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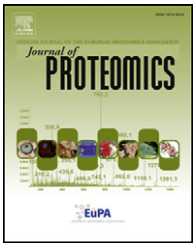

Blood modifications associated with end stage renal disease duration, progression and cardiovascular mortality: a 3-year follow-up pilot study

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ABSTRACT

Chronic kidney disease is a risk factor for cardiovascular mortality. This study uncovers pieces of hematological and erythrocyte protein variability observed in end stage renal disease (ESRD) in relation to disease progression/duration and mortality. Using a variety of experimental approaches, erythropoietin/dialysis-treated patients were compared to healthy individuals and had been followed for 36 months. During that period, half of the patients died from cardiovascular diseases. The high levels of uremic toxins in those patients were associated with damaged erythrocytes, bad tolerance and poor response to hemodialysis therapy. The postmortem study revealed significant variation in alkaline phosphatase, duration of dialysis, erythrocyte transformation and intracellular hemoglobin concentration compared to the survived patients. The erythrocyte proteins showed substantial remodeling characteristic of pathologic regulation of cell hydration and susceptibility to the dialysis-induced oxidation defects. According to the follow-up study, duration of hemodialysis was associated with a trend towards increased intracellular hemoglobin concentration, membrane expression of glucose transporter-1 and stomatin as well as lower levels of circulating stomatocytes. The uremic index variation in long survived patients is accurately reflected in plasma and erythrocyte oxidative stress modifications.

Abbreviations: ALP, alkaline phosphatase; aquaporin 1, Aqp1; DHA, dehydroascorbic acid; Epo, erythropoietin; ESRD, end stage renal disease; FRAP, ferric reducing ability of plasma (or antioxidant power); Hb, hemoglobin; HCT, hematocrit; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; γGT, gamma-glutamyltransferase; GLUT1, glucose transporter 1; MCH, mean cell Hb; MCHC, mean cell Hb concentration; MCV, mean cell volume; PCI, Proteome Carbonylation Index; Prx2, peroxiredoxin 2; RBCs, red blood cells; RDW, red cell distribution width; SGOT, serum glutamate-oxaloacetate transaminase; SGPT, serum glutamate-pyruvate transaminase; TAC, Total Antioxidant Capacity.

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The ESRD patients exhibit impressive compensatory responses to the chronic challenges of the uremic milieu.

Biological significance

This study demonstrates novel blood modifications probably associated with the duration of erythropoietin/hemodialysis treatment, disease progression and cardiovascular mortality in end stage renal disease. The observed variability adds new pieces to the erythrocyte pathophysiology puzzle in end stage renal disease and suggests novel hematologic and proteomic factors for consideration in future large scale studies on cardiovascular morbidity and mortality candidate biomarkers in uremic patients.

1. Introduction

The uremic syndrome is related to a complex set of pathophysiological disturbances resulting in increased morbidity and mortality rate. The major cause of death in all forms of chronic kidney disease including the end stage renal disease (ESRD) on hemodialysis (HD) [1] is cardiovascular diseases. The high risk of cardiovascular morbidity and mortality in these patients is attributed to a complex interplay of traditional (e.g. age, dyslipidemia, hypertension, and diabetes mellitus) and novel or uremia-specific cardiovascular risk factors, including uremic toxins, uremic bone disease, disturbed calcium-phosphate metabolism [2], inflammation [3], endothelium dysfunction [4], oxidative stress [5] and anemia [6]. At the advanced stage of the disease, retention of uremic toxins, metabolic alterations and HD may further contribute to the high risk of mortality.

Anemia in ESRD is the consequence of reduced red blood cell survival and functional erythropoietin (Epo) deficiency. Erythrocytes in hemodialysis patients undergo shear stress generated during blood flow through the dialyzer and peristaltic pumps and metabolic stress caused by the unfavorable plasma environment that is characterized by metabolite accumulation and loss of glucose [7]. The ESRD further represents a high pro-oxidant activity disease due to contributing factors like advanced age, chronic inflammation and dialysis material biocompatibility issues [6]. More specifically, erythrocytes are subjected to enhanced oxidative stress as a result of reduced cellular and plasma anti-oxidant factors and inadequate glutathione-defense system. Although hemodialysis partially improves the endogenous ROS levels, the glutathione antioxidant system as well as the RBC membrane protein defects [8], it has been associated with oxidation of plasma ascorbic to dehydroascorbic acid [9] and aggravation of protein carbonylation [10,11].

Prognosis, risk stratification and monitoring the effects of treatment are fundamental elements in the clinical handling and therapy guidance of uremic patients. A variety of blood biochemical risk markers have been consistently linked to cardiovascular disease and reduced survival in patients on dialysis [12,13]. However, biomarker identification in this group of patients has been proven to be a difficult task in that well-known associations between established risk factors in the general population do not exist or appeared reversed in ESRD [13], while some of the novel risk factors for cardiovascular disease seem to play a more important role for morbidity and mortality in uremic patients than in the general population [13–15]. Based on these peculiarities, a multi-marker approach reinforced by additional proteomic tools have been strongly proposed in renal disease biomarker area, as a safe way to refine prognosis in patients on HD, after their full-scale evaluation in large longitudinal studies and clinical trials [15].

It has recently been shown that the RBCs of non-diabetic ESRD patients on HD show substantial membrane remodeling and overexpression of cellular stress and senescence markers [8]. In the present follow-up study, we re-examined the same group of patients three years later in order to retrace blood modifications probably associated with the duration of HD and the progression of the disease, compared to healthy controls studied for the same period. Furthermore, and since half of the patients passed away in the meanwhile by cardiovascular diseases, we retrospectively assess a series of blood and erythrocyte factors as novel candidate markers of increased cardiovascular mortality in ESRD patients.

2. Materials and methods

2.1. *Material supplies*

Antibodies against band 3, actin, spectrin and human IgGs as well as HRP-conjugated secondary antibodies and all chemicals (unless otherwise stated) were obtained from Sigma-Aldrich (Munich, Germany). Electron microscopy grade glutaraldehyde solution was from Serva (Heidelberg, Germany). Antibodies against hemoglobin (Hb) and flotillin-2 were from Europa Bioproducts (UK) and BD Transduction Laboratories (CA, USA), respectively. Primary antibodies against CD47, HSP70, calpain-1 (μ-calpain), clusterin-α (secretory Apolipoprotein J) and band 3 were from Santa Cruz Biotechnology (CA, USA). Antibodies against peroxiredoxin 2 (Prx2), adducin alpha and glucose transporter 1 (GLUT1) were from Acris GmbH (Herford, Germany). The Oxyblot® detection kit was obtained from Millipore (Temecula, CA) and 5-(and-6) chloromethyl-2′,7′-dichloro-dihydro-fluorescein diacetate, acetyl ester (CMH_2DCFDA) was from Invitrogen, Molecular Probes (Eugene, OR). HRP-conjugated antibodies to rabbit IgGs and ECL Western blot detection kit were from GE Healthcare (Buckinghamshire, UK). HRP-conjugated antibodies to mouse IgGs were from DakoCytomation (Glostrup, Denmark).

