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Linkage of typically cytosolic peroxidases to erythrocyte membrane – A possible mechanism of protection in Hereditary Spherocytosis

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A R T I C L E I N F O

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A B S T R A C T

Hereditary Spherocytosis (HS) is a non-immune hemolytic anemia associated to oxidative stress (OS), namely to the linkage of cytosolic antioxidant enzymes to the erythrocyte membrane. Our aims were to evaluate erythrocyte OS changes and the membrane linkage of peroxiredoxin 2 (Prx2), glutathione peroxidase (GPx) and catalase (CAT) in unsplenectomized (*unspl*) and splenectomized (*spl*) HS patients and to search for associations with clinical severity (in *unspl* HS patients). We studied 114 HS patients (74 *unspl* and 40 *spl*) and 30 healthy individuals and we evaluated membrane bound hemoglobin (MBH), membrane lipid-peroxidation (LPO), enzymatic activities of GPx and CAT and the amounts of membrane bound Prx2, GPx and CAT, as well as, clinical and analytical parameters for characterization of HS. We found that *unspl* HS patients showed clear signs of anemia and in *spl* HS, a correction to this anemia was observed; the latter patients presented higher levels of OS biomarkers, namely, MBH and LPO. CAT was detected in the membrane of all individuals (control and HS groups), while GPx and Prx2 were only present in HS patients; moreover, their linkage to the membrane (in HS) appears to be cumulative since membrane bound peroxidases amount was higher as the number of peroxidases detected increased. MBH increased with the number/amount of membrane bound peroxidases, however LPO levels remained similar. In conclusion, our data suggest that the binding of these typically cytosolic peroxidases to erythrocyte membrane may be part of a mechanism of membrane protection to maintain its integrity by possibly regulating LPO.

1. Introduction

Hereditary Spherocytosis (HS) is a non-immune hemolytic anemia caused by molecular defects in either one of the genes that encode four erythrocyte membrane proteins – spectrin, ankyrin, band 3 and protein 4.2. The defect in one of these proteins (primary protein deficiency) may lead to secondary protein deficiencies in proteins with which that protein interacts [1]. These proteins are involved in the attachment of the lipid bilayer to the cytoskeleton network (vertical interaction); a deficiency in one or more of those proteins leads to membrane destabilization, favoring membrane vesiculation [2–4]. This loss of membrane portions mainly occurs when red blood cells (RBCs) traverse the splenic microvasculature, leading to the formation of more dense and rigid cells, the spherocytes, which are more prone to removal by splenic macrophages.

The rate of removal/hemolysis determines the severity of the

Abbreviations: HS, Hereditary Spherocytosis; OS, oxidative stress; Prx2, peroxiredoxin 2; GPx, glutathione peroxidase; CAT, catalase; *unspl*, unsplenectomized; *spl*, splenectomized; MBH, membrane bound hemoglobin; LPO, lipid-peroxidation; RBC, red blood cells; Hb, hemoglobin; CHT, cryohemolysis test; OFT, osmotic fragility test; G3PD, glyceraldehyde-3-phosphate dehydrogenase; metHb, methemoglobin; GSH, glutathione; Trx, thioredoxin; NADPH, reduced nicotinamide adenosine dinucleotide phosphate; UI, unbalance index; RPI, reticulocyte production index; EPO, erythropoietin; EDTA, ethylenediaminetetra-acetic acid; MCV, mean cell volume; MCHC, mean cell hemoglobin concentration; RDW, red cell distribution width; TBARS, thiobarbituric acid reactive substances

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anemia; thus, hemoglobin (Hb) concentration, reticulocyte number and bilirubin levels are hallmarks of HS clinical outcome [3]; splenectomy usually corrects the anemia and, consequently, the symptoms experienced by the patients [2–4]. Cryohemolysis (CHT) and osmotic fragility tests (OFT) are used as diagnosis tools for HS, as they reflect erythrocyte membrane destabilization/fragility [5,6].

In previous works [1,7], using standardized electrophoretic membrane protein analysis to identify and quantify protein deficiencies, we showed that the protein deficit (for isolated protein deficiencies) or the unbalance between primary and/or secondary protein deficiencies ratios were associated to HS clinical severity. These evaluations are helpful in providing additional data about clinical outcome of HS [3,4]. The electrophoretic profiling of RBC membrane proteins allows the quantification of the major erythrocyte membrane proteins, including glyceraldehyde-3-phosphate dehydrogenase (G3PD), which has been proposed as a marker of membrane destabilization and of oxidative stress (OS) [8–10].

Due to its function as oxygen (O_2) transporter, Hb is a target for OS damage; when O_2 dissociates from Hb, about 3% suffers auto-oxidation, from which results the production of superoxide anion that, after dismutation, generates hydrogen peroxide (H_2O_2). The other byproduct of Hb auto-oxidation is methemoglobin (metHb), which is a non-functional form of Hb that can be further oxidized to hemichromes (denatured Hb precipitates), if not regenerated by RBC's metHb reductases. The accumulation of metHb and H_2O_2 might lead to production of other oxygen species, such as hydroxyl radical and ferrylhemoglobins that are able to start lipid-peroxidation (LPO) on the erythrocyte membrane [11–13].

To counteract the deleterious effects of reactive oxygen species (ROS), the RBC is equipped with a great variety of antioxidant agents, namely, glutathione (GSH), vitamins C and E, and antioxidant enzymes, such as, superoxide dismutase, catalase (CAT), glutathione peroxidase (GPx) and peroxiredoxin 2 (Prx2) [14,15]. Prx2 and GPx are believed to detoxify the cell of low levels of H_2O_2, whereas CAT seems to play an important role in detoxifying high H_2O_2 amounts, of exogenous and/or endogenous origin; Prx2 and GPx are also capable of reducing hydroperoxides and peroxynitrites [15–20]. These enzymes have different turnover and activity mechanisms. CAT appears to act independently [18] whereas, GPx activity is dependent on glutathione reductase and NADPH for regeneration [19,21,22]. Similarly, Prx2, a typical 2-cys peroxiredoxin, with a thiol-dependent H_2O_2 scavenger activity, depends on thioredoxin (Trx)/Trx reductase/NADPH reducing system; Prx2 is active in its reduced monomeric form that, when performing ROS scavenging, is oxidized to an inactive dimer, until regeneration to the reduced monomeric form by Trx [14,20,21,23]. The linkage of Prx2 to the erythrocyte membrane has been reported by several authors [1,7,8,24–29], while very few studies described the same for GPx [25,29,30] and CAT [25,31,32].

The role of these antioxidant enzymes, commonly cytosolic proteins, in the erythrocyte membrane remains unclarified. In HS, with erythrocytes presenting a destabilized membrane structure, some studies have shown oxidative changes in RBC membrane, namely, increased membrane bound hemoglobin (MBH) [7,33], membrane bound Prx2 [1,7,8] and membrane bound CAT [31]; however, the connection (if any) of these oxidative changes with the clinical severity of HS is still unknown. Furthermore, how RBCs from HS splenectomized patients face these oxidative challenges along their life span is still poorly elucidated.

Our aims were to evaluate oxidative changes in RBCs from unsplenectomized (*unspl*) and splenectomized (*spl*) HS patients and to evaluate possible associations with clinical severity in the *unspl* HS patients, as well as, clarify the linkage of Prx2, GPx and CAT to erythrocyte membrane. Thus, we studied, as biomarkers of OS changes in RBCs, membrane bound hemoglobin (MBH), membrane LPO, GPx (whole cell) and CAT (membrane) enzymatic activities and G3PD, Prx2 (dimer and monomer forms), GPx and CAT membrane amounts; erythrocyte membrane destabilization/fragility was assessed by protein deficiencies underlying HS (unbalance index (UI) determined by isolated or ratio between protein deficits), CHT and OFT; Hb concentration, hematimetric indexes, reticulocyte number, reticulocyte production index (RPI), erythropoietin (EPO) and bilirubin plasma levels were measured as anemia severity biomarkers.

2. Materials and methods

2.1. Subjects

This study followed a protocol approved by the Ethic Committees of the participant Hospitals (Hospital Maria Pia, Hospital Santo António from Centro Hospitalar do Porto and Hospital S. João, Porto). All subjects (or their legal representative) gave their informed consent to participate in the study.

We studied 144 individuals from the north region of Portugal, including 30 healthy individuals (the control group) and 114 subjects diagnosed with HS, according to hematological and biochemical data and to clinical/family history; 74 HS patients were unsplenectomized (*unspl*) and 40 were splenectomized (*spl*). The three groups were matched for gender but not for age (43%/57%, 46%/54% and 38%/62% male/female and 22 ± 14, 16 ± 15 and 30 ± 14 years for control, *unspl* and *spl* patients groups, respectively).

