160) |
|------------------------------------|--------------------------|--------------------------|---------------------------|-----------------------------|--------------------------|------------------------------|----------------------------|----------------------------|
| Non detectable Prx2 [n (%)] | 71 (65%) | 23 (66%) | 8 (57%) | 1 (50%) | 46 (64%) | 38 (63%) | 19 (68%) | 103 (64%) |
| MBH x10⁻² (%) | 1.3 (0.7 – 3.7) | 0.9 (0.7 – 1.7) | 1.3 (0.8 – 2.6) | 1.7 | 0.8 (0.7 – 1.1) | 2.6 (0.9 – 4.4) | 2.4 (1.0 – 4.4) | 1.2 (0.8 – 3.4) |
| Detectable Prx2 [n (%)] | 38 (35%) | 12 (34%) | 6 (43%) | 1 (50%) | 26 (36%) | 22 (37%) | 9 (32%) | 57 (36%) |
| MBH x10⁻² (%) | 1.8 (1.0 – 3.5) | 1.3** (0.9 – 2.2) | 1.5 (1.0 – 5.7) | 0.5 | 1.5*** (1.1 – 1.9) | 1.7 (1.0 – 4.0) | 4.0 (1.9 – 6.9) | 1.8 (1.1 – 3.5) |

p<0.01, *p<0.001 non detectable vs. detectable

Table IV – Membrane bound haemoglobin (MBH) and glyceraldehyde-3-phosphate dehydrogenase (G3PD) values according to HS clinical severity for unsplenectomised and splenectomised HS patients.

| | MBH x10 ⁻² (%) | | G3PD value | |
|--------------------|---------------------------------|--|---|--|
| | <i>unspl HS patients</i> | <i>spl HS patients</i> | <i>unspl HS patients</i> | <i>spl HS patients</i> |
| Mild HS | 0.99 (0.72 – 1.37) (n=66) | 4.15 ^{YYY} (2.74 – 7.55) (n=6) | 5.7 (4.6 – 6.8) (n=66) | 6.0 (4.6 – 6.6) (n=6) |
| Moderate HS | 0.92 (0.77 – 1.42) (n=31) | 4.15 ^{YYY} (3.42 – 5.91) (n=29) | 6.9 ^{**} (5.1 – 7.8) (n=31) | 5.2 ^{YY} (3.9 – 6.6) (n=29) |
| Severe HS | 1.36 (0.90 – 1.62) (n=11) | 4.40 ^{YYY} (3.13 – 6.50) (n=17) | 8.0 ^{***SS} (7.1 – 8.4) (n=11) | 6.6 ^{SSYY} (5.4 – 7.3) (n=17) |

Data presented as median (inter-quartile range)

MBH – membrane bound haemoglobin

G3PD – glyceraldehyde-3-phosphate dehydrogenase

unspl – unsplenectomised HS patients; spl – splenectomised HS patients

p<0.01, *p<0.001 HS moderate or severe HS vs. mild HS

§p<0.05, §§p<0.01 HS severe HS vs. moderate HS

YYp<0.01, YYYp<0.001 unspl HS patients vs. spl HS patients