Bradford protein assay was obtained from Bio-Rad (Hercules, CA). Antibodies against aquaporin 1 (Aqp1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Cell Signaling Technology (Beverly, MA) and Abnova, respectively. Western lighting Plus ECL was from Perkin Elmer (CA, USA). mAb against stomatin and antiserum against protein 4.1R and pallidin (band 4.2) were kindly provided by Prof. R. Prohaska (Institute of Medical Biochemistry, University of Vienna, Austria) and Prof. J. Delaunay (Laboratoire d'Hématologie, d'Immunologie et de Cytogénétique, Hopital de Bicetre, Le Kremlin-Bicetre, France), respectively.

2.2. Study planning and subjects

The demographic characteristics as well as the hematological and serum biochemical profiles of healthy controls (n = 12) and ESRD patients on HD (n = 12) studied in 2009, have previously been reported [8]. Briefly, the patients were on Epo treatment and standard HD therapy (thrice a week) with highly biocompatible polyarylethersulfone (n = 6) or acrylonitrile (n = 6) synthetic dialyzers (Gambro-Hospal Ltd). They had no diabetes mellitus, autoimmune diseases, malignancies, infections and hematological disorders but they were all clinically stable at the time of investigation. For the follow-up study, the same groups of patients and healthy controls were invited for re-evaluation 3 years after the initial examination (2009–2012). Unfortunately, six of the patients had passed away (by cardiovascular diseases and acute thrombotic events) while two of the healthy controls did not renew their consent to take part in the investigation. As a result, blood samples were collected from the ten healthy subjects (n = 10) as well as from the six uremic patients that were alive during 2012 (n = 6). During the precedent three year period there was no significant change in Epo medication (Darbepoetin, EpoA or Epo B) or dialysis filters (synthetic, highly biocompatible filters of polyarylethersulfone, acrylonitrile or polyamide type). For the postmortem observational study, the ESRD patients were classified for comparative re-examination on the basis of their survival in subgroups A and B: Group A consisted of those who passed away while Group B consisted of those who were alive during 2012. The hematological, serum biochemical, cell morphology and the majority of the protein expression data represent those originally collected in 2009, currently being classified and re-evaluated a posteriori, following the survival criterion (Groups A and B). However, additional analyses have been performed on the appropriately stored blood plasma and membrane extraction samples (e.g. immunoblots for adducin and GLUT-1, as well as the FRAP assay) in all patients and healthy subjects originally studied in 2009. The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Research Bioethics and BioSecure Committee of the Faculty of Biology/University of Athens. All of the donors gave their written informed consent before their participation in this study.

2.3. Hematological and biochemical analysis

Blood samples were collected in EDTA anticoagulant before, 20 min after the start and immediately after the completion of the HD session. Red blood cell (RBC) count, hematocrit (HCT), Hb concentration and RBC indexes (mean cell volume, MCV; mean cell Hb, MCH; mean cell Hb concentration, MCHC; RBC distribution width, RDW) were measured by using an automatic blood cell counter (Sysmex K-4500, Roche). Standard biochemical tests in the serum (urea, creatinine, etc.) were performed using an automatic analyzer (Hitachi 902, Roche). Electrolyte estimation was performed with the electrolyte analyzer 9180 (Roche).

2.4. Total Antioxidant Capacity (TAC) of plasma

The measure of Total Antioxidant Capacity (TAC) considers the cumulative and synergistic action of all the antioxidants present in plasma, thus providing an integrated parameter of known and unknown antioxidants, as well as insight into the oxidant/antioxidant balance present in vivo. Freshly isolated and/or stored plasma samples were used from all ESRD patients and standard controls. TAC of plasma was measured by the ferric reducing ability of plasma (FRAP) assay as previously described [16]. Briefly, 40 μl plasma was mixed with working FRAP solution and samples were incubated at 37 °C. Working FRAP solution was freshly prepared by mixing acetate buffer (pH 3.6, 300 mmol/l), 2,4,6-tripyridyl-*s*-triazine (TPTZ, 10 mmol/l) in HCl (40 mmol/l) and $FeCl_3$ (20 mmol/l). After 4 min, absorbance was measured at 593 nm versus blank, containing only working FRAP solution. Ascorbic acid standards (100 μM–1000 μM) were tested in parallel. To determine the uric acid-independent antioxidant capacity, the plasma aliquots were treated with 0.005 U uricase and processed as mentioned above [17].

2.5. Determination of intracellular ROS by fluorometry

ROS accumulation in RBCs was detected with the membrane permeable, non-fluorescent and redox-sensitive dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CMH_2DCFDA) according to the manufacturer's guidelines with minor modifications, as previously reported [18]. More specifically, leukocyte-depleted (by cellulose columns, see below) and thoroughly washed RBCs (in triplets) were loaded with 5 μM CM-H_2DCFDA for 30 min at 25 °C. The production of fluorescent dichlorofluorescein (DCF) was measured using the VersaFluor™ Fluorometer System from Bio-Rad at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The intensity records were normalized to the RBC protein level and finally to the corresponding control values (healthy subjects, 100%).

2.6. RBC membrane total protein and protein carbonyl analysis

RBCs were isolated by the method of Beutler [19]. Purified RBC lysis was performed with hypotonic (5 mmol/l) sodium phosphate buffer (pH 8.0) containing a cocktail of protease inhibitors. Membrane fractions were prepared as previously described [20] and total protein concentration of the membrane fractions was determined using the Bradford protein assay with BSA as a standard. Equal amount (12–25 μg) of RBC total membrane protein was loaded in Laemmli gels, transferred to nitrocellulose

membranes electrophoretically and probed with primary and HRP-conjugated secondary antibodies. Immunoblots were developed using an ECL reagent kit and the relative amount of each protein was quantified by scanning densitometry. For the protein carbonylation analysis, purified RBC plasma membrane proteins were processed for the detection of carbonyl groups, using the OxyBlot detection kit as per manufacturer's specifications. For quantification purposes, the Proteome Carbonylation Index (PCI) was calculated as previously described [8].

2.7. Scanning electron microscopy

For the analysis of RBCs' morphology with a scanning electron microscope, purified RBCs were fixed with 2% glutaraldehyde and post-fixed with 1% osmium tetroxide in 0.1 mmol/l sodium cacodylate buffer, pH 7.4. Fixed cells were successively dehydrated in ascending ethanol series and allowed to settle on standard microscopic cover glasses. Finally, RBCs were coated with gold–palladium (Tousimis Samsputter-2a, Rockville, Maryland) before being examined in a microscope (Philips SEM515).