All the *unspl* patients presented clear signs of hemolytic anemia and were classified as presenting Mild ($n = 47$) or Moderate/Severe ($n = 27$) HS, according to the *Guidelines for the diagnosis and clinical management of Hereditary Spherocytosis – 2011 Update* [3]. The *spl* patients were submitted to splenectomy more than one year before this study; the *unspl* severe patients did not receive blood transfusions in the 4 months before this study. To exclude immune hemolysis, a direct antiglobulin test was performed for all patients [34].

The identification and quantification of membrane protein deficiencies underlying each case of HS was performed prior to this study, by standard methods [1]. Spectrin, ankyrin and band 3 protein defects were identified in 11 (7 *unspl* and 4 *spl*), 25 (16 *unspl* and 9 *spl*) and 80 (53 *unspl* and 27 *spl*) HS patients, respectively; thus, the prevalence of these membrane protein deficiencies underlying HS was 9.5%, 21.5% and 69%, respectively. This frequency was similar for all HS groups: total patients, *unspl* patients or *spl* patients (Pearson Chi-Square frequency analysis: $p = .970$).

2.2. Assays

2.2.1. Blood sample processing

Blood samples (using EDTA and lithium-heparin as anticoagulants) were collected and processed in order to obtain whole blood (EDTA), plasma (lithium-heparin) and erythrocyte membrane suspensions (EDTA). Plasma samples were obtained from whole blood by centrifugation (1100 *g*, 10 min, 4 °C) and stored at −80 °C until assayed. To ensure the minimal variability in RBC membrane preparation, the protocol was performed by the same individual during the course of this study. To prepare erythrocyte membranes, plasma and leukocytes were isolated from RBCs and discarded after centrifugation, using a double density gradient (Histopaque 1.077 and 1.119, Sigma-Aldrich). Erythrocytes were washed in saline solution and, afterwards, they were submitted to hypotonic lyses, according to Dodge et al. [35]. The membranes were washed in Dodge buffer, adding phenylmethylsulphonyl fluoride in the first two washes, as a protease inhibitor (0.1 mM, final concentration).

2.2.2. Hematological and biochemical analysis

The hemoglobin (Hb) concentration, mean cell volume (MCV), mean cell hemoglobin concentration (MCHC) and red cell distribution width (RDW) were determined using an automated blood cell counter (Sysmex K1000). Reticulocyte count was evaluated by light-microscopy

counting on blood smears, after vital staining with new-methylene blue (Reticulocyte stain, Sigma-Aldrich) and the reticulocyte production index (RPI) was calculated according to Hillman & Ault [36].

Plasma total bilirubin concentration was measured in an auto-analyzer (Cobas Mira S, Roche), using a colorimetric method (Bilirubin kit, Randox Laboratories Ltd.,) and erythropoietin (EPO) plasma levels were determined using an enzyme-linked immunosorbent assay (EPO ELISA kit, Roche).

2.2.3. Cryohemolysis (CHT) and osmotic fragility (OFT) tests

The CHT was performed on fresh blood (EDTA) and the percentage of hemolysis calculated according to Streichman et al. [6]. The OFT was carried out on fresh and after 24 h of incubation at 37 °C of whole blood (lithium-heparin), according to Roper et al. [37]; the results are presented as the NaCl concentration that causes 50% hemolysis (P50).

2.2.4. Enzymatic activities of erythrocyte glutathione peroxidase (GPx) and catalase (CAT)

We determined the enzymatic activity of CAT in erythrocyte membranes using a commercially available spectrophotometric method (Catalase Assay Kit, Cayman Chemical Company). CAT activity was expressed as U/mg of sample total protein, in order to standardize data. Enzymatic activity of GPx was evaluated in whole blood (lithium-heparin), using a spectrophotometric method (RANSEL kit, Randox Laboratories Ltd) and was expressed as U/g of Hb, in order to standardize data. This GPx assay was not sensitive enough to quantify GPx activity in membrane samples. We were not able to evaluate Prx2 enzymatic activity.

The protein concentration of RBC membrane suspensions was determined by the Bradford's method [38].

2.2.5. Membrane bound hemoglobin (MBH)

MBH was measured in RBC membrane suspensions by spectrophotometry, after protein dissociation with Triton X-100 (5% *w/v* in Dodge buffer). The absorbance was measured at 415 nm and this value was corrected by the background absorbance at 700 nm; this value and protein concentration were used to estimate MBH percentage [29].

2.2.6. Erythrocyte membrane lipid-peroxidation (LPO)

Erythrocyte membrane LPO was determined using the thiobarbituric acid (TBA) test, according to Mihara and Uchiyama [39], with slight modifications. Briefly, 40.0 µl of membrane suspension was incubated with Triton X-100 (20% *w/v* in Dodge buffer) for 10 min at room temperature for protein dissociation, and then mixed with 180.0 µl of 1% H_3PO_4 (*v*/v) and 60.0 µl of 0.6% TBA (*w/v*) and heated in boiling water for 45 min. After immersion in ice for 10 min, TBA reactive substances (TBARS) were extracted with butanol (240.0 µl) and then measured by spectrophotometry (535 and 520 nm). The ratios of TBARS/total protein (mg) for each RBC membrane suspension sample were calculated to obtain normalized data.

2.2.7. Preparation of erythrocyte membrane suspensions for electrophoretic analysis

Erythrocyte membrane suspensions for SDS-PAGE and western-blot (reducing conditions) were treated with an equal volume of loading buffer [0.125 M Tris-HCl pH 6.8, 4% sodium dodecylsulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol] and heat denatured. To evaluate the redox form of Prx2 in the RBC membranes by western-blot, non-reducing conditions were used to prepare membrane samples (loading buffer without the reducing agent and heat denatured).

2.2.8. Electrophoretic studies for identification and quantification of RBC membrane proteins

RBC membrane proteins were separated by SDS-PAGE, using a 5–15% linear acrylamide gradient gel (8 µg protein/lane) and a 3.5–17% exponential acrylamide gradient gel (6 µg protein/lane), according to Laemmli [40] and Fairbanks et al. [41], respectively. Proteins were stained with Coomassie brilliant blue, and scanned (Darkroom CN UV/wl, *BioCaptMW version 99*, Vilber Lourmat). The relative amount of each major protein (percentage of total), was quantified by densitometry (*Bio1D++ version 99*, Vilber Lourmat). These studies allowed the identification and quantification of the major protein constituents of RBC membrane, including glyceraldehyde-3-phosphate dehydrogenase (G3PD), and the evaluation of the protein deficiency (or deficiencies) associated with each HS case [1]. The relative amount of membrane bound G3PD was obtained directly from gel image analyses and the values of protein deficiencies were used to calculate the Unbalance Index (UI), which is a measure of erythrocyte membrane protein structure destabilization: for isolated protein deficiencies UI = 1-(%protein deficit/100), and for combined protein deficiencies, we calculated the ratio between the values of protein deficiencies and, afterwards UI = |1- ratio| [1].

2.2.9. Immunoblot analysis of peroxiredoxin 2 (Prx2), glutathione peroxidase (GPx) and catalase (CAT)

The RBC membrane samples prepared in non-reducing conditions were subjected to SDS-PAGE, using a 5–15% linear acrylamide gradient gel (30 µg protein/lane) [40], followed by western-blotting. The Prx2 immunoblots were probed with a monoclonal antibody anti-human Prx2 produced in mouse (dilution 1:5000) (MA0144; AbFrontier) and with an anti-mouse IgG conjugated to peroxidase (Sigma-Aldrich), as the secondary antibody (dilution 1:5000). To develop the immunoblot, we used an enhanced chemiluminescence method (WesternBright ECL HRP substrate, Advansta) and ChemiDoc™ Touch Imaging System (Bio-Rad). The Prx2 linked to RBC membrane (dimer and monomer forms) was determined by densitometry (Image Lab *version* 5.2.1, Bio-Rad), as the ratio between the value of Prx2 from each sample and the value of a Prx2 internal control, which was loaded in all gels.

The GPx and CAT immunoblots were carried out as described for Prx2 with the following alterations: RBC membranes samples were prepared in reducing conditions; for GPx immunoblots we used as the primary antibody, a monoclonal antibody anti-human GPx1 produced in mouse (dilution 1:100) (sc-130,160; Santa Cruz Biotechnology, Inc.) and for CAT immunoblots, a monoclonal antibody anti-human catalase produced in mouse (dilution 1:1000) (C0979; Sigma-Aldrich); an anti-mouse IgG conjugated to peroxidase (Sigma-Aldrich) was used as secondary antibody for both immunoblots (dilution 1:1000 and 1:2000, respectively). The values of GPx and CAT were determined as the ratio between the value of GPx and CAT from each sample and the value of GPx and CAT internal controls, which were loaded in all gels.

2.3. Statistical analysis

For statistical analysis we used the IBM SPSS Statistics software, version 24.0, for Windows (SPSS Inc.). Since most of our variables presented a non-Gaussian distribution, we present all our data as median values (inter-quartile range). To evaluate the differences between groups, we used the non-parametric Kruskal-Wallis H test and, when statistical significance was achieved, single comparisons (two groups) were made by using the Mann-Whitney *U* test (continuous variables). As some of the studied parameters can be affected by subject's age and our studied groups were not matched for age, we performed a co-variance adjustment test (univariate ANCOVA) for this covariable to confirm the statistical significance of the results (non-Gaussian distributed variables were normalized prior to this analysis). Pearson Chi-Square was used for frequency analysis between groups (categorical variables). Spearman's rank correlation coefficient was employed to evaluate relationships between sets of data. A $p < .05$ value was considered as statistically significant.

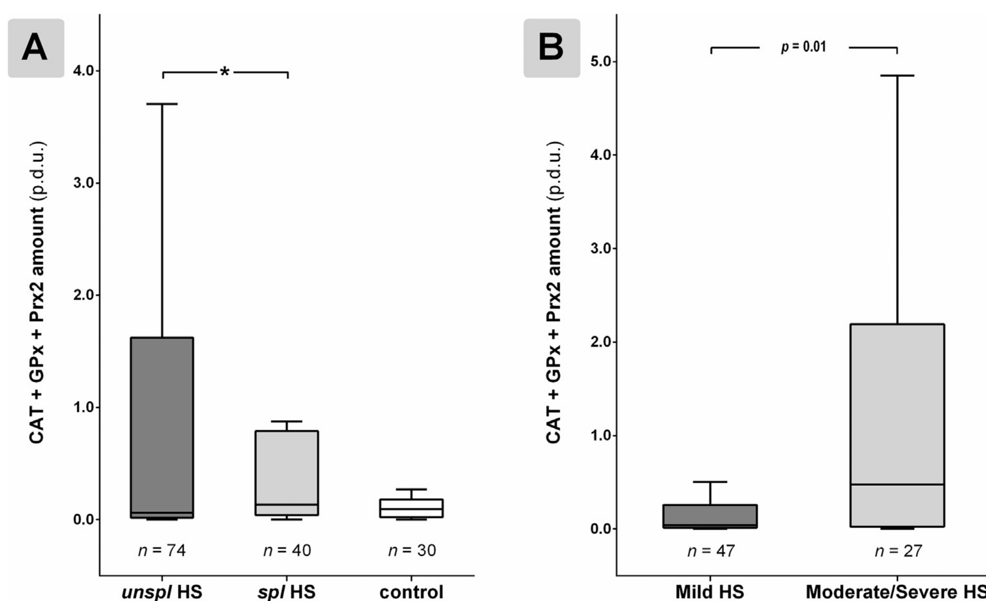

Fig. 1. Membrane detected total amount of Catalase + Glutathione Peroxidase + Peroxiredoxin 2 for: A) control, unsplenectomized and splenectomized Hereditary Sherocytosis patients groups and B) unsplenectomized HS patients according to clinical severity. Data presented as median (interquartile range). $p < .05$ value was considered as statistically significant (Mann-Whitney U test). *acquires statistical significance after adjustment for age (univariate ANCOVA). HS – Hereditary Spherocytosis; unspl – unsplenectomized; spl – splenectomized; CAT – catalase; GPx – glutathione peroxidase; Prx2 – peroxiredoxin 2; p.d.u. – procedure defined unit.

3. Results

In previous studies by our group, we found that Prx2 was linked to RBC membrane in about one third of HS patients [1,7] and that GPx was also binds to RBC membrane under induced OS conditions [29]. In the present work, we evaluated the linkage of Prx2 and of the other two cytosolic peroxidases – GPx and CAT – to the RBC membrane and we found that CAT was detected in RBC membranes of all studied individuals (control group and HS patients), while GPx and Prx2 were only detected in HS patients. In 63 (55%) HS patients CAT was the only membrane bound enzyme and the remaining HS patients (45%) presented at least two of these three enzymes linked to their RBC membranes. Specifically, membrane linked CAT + GPx was found in 18 (16%) and in 33 (29%) HS patients CAT + GPx + Prx2 were detected in the membrane. Our data show that CAT could be detected alone bound to the membrane (both in the control and HS groups), GPx was detected when CAT or CAT and PRx2 were also linked to the membrane and, finally, PRx2 was only detected when both CAT and GPx were bound to the RBC membrane.

In Fig. 1 we show the sum of the amounts of CAT, GPx and Prx2 detected in RBC membrane of control, *spl* and *unspl* HS patients groups (Fig. 1A) and of *unspl* HS patients according to disease severity – Mild and Moderate/Severe HS (Fig. 1B). The control group presented the lowest amounts of membrane-linked peroxidases, while in *spl* and, especially, in *unspl* HS patients the total amount of CAT + GPx + Prx2 greatly varied with a tendency for higher values (Fig. 2A). Analyzing the data according to HS clinical severity, we found that Moderate/Severe HS patients showed significantly higher amount of these enzymes in the membrane than Mild HS patients (Fig. 1B).

We performed the evaluation of OS, RBC membrane destabilization and fragility, hematological and biochemical parameters for control, *unspl* and *spl* HS groups (Table 1, left side). When compared to control, we found that MBH, LPO, CHT, OFT, MCHC, RDW, reticulocytes, RPI and bilirubin concentration were significantly higher for both *unspl* and *spl* HS patients. The *unspl* HS patients showed, also when compared to control, significantly higher values of EPO and G3PD, and significantly lower values of Hb and MCV, while the *spl* HS patients presented significantly higher values for Hb and similar G3PD, EPO and MCV levels, when compared to control. GPx and CAT activities presented no statistically significant differences between groups, as well as, the UI. After adjusting for subject's age, the statistical significance was lost for G3PD and MCV between *unspl* patients and control, and for Hb between *spl* patients and control.

We also showed that *spl* HS patients, when compared to *unspl* HS patients, presented significantly higher values of MBH, LPO, OFT, Hb and MCV and, significantly lower values of G3PD, RDW, reticulocytes, RPI, EPO and bilirubin; after adjusting for age, the significance for G3PD and MCV was lost (Table 1, left side).

We evaluated the relationships between the OS biomarkers on study and we found that, for control and both *unspl* and *spl* HS patients, MBH was positively correlated with CAT membrane activity ($r = 0.456$, $p = .013$, $n = 30$; $r = 0.372$, $p = .001$, $n = 74$; $r = 0.414$, $p = .009$, $n = 40$; control, *unspl* and *spl* HS groups, respectively) and with membrane bound CAT ($r = 0.489$, $p = .007$, $n = 30$; $r = 0.440$, $p < .001$, $n = 74$; $r = 0.555$, $p < .001$, $n = 40$; control, *unspl* and *spl* HS groups, respectively); whole blood GPx activity was positively correlated with CAT membrane activity ($r = 0.549$, $p = .002$, $n = 30$; $r = 0.331$, $p = .005$, $n = 74$; $r = 0.436$, $p = .005$, $n = 40$; control, *unspl* and *spl* HS groups, respectively) and CAT activity was positively correlated with membrane bound CAT ($r = 0.805$, $p < .001$, $n = 30$; $r = 0.859$, $p < .001$, $n = 74$; $r = 0.810$, $p < .001$, $n = 74$; control, *unspl* and *spl* HS groups, respectively). Moreover, we also found that for *unspl* HS patients, LPO was positively correlated with MBH ($r = 0.287$, $p = .013$, $n = 74$) and whole blood GPx activity was positively correlated with membrane bound CAT ($r = 0.402$, $p < .001$, $n = 74$). We also evaluated associations of reticulocyte number with OS biomarkers – MBH, LPO, whole-cell GPx activity, CAT membrane activity, CAT membrane amount, GPx membrane amount and Prx2 membrane amount – and found no statistically significant correlations between them in either the control, *unspl* or *spl* HS patients groups (*data not shown*).

In Table 1, we also present data according to HS clinical outcome for *unspl* patients. When comparing Mild HS patients with Moderate/Severe HS patients, we found that Mild HS patients showed significantly lower values for all studied parameters, except for LPO, Hb and MCV, with the latter two presenting significantly higher values and LPO showing similar values (Table 1, right side).