2.8. Scanning densitometry and statistical analysis

Presented experiments have been repeated at least two times, unless otherwise stated. Data points correspond to the mean value; error bars denote SD. Quantitation of gels and immunoblots was performed by lengthwise scanning densitometry using an image-processing program (Gel Analyzer v.1.0, Biosure, Athens, Greece). The electrophoretic protein bands were quantified in units of intensity, and the relative amount of each band was given as a percentage of total. Reference antibodies against 4.1R or actin proteins were used as internal loading controls for immunoblots. Individual relative protein levels were quantified as a ratio against reference band(s) or percentage of total immunoblotting bands per patient. This relative proportion, further normalized to the controls data (relative % of healthy subjects, Table 2), or to the relative protein expression determined in 2009 analysis of the same samples (100%), is presented in the tables. For statistical analysis the MS Excel and the Statistical Package for Social Sciences (IBM SPSS; version 19.0 for Windows; administrated by NKUA) were used. Significance was evaluated using the one-way ANOVA. Clinical and hematological quantitative variable comparisons between groups of subjects

Table 1 – Demographic characteristics, hematological and serum biochemical data for healthy subjects and ESRD Group A and B patients.

	Group A (n = 6)	Group B (n = 6)	Controls (n = 12)	p < 0.05 A vs. B
Age (years)	72.2 ± 9.9	59.2 ± 13.9	45.0 ± 11.5	
Gender (M/F)	4/2	4/2	7/5	
Time on HD (months)	**40.8 ± 6.6**	**24.0 ± 9.2**	–	0.029
WBCs ($\times 10^9$/l)	6.72 ± 1.49	7.7 ± 3.3	5.78 ± 1.34	
RBCs ($\times 10^6$/μl)	4.2 ± 1.2	**3.9 ± 0.3**	4.6 ± 0.8	
Hb (gr/dl)	13.2 ± 2.9	**11.1 ± 1.8**	12.6 ± 1.0	
Hct (%)	39.6 ± 8.5	**35.1 ± 4.8**	38.9 ± 2.4	
MCV (fl)	97.2 ± 7.5	89.2 ± 6.8	88.9 ± 3.5	0.049
MCH (pg)	**32.3 ± 2.6**	28.2 ± 3.0	29.18 ± 1.46	0.042
MCHC (gr/dl)	33.2 ± 0.8	**31.6 ± 1.2**	32.8 ± 0.8	0.021
RDW-CV (%)	**15.9 ± 0.6**	**16.5 ± 0.8**	13.41 ± 0.39	
PLTs ($\times 10^3$/μl)	205.0 ± 54.5	249.8 ± 78.6	258.2 ± 23.5	
Glucose (mgr/dl)	87.0 ± 7.9	100.0 ± 15.9	85.2 ± 5.8	
Urea (mg/dl)	**195.6 ± 63.3**	**154.6 ± 27.9**	29.11 ± 3.45	
Urea (mg/dl) post-HD	**67.8 ± 28.4**	**55.0 ± 15.1**	–	
Creatinine (mgr/dl)	**12.83 ± 2.24**	**9.34 ± 2.64**	0.74 ± 0.21	0.049
Creatinine (mg/dl) post-HD	**3.56 ± 0.37**	**3.18 ± 1.29**	–	
Cholesterol (mg/dl)	130.0 ± 25.2	169.0 ± 38.8	138.34 ± 19.56	
Uric acid (mg/dl)	6.06 ± 1.45	6.93 ± 1.49	4.64 ± 1.26	
Triglycerides (mg/dl)	174.6 ± 86.2	**232.6 ± 76.0**	150.3 ± 34.9	
Potassium (mEq/l)	**5.54 ± 1.53**	**5.38 ± 0.96**	4.32 ± 0.36	
Potassium (mEq/l) post-HD	4.32 ± 0.98	3.78 ± 0.19	–	
Sodium (mEq/l)	142.0 ± 2.8	138.8 ± 3.4	142.2 ± 3.1	
Iron (μg/dl)	**52.7 ± 9.2**	**55.3 ± 14.8**	73.2 ± 56.1	
Calcium (mg/dl)	9.13 ± 0.13	9.35 ± 0.29	9.46 ± 0.21	
Phosphorus (mg/dl)	**4.60 ± 0.45**	**6.94 ± 3.20**	3.54 ± 1.11	
Albumin (g/dl)	4.30 ± 0.29	4.03 ± 0.24	3.75 ± 0.22	
SGOT (IU/l)	**49.0 ± 20.8**	19.8 ± 6.5	22.3 ± 2.1	0.013
SGPT (IU/l)	30.0 ± 11.1	19.8 ± 7.5	25.8 ± 11.0	
γ-GT (IU/l)	22.8 ± 2.3	19.8 ± 7.3	12.9 ± 2.5	
ALP (IU/l)	**117.5 ± 36.9**	66.0 ± 29.0	82.3 ± 35.8	0.049
Amylase (IU/l)	**155.0 ± 38.2**	107.0 ± 40.1	62.2 ± 25.6	

2009 measurements. Results are presented as mean ± SD.
Bold: pathologic values.

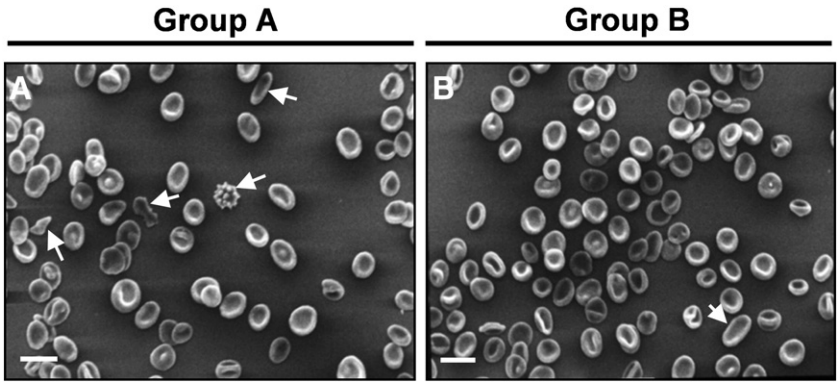

Fig. 1 – Representative scanning electron microscopy micrographs showing the degree of irreversible transformation (arrows) of pre-HD erythrocytes collected in 2009 from Group A (A) and B (B) patients. Scale bars, 10 μm.

were performed by the independent *t*-test or the chi-squared test. Significance was accepted at $p < 0.05$.