Concerning the linkage of Prx2, GPx and CAT to the RBC membrane in HS patients, we analyzed and compared the frequencies (Pearson Chi-Square) according to splenectomy, to the type of protein deficiency underlying HS and to the clinical severity of HS for individuals with membrane bound CAT, membrane bound CAT + GPx or membrane bound CAT + GPx + PRx2, respectively (Table 2). Regarding splenectomy, we found that the frequencies of *unspl* vs. *spl* HS patients were

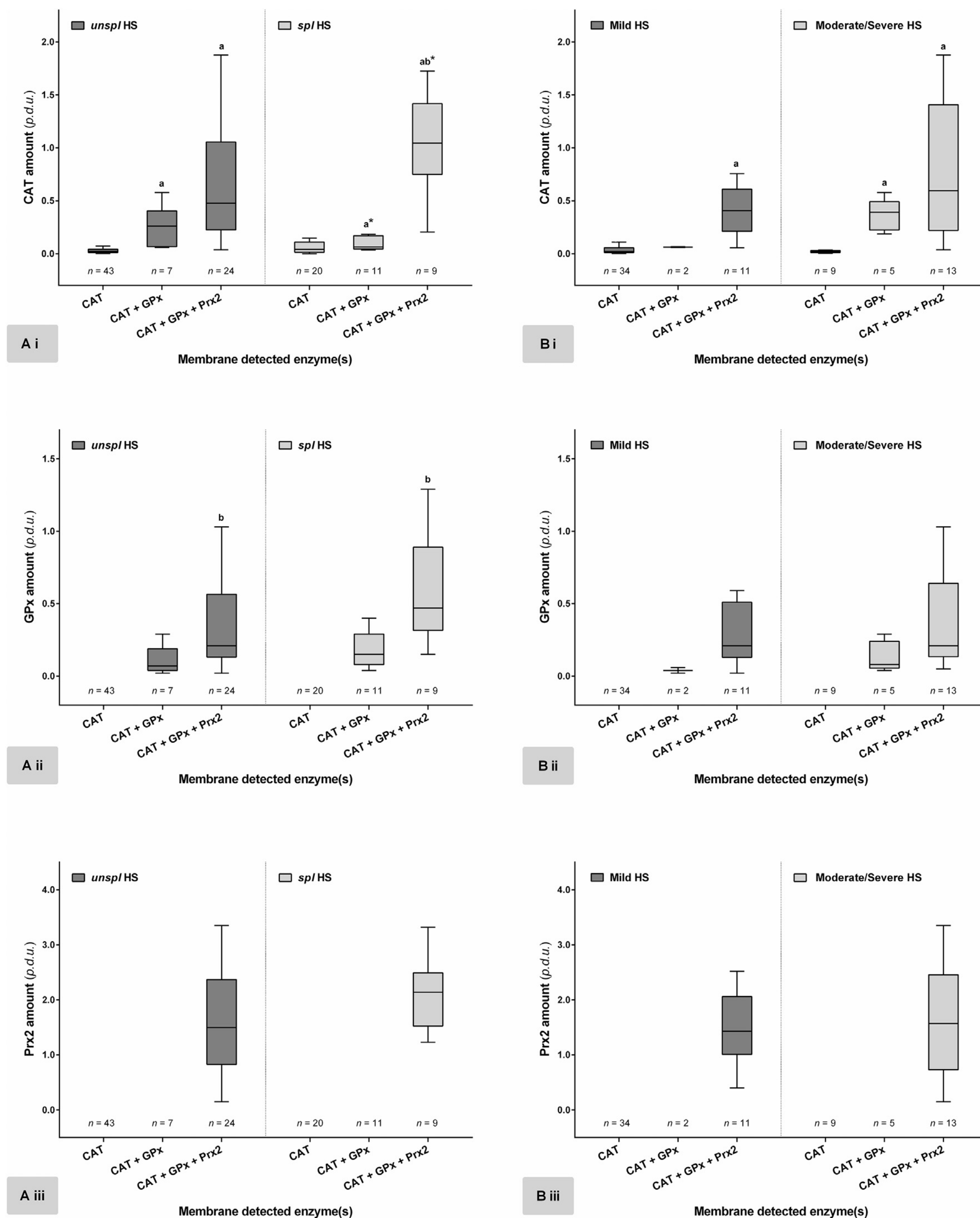

Fig. 2. Membrane detected individual amounts of catalase (i), of glutathione peroxidase (ii) and of peroxiredoxin 2 (iii) according to the number of membrane bound peroxidases for: A) unsplenectomized and splenectomized Hereditary Sherocytosis patients and B) unsplenectomized HS patients according to clinical severity. Data presented as median (inter-quartile range). $p < .05$ value was considered as statistically significant (Mann-Whitney U test): a – CAT vs CAT + GPx and CAT + GPx + Prx2 HS patients; b – CAT + GPx vs CAT + GPx + Prx2 HS patients; * – unspl HS vs. spl HS patients. HS – Hereditary Spherocytosis; unspl – unsplenectomized; spl – splenectomized; CAT – catalase; GPx – glutathione peroxidase; Prx2 – peroxiredoxin 2; p.d.u. – procedure defined unit.

significantly different between the groups with varying number of membrane detected peroxidases, with *unspl* HS patients showing higher prevalence of membrane bound CAT and of CAT + GPx + PRx2, while *spl* HS patients showed higher prevalence of membrane bound CAT + GPx. The frequencies, according to the type of protein deficiency underlying HS, were similar for HS patients with one, two or three RBC membrane bound enzymes (Table 2); we performed this analysis for total HS population, as we found that that the prevalence of protein defects responsible for HS was similar for total, *unspl* or *spl* HS patients. When evaluating frequencies according to clinical severity of HS (only applicable for *unspl* HS patients), we found a significant difference in frequencies of Mild vs. Moderate/Severe HS according to the number of membrane linked enzymes: membrane bound CAT was predominant in Mild HS cases (79%), while Moderate/Severe HS patients showed membrane CAT + GPx (71%) and CAT + GPx + PRx2 (54%) as the most prevalent membrane linked enzymes (Table 2).

Considering the role of these enzymes in the antioxidant defense of RBC and the significantly different frequencies in membrane linkage

Table 1

Oxidative stress, erythrocyte membrane destabilization/fragility, hematological and biochemical data for control, unsplenectomized and splenectomized Hereditary Spherocytosis patients groups and for unsplenectomized patients according clinical severity.

	Control (n = 30)	unspl HS (n = 74)	p(a)	spl HS (n = 40)	p(a)	p(b)	Mild HS (n = 47)	Moderate/Severe HS (n = 27)	p(c)
MBH (%)	0.78 (0.46–0.92)	1.03 (0.76–1.45)	< 0.001	4.92 (3.47–6.52)	< 0.001	< 0.001	0.98 (0.73–1.26)	1.36 (0.77–1.80)	0.028
LPO x10^{-2} (TBARS/mg$_{prot}$)	2.54 (2.06–3.28)	4.48 (3.41–8.23)	< 0.001	7.30 (5.16–22.7)	< 0.001	< 0.001	4.15 (3.20–7.53)	5.24 (3.58–10.4)	0.117
Whole Erythrocyte GPx Activity (U/gHb)	36.7 (30.7–44.2)	38.6 (24.6–52.2)	0.949	28.4 (17.7–47.8)	0.073	0.061	33.8 (24.6–46.2)	49.0 (22.2–67.4)	0.043
Erythrocyte Membrane CAT Activity (U/mg$_{prot}$)	0.43 (0.23–0.94)	0.52 (0.19–2.45)	0.589	0.50 (0.30–3.00)	0.086	0.238	0.39 (0.18–0.86)	0.88 (0.31–8.54)	0.014
G3PD membrane relative amount (%)	5.33 (4.38–5.96)	6.27 (4.78–7.04)	0.010*	5.42 (4.11–6.59)	0.530	0.038*	5.55 (4.55–6.43)	7.25 (6.84–7.87)	< 0.001
Unbalance Index	–	0.21 (0.11–0.42)	–	0.24 (0.14–0.46)	–	0.540	0.16 (0.11–0.30)	0.37 (0.21–0.57)	0.001
Cryohemolysis Test (%)	2.40 (1.68–3.54)	18.4 (4.13–31.6)	< 0.001	16.5 (10.2–22.1)	< 0.001	0.660	9.63 (1.85–25.1)	30.3 (19.1–42.1)	< 0.001
Osmotic Fragility Test (Fresh)	0.44 (0.43–0.46)	0.48 (0.45–0.50)	< 0.001	0.50 (0.49–0.51)	< 0.001	< 0.001	0.47 (0.44–0.49)	0.50 (0.48–0.52)	< 0.001
Osmotic Fragility Test (24 h incubation)	0.53 (0.51–0.56)	0.61 (0.54–0.70)	< 0.001	0.74 (0.68–0.80)	< 0.001	< 0.001	0.57 (0.52–0.65)	0.69 (0.60–0.76)	0.001
Hb (g/dL)	13.4 (12.8–14.1)	12.5 (11.5–13.3)	< 0.001	14.5 (13.4–15.6)	0.002*	< 0.001	12.8 (12.2–13.8)	11.1 (9.70–12.0)	< 0.001
MCV (fL)	88.8 (82.8–93.0)	84.0 (79.8–89.1)	0.018*	89.0 (84.5–91.4)	0.991	0.007*	86.2 (82.7–90.0)	80.7 (74.8–84.0)	< 0.001
MCHC (g/dL)	32.6 (32.2–33.2)	34.6 (33.4–36.8)	< 0.001	35.2 (34.2–36.8)	< 0.001	0.177	34.4 (33.1–35.3)	36.3 (34.5–37.4)	0.003
RDW (%)	12.8 (12.6–13.6)	16.8 (14.6–19.4)	< 0.001	14.8 (12.8–15.8)	0.001	< 0.001	15.4 (14.4–17.6)	19.3 (17.7–23.8)	< 0.001
Reticulocytes (x10^9/L)	41.5 (24.5–69.5)	197 (60.0–363)	< 0.001	83.0 (65.2–116)	< 0.001	0.001	90.0 (53.0–212)	368 (267–544)	< 0.001
RPI	0.84 (0.48–1.34)	3.09 (1.15–4.58)	< 0.001	1.65 (1.21–2.18)	< 0.001	0.002	1.66 (0.91–3.48)	4.48 (3.34–5.90)	< 0.001
EPO (mU/mL)	9.32 (7.12–12.3)	23.4 (12.2–37.4)	< 0.001	8.84 (6.46–11.8)	0.627	< 0.001	13.6 (9.42–29.9)	32.9 (24.4–114)	< 0.001
Bilirubin (μmol/L)	6.93 (5.05–10.2)	25.1 (9.84–53.4)	< 0.001	13.4 (10.3–20.9)	< 0.001	0.004	15.7 (6.67–32.3)	45.2 (34.2–74.9)	< 0.001

Data presented as median (inter-quartile range). $p < .05$ value was considered as statistically significant (Mann-Whitney U test): (a) – control group vs. HS patients; (b) – unsplenectomized vs. splenectomized HS patients; (c) – Mild vs Moderate/Severe HS patients; *loses statistical significance after adjustment for age (univariate ANCOVA).

HS – Hereditary Spherocytosis; unspl – unsplenectomized; spl – splenectomized; MBH – membrane bound hemoglobin; LPO – lipid-peroxidation; TBARS – thiobarbituric acid reactive substances; GPx – glutathione peroxidase; CAT – catalase; G3PD – glyceraldehyde-3-phosphate dehydrogenase; Hb – hemoglobin; MCV – mean cell volume; MCHC – mean cell hemoglobin concentration; RDW – red cell distribution width; RPI – reticulocyte production index; EPO – erythropoietin.

Table 2
Distribution of Hereditary Sherocytosis patients according to splenectomy, to protein defect and to clinical severity for catalase (CAT), glutathione peroxidase and catalase (CAT + GPx) and catalase, glutathione peroxidase and peroxiredoxin 2 (CAT + GPx + Prx2) detection in the erythrocyte membrane, respectively.

CAT detected (63 HS patients)	CAT + GPx detected (18 HS patients)	CAT + GPx + Prx2 detected (33 HS patients)	p (Pearson χ^2)
Frequency according to splenectomy			
43 unspl HS patients (68%)	7 unspl HS patients (39%)	24 unspl HS patients (73%)	0.038
20 spl HS patients (32%)	11 HS spl patients (61%)	9 HS spl patients (27%)	
Frequency according to protein defect underlying HS			
6 Spectrin deficiency (9%)	1 Spectrin deficiency (6%)	4 Spectrin deficiency (12%)	0.461
10 Ankyrin deficiency (16%)	4 Ankyrin deficiency (22%)	10 Ankyrin deficiency (30%)	
47 Band 3 deficiency (75%)	13 Band 3 deficiency (72%)	19 Band 3 deficiency (58%)	
Frequency according to HS clinical severity (applicable only for unspl patients)			
34 Mild HS (79%)	2 Mild HS (29%)	11 Mild HS (46%)	0.003
9 Moderate + Severe HS (21%)	5 Moderate + Severe HS (71%)	13 Moderate + Severe HS (54%)	

HS – Hereditary Spherocytosis; unspl – unsplenectomized; spl – splenectomized; $p < .05$ was considered as statistically significant (Pearson χ^2).

observed in *unspl and spl* patients, and in *unspl* HS patients with Mild or Moderate/Severe HS (Table 2), we analyzed OS, RBC membrane destabilization and fragility, hematological and biochemical data according to the type(s) and number of enzyme(s) bound the RBC membrane. The results for *unspl* and *spl* HS patients with membrane bound CAT, membrane bound CAT + GPx or membrane bound CAT + GPx + Prx2 are presented in Table 3 and Fig. 2A; for *unspl* HS patients according to the different clinical outcome, the results are presented in Table 4 and Fig. 2B.

We found that in both *unspl* and *spl* HS patients (Fig. 2A) and in both Mild and Moderate/Severe HS (Fig. 2B) the amount of membrane bound CAT (Fig. 2i) and GPx (Fig. 2ii) significantly increased with the number of membrane-bound enzymes. Concerning membrane bound Prx2, Fig. 2 presents the total amount of Prx2 linked to the membrane instead of the dimer and monomer (i.e. oxidized and reduced) forms, since we found that the frequency of the ratio dimer/monomer was similar independently of splenectomy (Pearson Chi-Square frequency analysis: $p = .543$) and of clinical outcome (Pearson Chi-Square frequency analysis: $p = .590$) – approximately 2/3 of Prx2 detected in the membrane was in the oxidized form for all groups.

When comparing *unspl* vs. *spl* HS patients, we showed that CAT amount was significantly increased in *unspl* HS patients for the CAT + GPx group and significantly decreased for the CAT + GPx + Prx2 group, showing no difference in the CAT group (Fig. 2Ai); for GPx and Prx2 amounts no statistically significant differences were observed although the *spl* HS patients exhibited a tendency for higher values (Fig. 2Aii and 2Aiii, respectively). Between Mild and Moderate/Severe HS no significant differences were found in the amounts of CAT (Fig. 2Bi), GPx (Fig. 2Bii) or Prx2 (Fig. 2Biii), even though the latter group presented with a trend to higher levels.

Both *unspl* and *spl* HS patients (Table 3) showed that erythrocyte membrane CAT activity significantly increased with the number of membrane detected enzymes; whole-RBC GPx activity was significantly decreased in CAT group, when compared with the other two groups and presented the highest value in the CAT + GPx group; MBH was significantly increased in the CAT + GPx + Prx2 group, as compared to MBH values when CAT + GPx were linked to the membrane; LPO, UI, Hb and RDW presented no significant changes between groups; and GP3D presented the highest values when CAT + GPx were linked to the membrane and the lowest when CAT alone was bound to the membrane. For *unspl* HS patients CHT, OFT, MCHC, RDW, reticulocytes, RPI, EPO and bilirubin showed the lowest values in the CAT group when compared with the two other groups and the opposite was observed for MCV; the *spl* patients showed no significant differences in these parameters (Table 3).

Concerning *unspl* HS patients according to HS severity (Table 4), we found that in Mild and Moderate/severe HS patients, MBH showed increasing values with the number of bound enzymes, presenting CAT + GPx + Prx2 group the highest values, and LPO showed similar values independently of membrane bound peroxidases; whole-RBC GPx activity showed significantly higher values in CAT + GPx + Prx2 group, as compared to CAT group; erythrocyte membrane CAT activity showed significant increasing values with the number of peroxidases detected, presenting CAT + GPx + Prx2 group a noticeably high value; UI, CHT and OFT presented a trend for the highest values in CAT + GPx group. In Mild HS patients, MCHC increased with membrane peroxidases linkage and reticulocytes were significantly higher for CAT + GPx group; in Moderate/Severe HS, RDW decreased with the increasing number of peroxidases detected in RBC membrane.

4. Discussion

HS is the most common non-immune congenital hemolytic anemia, caused by erythrocyte membrane protein deficiencies that lead to membrane destabilization and vesiculation, with development of spherocytes that are smaller and less deformable RBCs and, thus, more prone to premature sequestration and destruction in the spleen [2–4]. In this condition, the erythrocytes are metabolically stressed to assure stability and deformability of the altered membrane structure and to face the oxidative changes associated to cell ageing process. Actually, oxidative injuries and changes in RBC membrane proteins associated with cell senescence were described in RBCs from HS patients, and are particularly enhanced in *spl* patients [1,7,33]; however, how and/or if these changes influence the clinical outcome of the disease and how RBCs from *spl* HS patients deal with these changes along their longer life-span is still unclear.