3. Results

3.1. Post-mortem study: blood and RBC protein diversification between the patient groups

The hematological and serum biochemical profiles of deceased (before 2012, Group A) and alive (to 2012, Group B) ESRD patients are presented in Table 1. According to that classification of 2009 data, the Group A of ESRD patients was characterized by statistically longer mean duration on HD therapy and increased levels ($p < 0.05$) of RBC indexes (either above or within the normal range, however, in most of the cases), related to both cellular volume (MCV) and intracellular Hb concentration (MCH and MCHC). Unlike Group B patients, Group A ones were not anemic but exhibited higher pre-dialysis levels of creatinine. The liver damage biomarkers serum glutamate-oxaloacetate transaminase (SGOT) and alkaline phosphatase (ALP) were also statistically different between the two groups, although ALP values in some patients varied within the normal range.

At the cellular morphology level, SEM analysis of the pre-dialysis collected RBCs had previously revealed severe variations from normal discocyte shape towards intent anisocytosis and poikilocytosis [8]. Following the well established criteria for the classification of reversible and irreversible transformations of circulating RBCs detected by electron microscopy [21,22], we retrospectively found that the spherocytic modifications of RBCs (spherocytes, spheroechinocytes and spherostomatocytes) along with those associated with mainly degenerative shapes (dacryocytes, ovalocytes, elliptocytes, schistocytes etc.) were more frequently met in the peripheral blood of Group A patients compared to the Group B ones (3.5 ± 1.2% vs. 1.9 ± 0.3%, respectively, $p < 0.05$, Fig. 1). The percentage of irreversibly transformed RBCs in the control group of healthy subjects

Table 2 – Summary of the differentially expressed proteins in the membrane of ESRD patients Group A and B erythrocytes with respect to the HD session.

	Group A (n = 6)			Group B (n = 6)			Controls (n = 12)
	Pro	20 min	Post	Pro	20 min	Post	
a-Adducin	**84 ± 11**	94 ± 10 *	90 ± 10	**72 ± 15**	**78 ± 12**	**79 ± 12**	100 ± 8
Aberrant bands [a]	**135 ± 17**	**131 ± 23** *	**124 ± 20** *	**121 ± 21**	112 ± 17	106 ± 14	100 ± 7
Aquaporin-1	**171 ± 22** *	**171 ± 36** *	111 ± 20	**141 ± 20**	107 ± 19	120 ± 25	100 ± 17
Band 3	86 ± 17 *	**82 ± 10** *	**83 ± 17**	95 ± 26	94 ± 28	88 ± 26	100 ± 7
Band 8 [a]	**186 ± 27** *	**182 ± 19** *	**160 ± 19**	**154 ± 12**	127 ± 16	**156 ± 12**	100 ± 26
Calpain 1	**203 ± 15**	**203 ± 18** *	**237 ± 25** *	**186 ± 35**	**169 ± 27**	**169 ± 25**	100 ± 19
CD47	90 ± 22	**52 ± 13** *	**56 ± 18**	78 ± 22	82 ± 23	**67 ± 17**	100 ± 20
GAPDH	99 ± 16 *	105 ± 12 *	90 ± 12 *	**44 ± 11**	**45 ± 14**	**36 ± 15**	100 ± 12
GLUT1	**75 ± 12** *	102 ± 9 *	**72 ± 14**	**51 ± 12**	**65 ± 9**	**49 ± 12**	100 ± 9
Pallidin (band 4.2)	**65 ± 13** *	**65 ± 9** *	**51 ± 12** *	101 ± 12	101 ± 17	87 ± 14	100 ± 15
Prx2	**266 ± 22**	**319 ± 15** *	**436 ± 25** *	**277 ± 27**	**362 ± 34**	**372 ± 40**	100 ± 18
Clusterin	**74 ± 17** **	**70 ± 20** *	**66 ± 21**	**56 ± 21**	**54 ± 20**	**63 ± 16**	100 ± 19
Spectrin 150 kDa	**149 ± 22** **	117 ± 10	**135 ± 18** *	110 ± 15	99 ± 18	99 ± 19	100 ± 11
Spectrin 120 kDa	**177 ± 27** *	**142 ± 21** *	**142 ± 16** *	106 ± 14	115 ± 16	97 ± 19	100 ± 16

Immunoblotting densitometry results (in the majority of them). Data represent averaged (n = 6 or n = 12) relative membrane protein expression ± SD after normalization to the controls (100% of expression).
Bold: $p < 0.05$ ESRD patients vs. controls.
* $p < 0.05$ Group A vs. Group B.
[a] SDS-PAGE densitometry data.

collected at the same period (2009) was 0.9 ± 0.3% (data not shown).

Since the hematological and morphological examination revealed subtle but significant variation of probable diagnostic value between the two subgroups, it was therefore questioned whether there was a similar variation at the RBC membrane protein level with respect to the HD procedure. Although usually pathologically affected compared to the healthy controls (100% of expression), similar membrane expression of stomatin, actin, HSP70, ubiquitinylated components, spectrin–Hb complex and membrane bound Hb and IgGs was observed between Group A and B patients (data not shown). On the opposite, the expression of the proteins shown in Table 2 was significantly different in Group A compared to the Group B patients. Without exception, in the 2009 samplings only the Group A RBC membranes were deficient in band 3 and pallidin proteins contained fragmented spectrin (fragments with molecular weight of 150 kDa and 120 kDa) and normal amount or minimally affected GAPDH and alpha-adducin proteins (Table 2 and Fig. 2). Moreover, the same samples were characterized by excess of Aqp1, band 8, calpain 1 and peroxiredoxin 2 (Prx2) proteins compared to the Group B membranes, before, during and/or after the HD procedure (Table 2). The RBC membranes of all ESRD patients, especially of Group B ones ($p < 0.05$), were further deficient in glucose transporter 1 (GLUT1) and clusterin proteins. Soon after the completion of the HD session, Group A erythrocytes contained electrophoretically and immunologically detected aberrant bands, increased membrane binding of calpain and severe loss of CD47 "marker of self", compared to the pre-HD values (Table 2).

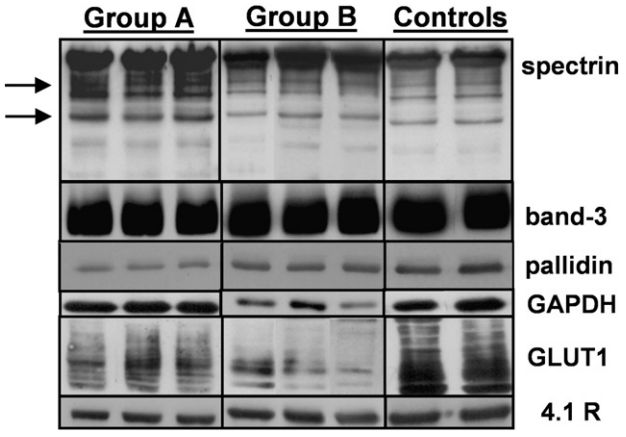

Fig. 2 – Representative immunoblot analysis of RBC membrane proteins in six ESRD patients (three of Group A and three of Group B) vs. two healthy controls performed in 2009 samplings. The ESRD samples were prepared from RBCs collected before the HD sessions and probed with polyclonal and mAbs against the proteins indicated to the right of the blots. Arrows indicate the aberrant immunoblotted bands of spectrin proteolytic fragments, especially developed in Group A samples. Group A patients' membranes were also obviously deficient in band-3 and pallidin proteins compared to Group B ones, which contain comparatively less GAPDH and glucose transporter components. The densitometric and statistic analysis of the data is presented in Table 2.

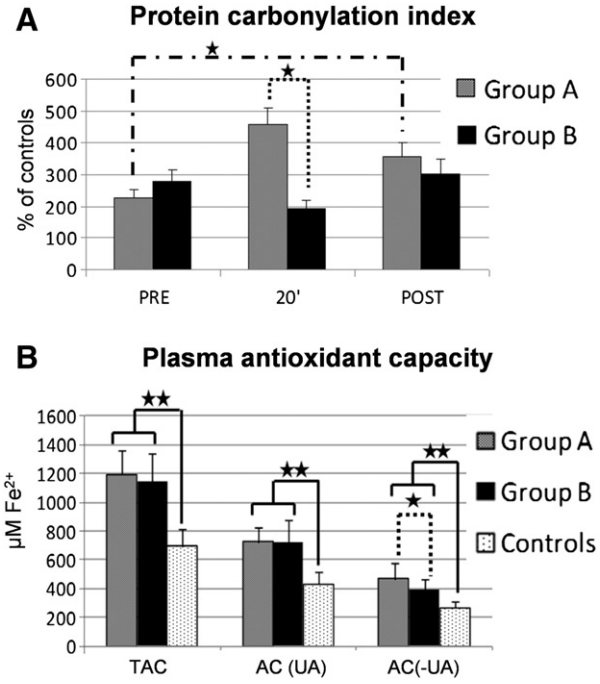

Fig. 3 – Bar graphs showing the variation in the RBC membrane Proteome Carbonylation Index (A) and plasma pre-HD Total Antioxidant Capacity (B) in ESRD patients and healthy controls. Data represent averaged (n = 6 for patients groups and n = 12 for healthy controls) values (of relative protein carbonyl expression and μM Fe^{2+}, respectively) ± SD. ESRD PCI values were normalized against the PCI of the controls (100%). Pre, 20 min and post: before, during and after the HD. TAC, AC(UA), AC(–UA): total, urate-dependent and urate-independent plasma antioxidant capacity. (*) $p < 0.05$. (**) $p < 0.01$.

The differential effect of HD on the two subgroups' RBCs collected in 2009 was additionally manifested by the comparative analysis of the membrane Proteome Carbonylation Index (PCI). PCI, that is a measure of the protein oxidative assaults, was substantially high ($p < 0.01$) for the Group A RBCs collected during the HD session (Fig. 3A). Furthermore, the post-HD PCI index was significantly different compared to the pre-HD index only in the case of the high mortality group (Fig. 3A), signifying that at a high mortality risk state the erythrocytes are particularly susceptible to the HD-associated oxidative damage.

As expected [23], Total Antioxidant Capacity of plasma, measured by the FRAP assay, was increased in the pre-HD collected plasma of all ESRD patients compared to healthy controls (Fig. 3B). Since FRAP-effector components, like the uric acid [24] are subjected to extreme variation in uremic patients on HD, we repeated the measurement in uricase-treated plasma samples. According to the results shown in Fig. 3B, both the urate-dependent and urate-independent antioxidant capacities were higher in ESRD patients than in controls ($p < 0.01$). A small but significant increase was observed in the urate-independent plasma antioxidant capacity of Group A compared to Group B patients.

Table 3 – Modifications in the hematologic and serum biochemical profile of the survived ESRD patients (H1 to H6) after three more years on HD (follow-up study).

	Patients					
	H1	H2	H3	H4	H5	H6
RBCs	**83.1***–	**87.8***–	107.2	**102.0**–	**88.1***–	98.0
Hb	**90.3***–	**107.8**–	**112.7**–	**111.1**–	**87.2***–	**95.1**–
Hct	**83.0***–	**95.1**–	**105.6**–	**100.9**–	**88.0***–	**95.7**–
MCV	100.1	**108.5**+	98.5	**99.0**–	100.1	**98.3**–
MCH	**108.7***+	**122.8***+	105.1	140.7*	**98.6**+	**96.8**–
MCHC	108.6*	**113.3***–	106.7*	**110.0***–	**98.5**–	98.6
RDW-CV	**100.3**+	**93.1***+	**100.7**+	**101.8**+	**116.0***+	**101.2**+
Urea	**88.4**+	**87.3**+	**115.6**+	**110.1**+	**87.5**+	**96.8**+
Urea (post-HD)	91.1	75.5	**85.2**+	62.0	112.9	**110.2**+
Creatinine	**78.6***+	**85.3***+	**104.2**+	**88.2***+	**123.0***+	**72.6***+
Creatinine (post-HD)	**81.7**+	**100.7**+	**115.9**+	**93.1**+	**146.3***+	**69.3**+
Potassium	**121.4***+	114.1	72.3*	120.0*	**85.0**+	**103.4**+
Potassium (post-HD)	92.3	91.9	82.1	83.5	84.7	82.5
Uric acid	112.6	67.2*	86.4	71.6*	67.9*	69.9*
Cholesterol	68.9*	66.3*	96.0	110.2	96.5	85.5
Triglycerides	56.0*	28.6*	32.2*	**159.5***+	54.3*	93.7
Sodium	**100.0**–	99.8	**96.2**–	**93.5***–	97.4	98.5
Calcium	106.3	101.7	94.4	103.2	102.5	102.5
Phosphorus	137.9*	**106.3**+	**107.0**	**62.9***+	92.2	**91.4**+
Iron	183.0*	113.9	67.2*	**71.4***–	**77.2***	265.8*
Proteins	98.2	89.5	106.0	99.5	116.8*	109.7
Albumin	107.6	114.9	97.9	109.5	102.6	100.7
SGOT	103.1	163.6*	57.1*	70.0*	73.0*	60.4*
SGPT	157.1*	132.8*	85.4	95.0	51.6*	69.3*
γ-GT	81.0	196.4*	**435.0***+	98.0	**164.3***+	71.4*
ALP	101.2	91.3	103.3	109.8	288.9*	88.2

Data represent modifications in the parameters listed after normalization to the 2009 values (100%).
Bold: pathologic values either above (+) or below (–) the normal range (2012 measurement).
* Statistically significant difference ($p < 0.05$) in the rate of change of each parameter compared to the averaged rate of change of the same parameter in healthy controls for the same period (data not shown).