Our data confirms the association of HS with OS, as showed by the significant increase in MBH and membrane LPO in HS patients, particularly in *spl* patients in relation to healthy individuals (Table 1, left side). After splenectomy, erythrocytes are able to survive longer, explaining the correction of anemia, the reduction in erythropoietic stimuli (EPO) and hemolysis (bilirubin); however, the oxidative/senescence injuries accumulate in RBCs from HS *spl* patients along their extended life-span as shown by the significantly higher values of MBH (almost four-fold) and LPO (two-old) compared to *unspl* HS patients. The CH, OF and MCHC were not improved in HS *spl* patients, once their altered values arise from the intrinsic erythrocyte membrane protein defect(s) and, in spite of correction of anemia, our data still show increased membrane destabilization and RBC removal, as suggested by the increased OF and bilirubin levels, as well as, an increased EPO stimuli, as compared to control group (Table 1, left side). Thus, in *spl* HS patients, the accumulation of OS/senescence injuries are likely to play a more important role in triggering hemolysis, than the intrinsic membrane protein defect causing membrane destabilization/fragility, in accordance with other reports [33,42]. In line with this, we found, in HS patients and controls, significant positive correlations between the OS markers: the increase in MBH was associated with an increase in membrane bound CAT and CAT activity, being the latter ones positively

Table 3
Oxidative stress, erythrocyte membrane destabilization/fragility, hematological and biochemical data for unsplenectomized and splenectomized Hereditary Spherocytosis patients according to the number of membrane bound peroxidases.

	unspl HS						spl HS					
	CAT (n = 43)	CAT + GPx (n = 7)	p(a)	CAT + GPx + Prx2 (n = 24)	p(a)	p(b)	CAT (n = 20)	CAT + GPx (n = 11)	p(a)	CAT + GPx + Prx2 (n = 9)	p(a)	p(b)
MBH (%)	0.83 (0.68–1.20)	0.97 (0.75–1.37)	0.493	1.40 (1.09–1.82)	< 0.001	0.015	4.54 * (3.40–6.40)	4.05 * (3.29–5.09)	0.591	6.45 * (4.58–7.48)	0.090	0.033
LPO x10^{-2} (TBARS/mg_{prot})	4.24 (3.20–7.74)	4.15 (3.40–40.7)	0.493	4.92 (3.65–8.28)	0.281	0.813	7.29 * (5.16–39.2)	6.26 (4.34–14.1)	0.804	8.17 * (6.33–17.6)	0.814	0.425
Whole Erythrocyte GPx Activity (U/gHb)	30.2 (20.2–42.5)	51.7 (41.4–60.9)	0.013	48.7 (33.4–68.8)	0.001	0.850	19.3 * (12.9–27.3)	48.1 (28.3–53.9)	0.003	42.0 (26.6–49.7)	0.016	0.569
Erythrocyte Membrane CAT Activity (U/mg_{prot})	0.22 (0.14–0.48)	0.80 (0.54–2.83)	0.002	5.76 (2.20–12.2)	< 0.001	0.007	0.33 (0.25–0.48)	0.70 (0.37–1.43)	0.007	9.24 (3.39–24.9)	< 0.001	< 0.001
G3PD membrane relative amount (%)	5.70 (4.71–6.79)	7.02 (4.57–7.87)	0.203	6.83 (5.21–7.37)	0.083	0.539	4.80 * (3.11–6.02)	6.47 (5.46–7.37)	0.009	5.02 (4.13–6.87)	0.322	0.138
Unbalance Index	0.18 (0.11–0.35)	0.43 (0.15–0.59)	0.097	0.26 (0.11–0.43)	0.362	0.162	0.29 (0.19–0.50)	0.19 (0.11–0.54)	0.549	0.19 (0.12–0.27)	0.109	0.425
Cryohemolysis Test (%)	8.93 (1.85–23.1)	33.4 (12.0–43.9)	0.014	30.4 (19.3–37.5)	< 0.001	0.637	16.3 (7.34–22.1)	16.3 (9.4–26.4)	0.967	19.2 (13.0–24.1)	0.451	0.470
Osmotic Fragility Test (Fresh)	0.47 (0.44–0.50)	0.51 (0.47–0.62)	0.035	0.48 (0.46–0.51)	0.193	0.186	0.50 * (0.48–0.52)	0.49 (0.49–0.51)	0.606	0.51 * (0.50–0.52)	0.211	0.110
Osmotic Fragility Test (24 h incubation)	0.57 (0.52–0.67)	0.67 (0.62–0.81)	0.016	0.62 (0.56–0.75)	0.046	0.247	0.74 * (0.68–0.77)	0.73 (0.67–0.82)	0.967	0.76 * (0.67–0.84)	0.509	0.569
Hb (g/dL)	12.7 (11.7–13.6)	12.1 (11.5–13.0)	0.476	12.0 (10.9–12.9)	0.116	0.813	14.6 * (13.7–16.2)	14.0 * (13.2–15.4)	0.386	14.7 * (13.3–15.4)	0.571	0.790
MCV (fL)	86.0 (80.0–92.8)	84.0 (73.8–87.5)	0.193	82.8 (77.9–86.0)	0.032	0.962	89.0 (84.6–92.0)	89.0 * (88.0–94.0)	0.648	88.0 (77.6–89.2)	0.084	0.039
MCHC (g/dL)	34.4 (33.2–35.3)	36.8 (35.3–37.4)	0.015	36.2 (33.5–37.8)	0.030	0.523	35.5 * (34.0–36.4)	34.6 (33.6–34.9)	0.200	36.7 (35.4–38.0)	0.109	0.020
RDW (%)	15.5 (14.4–18.2)	19.0 (16.4–21.1)	0.203	18.0 (14.8–19.6)	0.229	0.508	15.2 (13.5–16.1)	13.3 * (11.8–16.6)	0.292	14.1 * (13.2–14.6)	0.050	0.879
Reticulocytes (x10^9/L)	126 (57.0–290)	361 (260–630)	0.003	297 (108–410)	0.052	0.156	76.5 (59.0–104)	77 * (52–116)	0.901	107 * (91–126)	0.059	0.119
RPI	2.27 (0.95–4.23)	4.46 (4.27–7.36)	0.005	3.71 (1.24–5.17)	0.147	0.073	1.51 (1.12–1.93)	1.51 * (0.99–2.28)	0.804	2.10 (1.65–2.36)	0.109	0.382
EPO (mU/mL)	14.8 (10.7–27.3)	33.5 (23.9–76.2)	0.014	30.4 (14.9–44.6)	0.012	0.479	8.31 * (6.36–10.6)	9.34 * (8.00–11.9)	0.375	9.93 * (5.14–14.4)	0.944	0.790
Bilirubin (μmol/L)	20.5 (7.35–45.2)	24.6 (19.0–66.7)	0.219	41.9 (15.8–71.0)	0.048	0.777	13.8 (10.5–23.1)	13.7 * (6.67–15.4)	0.363	13.2 * (8.04–16.1)	0.540	0.648

Data presented as median (inter-quartile range). $p < .05$ value was considered as statistically significant (Mann-Whitney U test): (a) – CAT vs CAT + GPx and CAT + GPx + Prx2 HS patients; (b) – CAT + GPx vs CAT + GPx + Prx2 HS patients; * – unspl HS vs. spl HS patients.
HS – Hereditary Spherocytosis; unspl – unsplenectomized; spl – splenectomized; MBH – membrane bound hemoglobin; LPO – lipid-peroxidation; TBARS – thiobarbituric acid reactive substances; GPx – glutathione peroxidase; CAT – catalase; G3PD – glyceraldehyde-3-phosphate dehydrogenase; Hb – hemoglobin; MCV – mean cell volume; MCHC – mean cell hemoglobin concentration; RDW – red cell distribution width; RPI – reticulocyte production index; EPO – erythropoietin.

Table 4
Oxidative stress, erythrocyte membrane destabilization/fragility, hematological and biochemical data for unsplenectomized Hereditary Spherocytosis patients according to clinical severity and to the number of membrane bound peroxidases.