3.2. *Follow-up study: blood and RBC protein diversification after three years on HD*

Three years after the initial examination, the Group B patients exhibited some variation in the hematologic profile (Table 1 and Table 3). The modifications observed were clearly associated with the ESRD and the HD therapy, since the rate of change of those parameters in the patients was statistically higher ($p < 0.05$) than that of co-studied healthy controls (asterisks in Table 3) for the same period of time. Uremic index variation, for better or for worse, in long survived ESRD patients, was accurately reflected in plasma and erythrocyte oxidative stress markers as well as in cellular morphology, irrespective of the dialysis period.

More specifically, although there was not a common variation profile among the patients, a trend towards increased MCH and MCHC indexes without a concomitant decrease in the mean cell volume was obvious (Table 3). Regarding the serum biochemical markers, the pre-HD levels of uremic solutes (urea, creatinine, potassium, urate) were stable or substantially improved in the majority of the patients. Compared to the initial evaluation, creatinine exhibited worse clearance in patient H5. Apart from gamma-glutamyltransferase (γGT) that was substantially increased in half of the patients, the serum biochemical profile was overall stable or improved. Notably, the comparatively increased concentration of pre-HD creatinine in patient H5 was correlated to a wide increase in ALP levels (Table 3). The respective profiles of healthy subjects showed negligible changes for the same period (data not shown).

In accordance with the hematological data, three years after the initial examination of the Group B patients, there was an apparent increase ($p < 0.01$) in the frequency of the normally shaped discocytes (from 44.9 ± 0.23% to 60.7 ± 1.57%, respectively) at the expense of irreversible and reversible RBC transformations (from 53.5 ± 1.04% to 39.1 ± 1.20%, respectively) (Fig. 4). It should also be mentioned that a decrease of more than 50% was estimated for the stomatocytes compared to their frequency in 2009. For the same period, there was no significant variation in the percentage of transformed RBCs among healthy controls (0.9 ±0.3% to 1.0 ± 0.25% for the 2009 and 2012 measurements, respectively).

It is well-established that uremia represents a pro-oxidant disease state [13]. As a result, we subsequently performed a follow-up evaluation of oxidative stress-related distortions in patients' plasma and RBCs. After 3 years on HD, the antioxidant capacity of plasma measured by the FRAP assay, remained essentially stable or was increased in HD patients with the exception of patients H2 and H5 (Table 4). Low plasma TAC values in those patients seemed to be correlated with worse levels of intracellular ROS compared

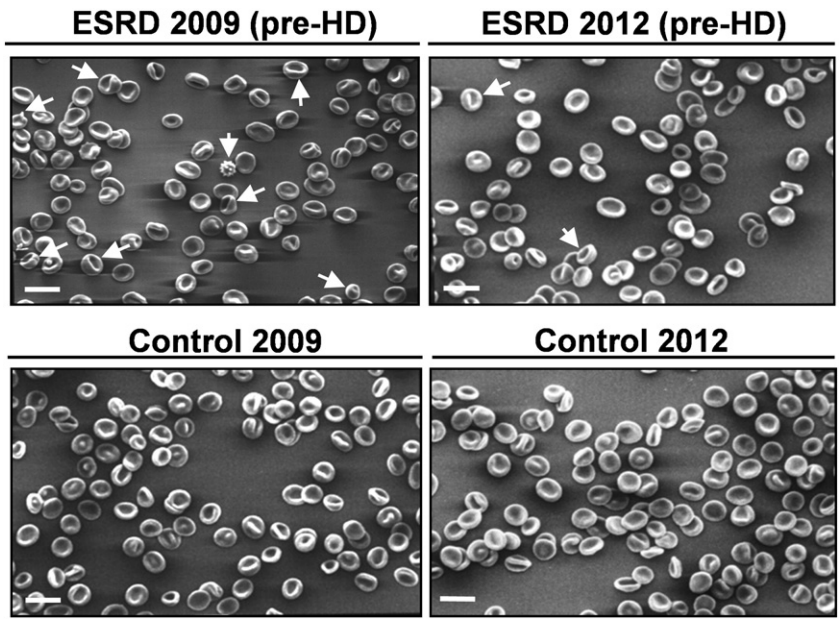

Fig. 4 – Representative scanning electron microscopy micrographs showing the follow-up evaluation of pre-HD RBC morphology in ESRD patients. Arrows indicate stomatocytes and other shape transformations. Scale bars, 10 μm.

to the initial examination (see below). The fluctuation in plasma TAC was attributed to shifts in urate (H2, H3) and/or urate-independent factors (H1, H5). The healthy controls exhibited lower values and minimal fluctuation in plasma TAC levels during the 3-year interval compared to the patients (Table 4).

The endogenous ROS levels in RBCs of ESRD patients after 3 years on HD were evaluated by a fluorimetric assay. Compared to the minor ROS modifications seen in healthy controls for the same period, most of the patients showed a significant decrease in the intracellular ROS accumulation (Table 5). On the other side, patient H5 exhibited a significant increase in RBC ROS, in accordance with the aggravation in serum biochemical status (Table 3, Table 4) and the expression of oxidation stress protein markers in RBC membrane (see below).

Finally, we performed a follow-up evaluation of RBCs' membrane protein modifications in healthy subjects and ESRD patients in Group B (Table 6). Compared to the rate of protein modifications for the same period in healthy controls, the majority of the patients exhibited statistically significant changes in the expression of GLUT1, stomatin and membrane-bound Hb (Table 6 and Fig. 5). Aqp1, flotillin 2, GAPDH and band 3 proteolysis varied significantly in more than half of the patients. Apart from patient H5, Prx2 expression was stable or significantly decreased in patients in Group B (Table 6 and Fig. 5). Moreover, calpain expression was pathologically elevated in only two patients, including patient H5. Alpha adducin, Hsp70, membrane-bound IgGs, band 3 dimerization and clusterin were increased in some cases. Protein carbonylation was proportionally decreased pre- and post-HD in all patients with the exception of patient H5 and, regarding the pre-HD value, of patient H6 too (Table 6).

4. Discussion

Chronic kidney disease is a risk factor for the development of cardiovascular complications, which gradually ends up in tenfold mortality rate after the beginning of the HD therapy [25]. The present study reports the results extracted from a three-year follow up examination of a well-characterized [8] ESRD patient group. It was conducted to gain more insight into the pathophysiology of RBCs and the candidate biomarkers of mortality risk in ESRD, as well as to identify new molecular changes probably associated with the duration and the progression of the disease. To these purposes, (i) we retrospectively analyzed the blood serum and RBC profile of the subgroup of patients that passed away by cardiovascular diseases soon after their initial examination in 2009 compared to those who survived and (ii) we performed a complete follow-up study of the same parameters in the survived patients after three more years on HD therapy.

4.1. *Post-mortem study*

Patients who died were characterized by elevated levels of uremic toxins and prolonged HD, compared to the levels seen in the survivor ones, verifying that both parameters represent risk factors for cardiovascular morbidity and mortality [13,26]. Serum ALP and SGOT level variation between the examined patient subgroups verifies the previously established pathogenic role of ALP in vascular calcification [27], inflammation [28] and cardiovascular mortality in ESRD [29], in common or independently of the serum liver enzyme levels [30].

A variation currently associated for the first time with both the disease duration and mortality in HD patients was the intra-erythrocyte Hb concentration. Despite discordance in

Table 4 – Follow-up evaluation of total (TAC), urate-dependent (UA) and urate-independent (uricase-treated, TAC-UA) antioxidant capacity of plasma in ESRD patients before HD and healthy controls.

	TAC		UA		TAC-UA	
	2009	2012	2009	2012	2009	2012
H1	910	1021*	602	592	308	429*
H2	1414	1237*	1019	739*	395	498
H3	1279	1445*	760	968*	519	477
H4	1236	1277	828	765	408	512*
H5	972	843*	634	565	338	278
H6	1055	1122	644	648	411	474
Average ESRD	1144 ± 195	1164 ± 210	765 ± 150	713 ± 148	397 ± 73	445 ± 86
Average controls	696 ± 117	742 ± 73	432 ± 88	485 ± 66	265 ± 47	258 ± 14

Antioxidant capacity of plasma was measured by the ferric reducing antioxidant power (FRAP) assay (μM Fe^{2+} equivalents). The ESRD plasma was characterized by significantly increased antioxidant capacity levels compared to healthy controls.
* $p < 0.05$, 2009 vs. 2012.

the field [31,32], high HCT [33] and blood Hb levels [34] have been proposed as mortality risk factors in HD, even if found within the physiological normal range, probably in relation to the increased risk of thrombosis, uncontrolled hypertension [35] and HD-related blood viscosity effect [36]. Similarly, in our study, blood Hb and HCT levels were high in the passed-away patients compared to the anemic survivors, but the statistically significant variation refers to the intracellular concentration of Hb. In the follow-up study as well, the duration of HD is associated with a trend to increased MCH and MCHC indexes. Increased concentration of Hb in RBCs under constant Epo supply is most probably correlated to cellular changes, namely, either cell surface loss or pathologic regulation of cell hydration. According to our results, both possibilities could occur in ESRD patients.

Increased microvesiculation leading to erythrocyte surface loss has been reported in ESRD [37]. It is probably driven by the uremic environment, the cardiovascular disease background [38] and the dialysis-associated mechanical stress [39]. Blood microparticles are thought as potent procoagulant factors and potential biomarkers of various diseases characterized by thrombotic and inflammatory events. Such as, the levels of the endothelial microparticles are tightly linked to arterial dysfunction in ESRD. Although we did not measure RBC microvesiculation in the present study, a wide range of membrane vesiculation promoting factors like shape distortion, intracellular calcium activity, metabolic and oxidative stress [40], as well as protein defects that weaken the adhesion of cytoskeleton to the membrane, were all encountered in higher levels in HD patients who passed away. In those cases, there is substantial membrane remodeling involving proteolysis, loss of essential components and oxidative defects, a subset of which were found only in Group A patients, suggesting that more severe uremia is required for those changes to be observed. The calcium-dependent membrane binding of calpain [41], which is associated with band 3 and spectrin proteolysis [42] was higher in Group A patients compared to Group B ones. Interestingly, low activity of membrane ATPases (Na^+–K^+, Mg^{2+} and Ca^{2+}) has been reported in chronic renal disease [43], with obvious consequences in both cell calcium and hydration regulation. In the same context, the membrane expression profile of Aqp1, which is the main water-transporter in RBCs, probably reflects a response to the osmotic provocations of the unstable and complex uremic environment, in order for ESRD RBCs to efficiently regulate their volume and hydration. In consistency, previous studies in chronic renal disease have reported increased resistance of RBCs to osmotic hemolysis [43].

Whatever its origin might be, the intracellular increase of Hb concentration could lead to problematic oxygen access, increased rate of Hb auto-oxidation and a series of Hb-mediated oxidative reactions to cells, as previously suggested in hereditary spherocytosis [44,45]. In our study the oxidative stress was substantially higher in the passed away ESRD patients. In fact, a variety of RBC oxidative indexes, namely, appearance of

Table 5 – Follow-up evaluation of RBC intracellular ROS levels in ESRD patients estimated by fluorometry.

	Patients					
	H1	H2	H3	H4	H5	H6
2009						
Pre-HD	**221**	80	**343**	**211**	**203**	**306**
Post-HD	**203**	**160**	**334**	**256**	**193**	**184**
2012						
Pre-HD	**124***	116	104*	114*	**300***	**146***
Post-HD	**162**	**162**	114*	**154***	**308***	**170**

Data present the endogenous RBC ROS levels (DCF fluorescence) in ESRD patients after normalization to the corresponding (2009 or 2012) control values (100%).
Bold: pathologic values compared to healthy controls.
* $p < 0.05$, 2009 vs. 2012.

Table 6 – Follow-up evaluation of the differentially expressed RBC membrane proteins in healthy subjects and survived ESRD patients in group B.

	Patients						Controls
	H1	H2	H3	H4	H5	H6	
a-Adducin	**80** * –	**106**–	392 *	**426** * –	**114**+	**108**+	104.0 ± 11.6
Aquaporin-1	109	**396** *	63 *	94 *	1288 *	1716 *	114.0 ± 9.5
Band 3 oligomers	**98**–	**88**+	1026 *	**101**–	20 *	**24** * –	96.4 ± 8.7
Band 3 proteolysis	**719** * +	**59** * +	**304** * +	**117** * +	**103**+	**54** * +	88.1 ± 5.2
Calpain	**22** * –	**37** * –	360 *	**102**–	222 *	99	94.4 ± 8.2
Flotillin-2	**147** * –	**93**–	163 *	**79**–	**90**+	**131** * +	87.4 ± 8.9
GAPDH	**156** * –	**72** * –	**90**–	**184** * +	**103**+	**117** * +	95.0 ± 6.2
GLUT1	**156** * –	**192** * –	**158** * +	**92**–	**250** * –	**240** * –	101.2 ± 9.4
Hsp70	45 *	**89**+	**98**+	**84**+	**327** * +	**141** * +	91.1 ± 8.0
IgGs	62 *	**89**+	**83**+	**78**+	**138** * +	**135** * +	82.2 ± 7.5
Membrane Hb	91	98	**216** * –	134 *	**165** * +	124 *	85.7 ± 9.3
Prx2	**35** * –	**78** * –	**94**–	**27** * –	**268** * –	**94**–	88.5 ± 8.5
PCI (pre-HD)	32 *	75 *	72 *	62 *	232 *	208 *	102 ± 23
PCI (post-HD)	75 *	48 *	61 *	18 *	332 *	86	n.d.
Clusterin	77	**116**+	**2700** * +	**112**+	121	**127**+	117.4 ± 10.1
Stomatin	**140** * –	**142** * –	**115**–	**102**–	**364** * –	**265** * –	109.6 ± 8.4