	Mild HS						Moderate + Severe HS					
	CAT (n = 34)	CAT + GPx (n = 2)	p(a)	CAT + GPx + Prx2 (n = 11)	p(a)	p(b)	CAT (n = 9)	CAT + GPx1 (n = 5)	p(a)	CAT + GPx + Prx2 (n = 13)	p(a)	p(b)
MBH (%)	0.83 (0.67–1.09)	0.93 (0.86 –)	0.534	1.17 (1.09–1.80)	0.001	0.060	0.92 (0.66–1.66)	0.97 (0.72–1.39)	0.947	1.59 (1.10–2.44)	0.095	0.055
LPO x10^{-2} (TBARS/mg_{prot})	4.08 (3.01–7.15)	3.78 (3.40 –)	0.782	5.73 (3.47–15.2)	0.214	0.324	6.43 (3.80–10.4)	23.8 (2.33–59.8)	0.386	4.55 (3.81–6.64)	0.640	0.349
Whole Erythrocyte GPx Activity (U/gHb)	31.0 (21.0–42.0)	57.5 (54.2 –)	0.019	43.2 (31.7–57.3)	0.022	0.167	25.5 (11.1–56.9)	46.5 (30.5–64.3)	0.317	60.7 (39.3–72.1)	0.042	0.522
Erythrocyte Membrane CAT Activity (U/mg_{prot})	0.23 (0.15–0.49)	0.55 (0.54 –)	0.112	3.74 (2.20–7.09)	< 0.001	0.032	0.14 (0.11–0.49)	0.96 (0.51–4.42)	0.014	9.63 (2.11–38.8)	< 0.001	0.045
G3PD membrane relative amount (%)	5.54 (4.64–6.34)	3.72 (2.88 –)	0.084	6.04 (4.53–6.89)	0.369	0.114	7.38 * (6.38–8.18)	7.25 (6.95–8.01)	0.947	7.21 * (6.24–7.69)	0.570	0.402
Unbalance Index	0.16 (0.11–0.25)	0.35 (0.13 –)	0.522	0.16 (0.08–0.39)	0.943	0.283	0.35 * (0.20–0.62)	0.44 (0.23–0.60)	0.947	0.37 (0.18–0.45)	0.764	0.522
Cryohemolysis Test (%)	7.14 (1.70–15.7)	28.9 (24.5 –)	0.062	20.7 (9.53–33.2)	0.017	0.430	23.1 * (10.8–38.5)	42.1 (9.64–44.6)	0.739	31.4 (22.5–39.3)	0.243	0.961
Osmotic Fragility Test (Fresh)	0.46 (0.43–0.49)	0.47 (0.46 –)	0.972	0.47 (0.45–0.50)	0.174	0.621	0.50 * (0.49–0.52)	0.54 (0.50–0.63)	0.125	0.48 (0.46–0.51)	0.124	0.049
Osmotic Fragility Test (24 h incubation)	0.56 (0.52–0.63)	0.63 (0.59 –)	0.189	0.62 (0.55–0.71)	0.229	0.693	0.69 * (0.57–0.73)	0.76 (0.62–0.85)	0.286	0.65 (0.60–0.76)	0.815	0.183
Hb (g/dL)	12.8 (12.4–13.9)	12.6 (12.1 –)	0.468	12.7 (11.9–14.1)	0.492	0.999	10.9 * (9.45–11.4)	11.5 (10.6–14.5)	0.071	11.1 * (9.8–12.4)	0.423	0.401
MCV (fL)	87.1 (82.8–93.0)	87.2 (86.4 –)	0.945	85.6 (81.7–88.0)	0.139	0.374	79.2 (73.9–90.6)	80.0 (73.6–85.8)	0.841	81.2 (75.0–83.5)	0.894	0.767
MCHC (g/dL)	33.9 (32.9–34.8)	35.8 (35.3 –)	0.084	36.3 (33.6–37.8)	0.030	0.843	36.0 * (35.0–37.3)	37.0 (35.0–39.2)	0.350	36.2 (33.4–37.7)	0.764	0.349
RDW (%)	15.2 (14.0–17.4)	19.2 (17.3 –)	0.097	15.5 (14.5–18.9)	0.383	0.166	23.2 * (19.8–27.1)	19.0 (14.9–21.6)	0.072	18.7 (17.1–20.2)	0.025	0.730
Reticulocytes (x10^9/L)	71 (52–156)	286 (224 –)	0.045	125 (46–422)	0.291	0.554	430 * (342–624)	393 (310–858)	0.739	354 (231–418)	0.161	0.153
RPI	1.43 (0.91–3.12)	4.41 (4.36 –)	0.053	2.26 (0.84–5.28)	0.476	0.324	4.91 * (4.15–7.08)	6.85 (3.56–10.7)	0.641	4.14 (2.90–5.22)	0.133	0.127
EPO (mU/mL)	12.6 (8.78–21.9)	35.3 (33.5 –)	0.073	17.0 (9.42–36.9)	0.205	0.236	33.7 * (21.1–156)	30.2 (22.6–114)	0.739	32.9 * (25.7–103)	0.973	0.588
Bilirubin (μmol/L)	11.8 (6.46–28.1)	22.7 (20.7 –)	0.407	22.2 (10.3–94.1)	0.136	0.999	52.8 * (41.2–85.5)	39.3 (16.2–94.9)	0.463	42.8 (25.4–65.1)	0.301	0.805

Data presented as median (inter-quartile range). $p < .05$ value was considered as statistically significant (Mann-Whitney U test): (a) – CAT vs CAT + GPx and CAT + GPx + Prx2 HS patients; (b) –CAT + GPx vs CAT + GPx + Prx2 HS patients; * – Mild HS vs. Moderate + Severe HS patients.
HS – Hereditary Spherocytosis; MBH – membrane bound hemoglobin; LPO – lipid-peroxidation; TBARS – thiobarbituric acid reactive substances; GPx – glutathione peroxidase; CAT – catalase; G3PD – glyceraldehyde-3-phosphate dehydrogenase; Hb – hemoglobin; MCV – mean cell volume; MCHC – mean cell hemoglobin concentration; RDW – red cell distribution width; RPI – reticulocyte production index; EPO – erythropoietin.

correlated with each other; furthermore, both membrane bound CAT and activity were correlated with GPx activity. These results show that membrane bound CAT is active and that the binding of Hb and CAT to the erythrocyte RBC membrane are intertwined with each other and with OS; indeed, neither GPx nor Prx2 correlated with MBH. Additionally, only *unspl* HS patients also showed a significant positive correlation of MBH with LPO, in accordance with other authors reporting their joint importance for band 3 clustering that may lead to splenic erythrocyte removal [43]. Moreover, since it is known that reticulocyte enzymatic activity greatly differs from mature and old erythrocytes [44] and the number of circulating reticulocytes is significantly higher in *unspl* HS patients in comparison with healthy individuals and *spl* HS patients, we evaluated the associations between the reticulocyte number and the OS biomarkers in these three groups and found no statistically significant correlations between them (*data not shown*). Thus, we can surmise that the number of reticulocytes does not directly influence the activities of whole-cell GPx or membrane CAT and it is also not associated to the amount of membrane bound cytosolic peroxidases.

In *unspl HS* patients (Table 1), the degree of anemia (decreasing values of Hb concentration) shows the severity of the disease, which is known to be associated with increasing number of circulating spherocytes, reticulocytes and bilirubin levels. Spherocytes are metabolically stressed to maintain membrane integrity, presenting premature and cumulative oxidative damages [24,33]. In accordance, we found that Moderate/severe HS patients, when compared with Mild HS patients, present increased anemia with higher erythropoiesis (as showed by the rise in EPO, circulating reticulocytes and RPI); increased number of spherocytes, explaining the increase in CHT, OFT, and in erythrocyte removal (increase in bilirubin levels); increased UI, reflecting the severity of protein defect(s) and increased oxidative membrane changes, showed by the rise in MBH and G3PD, that seems to associate with increased enzymatic activities of GPx and CAT in the whole cell and in the membrane, respectively (Table 1, right side).

Our group has previously reported the linkage of H_2O_2-scavenging cytosolic Prx2 to erythrocyte membrane in HS patients [1,7], and in normal erythrocytes under OS conditions [28]; later, we showed for the first time, the linkage of both Prx2 and GPx to erythrocyte membrane under H_2O_2 – induced OS in healthy individuals [29]. These findings led us to investigate the involvement of these antioxidant enzymes in membrane protection in *in vivo* models of OS, such as HS. In the present study, we found that the binding of CAT to RBC membrane occurs in all individuals (control group and HS patients), while GPx and Prx2 were not detected in RBC membrane of healthy individuals. GPx was linked to erythrocyte membranes of a larger number of HS patients than Prx2; actually, while GPx was detected in the membrane when CAT or CAT + PRx2 were linked to the membrane, PRx2 was only detected when both CAT and GPX were bound to the membrane. This is in accordance with our previous data suggesting that GPx is more sensitive than PRx2 to OS levels [29].

We also evaluated the total amount (sum) of CAT, GPx and Prx2 bound to the RBC membrane for control, *spl* and *unspl* HS groups (Fig. 1A) and for *unspl* HS patients according to disease severity (Fig. 1B). Our data unequivocally shows that the linkage of these enzymes to the membrane is associated both to the pathological condition studied (Fig. 1A) and to its clinical severity (Fig. 1B). Taken together, the results discussed so far (Table 1 and Fig. 1), demonstrate that CAT mobilized to the membrane is in its active form; however, as GPx activity was measured in whole erythrocyte samples and Prx2 activity was not assessed, we cannot conclude about their activity when linked to the RBC membrane.

To further clarify the binding of these three enzymes to the erythrocyte membrane in HS, we evaluated the frequencies in HS patients with one, two or three membrane-bound peroxidases, according to splenectomy, to protein deficiencies underlying HS and to severity of HS in *unspl* patients (Table 2). We found that the type of membrane protein defect underlying HS was not related to membrane binding of CAT, GPx or Prx2; however, it seems that both the enhancement of OS mediated changes in erythrocytes from *spl* HS patients due to a longer life-span than RBCs of *unspl* HS patients, and the severity of HS, with increasing membrane destabilization and anemia in *unspl* HS patients appear to trigger the mobilization of more antioxidant enzymes to the membrane. Indeed, the prevalence of *unspl* vs. *spl* HS patients and of Mild vs. Moderate/Severe HS patients significantly changes with the number of membrane-bound peroxidases (Table 2), alluding to hypothesis that these enzymes are present in the membrane as part of a mechanism to protect HS erythrocytes from OS damage or suffer themselves oxidative injury that leads to membrane binding.

Still concerning the binding of these three enzymes to the membrane and taken into account the different prevalences of membrane detected antioxidant enzymes, we decided to re-analyze our data according to the number of membrane bound peroxidases that HS patients presented. The most striking finding is that it is evident that the linkage of more proteins is accompanied by a substantial increase in their amount (Fig. 2), i.e., not only more peroxidases are detected in the membrane, their amount also rises exponentially, which is best noticeable in CAT levels (Fig. 2i). In *unspl* HS patients, when GPx (+CAT or + CAT+Prx2) linked to the membrane, we observed an increase in the activity of whole-RBC GPx (almost two-fold) and in *spl* HS patients it is about 2.3 times higher in the groups where GPx is bound to the membrane (Table 3) and, even though we cannot specifically assert that this increase in activity it is due to the membrane bound GPx, it is a noteworthy fact that it correlates with the amount of membrane detected GPx (Fig. 2Aii), which might suggest that GPx is still active while present in the membrane. As for the activity of membrane CAT in *unspl* HS patients (Table 3), it increases (almost four-fold) when GPx also linked to the membrane and this increase was even higher (about 26 times) when PRx2 (+CAT+GPx) was also detected in the membrane. Moreover, as occurred in *unspl* HS patients, the activity of membrane CAT increases with the linkage of GPx (two-fold) and GPx+PRx2 (28 times higher) in *spl* patients (Table 3), which is in accordance with the results found for the amounts of CAT detected in the membrane (Fig. 2i). These results establish that within our population of HS patients (*unspl* and *spl*) the number of membrane bound peroxidases clearly affects the amounts and activities of these membrane detected cytosolic enzymes.

When analyzing OS biomarkers, RBC membrane destabilization/fragility, hematological and biochemical data according to the number/type(s) of membrane linked peroxidases (Table 3), for *unspl* HS patients we found that the increase in MBH, but not in LPO, was associated with the progressive linkage of the three of enzymes to the RBC membrane and that this appears to enhance membrane destabilization, as showed by the increase in CHT, OFT and MCHC and the reduction in MCV (hallmarks of spherocytic cells). It is interesting to notice that the linkage of GPx (+CAT) to membrane seems to be especially important for membrane destabilization, while the linkage of PRx2 (+CAT+GPx) to the membrane appears to be critical for more CAT mobilization from cytosol to the membrane, which is corroborated by Rinnalducci et al. work [27], in which the authors describe a CAT/PRx2 complex in the RBC membrane. However, Prx2 linkage probably leads to an even higher membrane destabilization, as suggested by the increase in RBC removal shown by the higher bilirubin concentration. In spite of membrane destabilization and removal, the severity of anemia (Hb concentration) is maintained (compensated) by higher erythropoiesis through an increase in EPO levels, which triggers an increase in reticulocyte production and number (Table 3). Additionally, in accordance with the higher values of MBH and LPO in RBCs from *spl* HS patients (Table 3), we found significantly increased amounts of CAT (vs. *unspl* HS), and the median values of GPx and PRx2 were also higher in HS *spl* patients (Fig. 2A).

Recently, Welbourn et al. [45] reported that the reversible linkage of Hb to the RBC membrane becomes increasingly irreversible as its

oxidant state increases, which is associated to OS within the erythrocyte, and it prompts membrane LPO [13], in turn leading to spleen erythrocyte removal from circulation [43]. This mechanism is happening in un*spl* HS patients but in *spl* patients the cells are not removed from circulation as effectively and, more and more irreversible binding of Hb to the membrane occurs, as we observed in our patients (Table 3). Considering that Hb [43], Prx2 [26] and CAT [32] seem to share the same binding location in the membrane and that it was reported that CAT and Prx2 can form complexes in the cytosol that are able to bind to the membrane under OS conditions [27], competition for this binding site is likely to occur. The degree of increase in membrane bound CAT amount is much higher than the increase in MBH when Prx2 is detected in the membrane for both *unspl* or *spl* HS patients (Fig. 2Ai and Table 3), which suggests that the linkage of these peroxidases to the membrane might impede Hb linkage by directly occupying the location site or by preventing the irreversible Hb binding to the membrane by protecting it from oxidation. Moreover, bearing in mind that LPO values in both *unspl* and *spl* HS patients are roughly the same for the groups CAT, CAT + GPx and CAT + GPx + Prx2, in spite of other gradually oxidative changes (MBH, enzymes amounts and activities) (Table 3 and Fig. 2A), this fact strengthens the hypothesis of an antioxidant protective role of CAT, GPx and PRx2 in the RBC membrane, specifically by preventing LPO. The mechanism/binding site of GPx to the RBC membrane is still unknown and we cannot speculate about such issue.

When performing the sub-analysis of OS markers, RBC membrane destabilization, hematologic and biochemical data of according to clinical severity (only in *unspl* HS patients) and to the number of membrane bound peroxidases (Table 4 and Fig. 2B), we found that the results regarding the OS biomarkers were similar to the above reported for total *unspl* HS patients both either in Mild or Moderate/Severe HS patients, although enhanced in the latter group. This strengthens the potential role of these enzymes, especially of PRx2, to protect the membrane through the mobilization of more cytosolic enzymes to the membrane. Moreover, although MBH increases with the number of peroxidases bound to the membrane, LPO is roughly the same within Mild or Moderate/Severe HS (Table 4). Thus, one might suggest these peroxidases binding to the RBC membrane is intentional and could be part of an intricate mechanism of antioxidant protection and cell survival, by keeping in check LPO levels.

In the present study, we showed by direct measurement of CAT activity in the RBC membrane that CAT is active and functional in the membrane; in the case of Prx2, we were able to identify both dimer (oxidized, inactive) and monomer (reduced, active) forms of Prx2 in the membrane, showing that the enzyme is still capable of its redox-cycle of action, and, thus, still functional (at least partially). We were not able to assess directly or indirectly GPx membrane activity, although whole-RBC GPx activity showed correlations with GPx linkage to the membrane, suggesting that it might be active in the membrane. Thus, our data shows that the binding of these enzymes to the membrane might serve a crucial role in the antioxidant protection of HS erythrocytes and that it appears to be associated to decreasing (or regulating) the levels of LPO. In fact, Prx2 and GPx are known to act upon organic hydroperoxides [16,17,23], while CAT is an exclusive H_2O_2 scavenger [18]; thus, the former two may prevent LPO directly, and the later might do so indirectly, by acting upon H_2O_2 and avoiding Hb oxidation that eventually leads to LPO.

In conclusion, we show that HS is undoubtedly related to the OS induced changes within the erythrocytes, as these are much higher than in the control group; also, the linkage of GPx and PRx2 to the membrane seems to trigger the mobilization of more CAT from the cytosol to the membrane. Indeed, in both *spl* and *unspl* HS patients, the linkage of GPx and, particularly, of Prx2 to the RBC membrane was associated with an significant increase in membrane amount and activity of CAT, suggesting that as the RBCs become smaller and more fragile (with higher metabolic stress to maintain membrane integrity), the cytosolic peroxidases bind to the membrane in an effort to maintain membrane integrity, by inhibiting LPO specifically and, thereby delaying erythrocyte removal. In *spl* HS patients (without spleen removal of erythrocytes with oxidative injuries), the accumulation of these modifications is enhanced since it occurs for a longer period of time.

The linkage of typically cytosolic antioxidant enzymes to the RBC membrane appears to be an important mechanism of antioxidant defense in pathological erythrocytes that warrants further research, namely because of the emerging potential of Prx2 as a therapeutic target in the treatment of malaria [46]. A better understanding of the biology of these RBC antioxidant enzymes in health and disease deserves to be re-visited since our study shows that even when all seems similar (anemia degree and LPO levels) within HS individuals, it is not, in fact, all equal (number/type(s) of membrane bound cytosolic peroxidases) and we believe much is still unknown. Perhaps these mechanisms or the affinity for antioxidant cytosolic enzymes binding to the erythrocyte membrane might even have genetic/molecular basis.

Data statement

Data not available/The data that has been used is confidential.

Although the samples were anonymized it was agreed that raw data would remain confidential and would not be shared, due to a great number of the participants in this study being children.

CRediT authorship contribution statement

Susana Rocha: Conceptualization, Formal analysis, Funding acquisition, Validation, Investigation, Visualization, Writing - original draft, Writing - review & editing. Petronila Rocha-Pereira: Funding acquisition, Investigation, Validation. Esmeralda Cleto: Resources, Validation, Writing - review & editing. Fátima Ferreira: Resources, Validation, Writing - review & editing. Luís Belo: Formal analysis, Funding acquisition, Supervision, Writing - review & editing. Alice Santos-Silva: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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