Data represent pre-HD modifications in the relative membrane protein expression after normalization to the immunoblotting data collected in 2009 (100%).
Bold: pathologically increased (+) or decreased (–) protein levels for the 2012 evaluation in ESRD patients compared to the variation range of healthy controls (average ± SD).
* $p < 0.05$ 2009 vs. 2012, statistically significant difference in the rate of change of each parameter compared to the rate of change in healthy controls for the same period.

aberrant electrophoretic bands (representing proteolytic fragments of high molecular weight components), membrane binding of Prx2 as well as protein carbonylation [46–48] were significantly different in Group A patients compared to the Group B ones in relation to the HD procedure. The Prx2 protein is a molecular chaperone with a critical anti-oxidant function in RBCs [47,49,50]. Increased membrane binding of Prx2 has been reported under conditions of high calcium [51] or oxidative stress, including hereditary spherocytosis [52] and ESRD [8]. In a similar way, the cellular and extracellular protein carbonylation stress has been implicated in a wide variety of clinical complications in uremia, including atherosclerosis [46]. Although the HD exhibits negative effect on protein carbonylation in the majority of the ESRD patients [53], there was not a common HD-effect profile among them [8]. In the present study we clarify for the first time that this variation is probably associated with the cardiovascular mortality risk, since the PCI was deteriorating during and after the HD in the passed away patients while it was invariable or even improved in all the cases who survived. This finding suggests that in a background of high mortality risk the antioxidant defense of RBCs against the HD-related oxidative threats is severely affected.

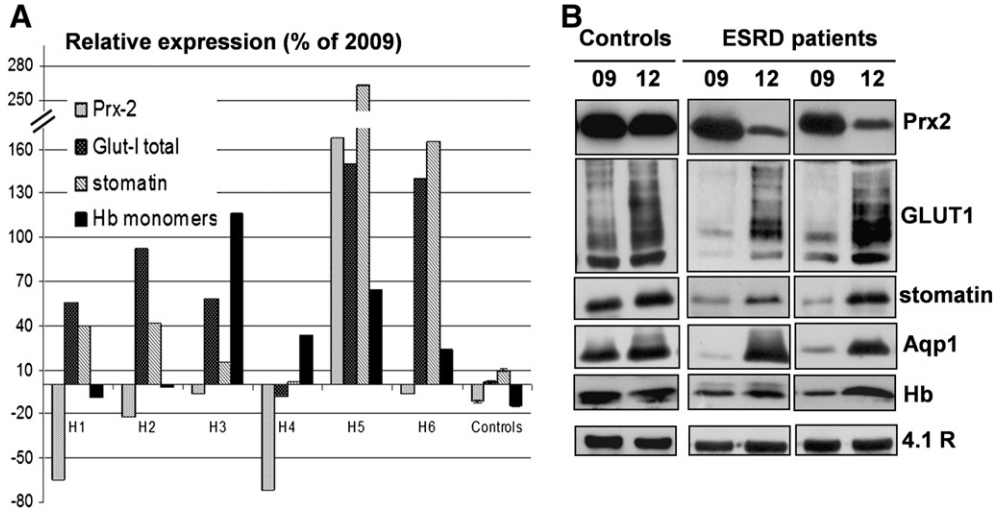

Fig. 5 – Bar graphs (A) and representative immunoblots (B) showing the follow-up evaluation of RBC membrane protein modifications in healthy subjects (average ± SD, n = 10) and ESRD patients. Data in (A) represent variation in the relative membrane protein expression after normalization to the 2009 immunoblotting data (100%).

Interestingly, the RBC membrane of Group B patients was characterized by low expression of GAPDH, GLUT1 and clusterin proteins compared to both the healthy subjects as well as Group A patients. GAPDH profile probably represents an adaptation to the metabolic and oxidative stress. Indeed, membrane binding inhibits enzyme activity and drives the usage of cellular glucose to the phosphate pentose pathway for the regeneration of reduced glutathione [54,55]. GLUT1 deficiency is reported for the first time in ESRD patients and as discussed later, it may be associated with numerous intracellular functions. The small loss of GLUT1 and adducin in Group A membranes compared to Group B ones could be a compensatory response for the observed loss of band 3, since all of these proteins exhibit a critical and probably synergistic structural role in RBC membrane [56]. Clusterin is a ubiquitously expressed molecular chaperone. Serum clusterin levels have been considered an index of underlying cardiovascular damage [57] while erythrocyte clusterin levels, a sensitive biomarker of senescence and cellular, including oxidative stress [58]. The currently reported for the first time clusterin downregulation in ESRD RBCs was not a surprising finding considering recent reports on clusterin expression in senescent, stressed or diseased erythrocytes [58]. Although it could be related to increased vesiculation of ESRD erythrocytes [37,59], its intergroup variability (Group A > Group B) suggests a different degree of protein absorbance from the serum. In support, decreased clusterin levels have been reported in the plasma of long-term HD survivors [60].

Finally, the elevated urate-independent plasma antioxidant capacity in the oxidatively challenged ESRD patients probably reflects a homeostatic production of low molecular weight antioxidants, especially of lipophilic ones like the vitamin E, which are not severely cleared during the hemodialysis.

4.2. *Follow-up study*

In the second part of the study, the follow-up examination of the patients after three additional years in HD, revealed stable or improved uremic toxin levels in the majority of them, suggesting a positive response to the HD therapy. Uremic toxin interaction with RBCs leads to local or extensive oxidative modifications to membrane components. In contrast to Epo that cannot affect this interaction, effective HD can ameliorate the toxin-associated cellular defects.

In dialyzed patients, the erythrocyte membrane proteins develop modifications as a probable compensative response to the chronic stress. Interestingly, the uremic index variation in long survived ESRD patients is accurately reflected not only in serum biochemicals but also in plasma and cellular stress factors, even in the erythrocyte shape. For instance, patient H5 who was presented with a relative increase in serum creatinine also exhibited a parallel increase in serum ALP, anisocytosis as well as deterioration in intracellular ROS, membrane-bound Prx2 and calpain, protein carbonylation and plasma TAC levels. The relative increase in γGT in half of the patients is correlated to a slight aggravation in creatinine clearance (post-HD values, Table 4), verifying its role as an independent risk factor of atherosclerosis and cardiovascular mortality [61]. Interestingly, the enormous increase of γGT in patient H3 was associated with an impressive increase in RBC membrane clusterin, reminding previous reports on serum clusterin as a cardiovascular disease biomarker [57].

Furthermore, at the RBC membrane level, there was a substantial variation in the membrane expression of several components after three years on HD. Augmented membrane binding of Hb is probably dictated by the intracellular Hb concentration levels, as previously observed in high MCHC patients [45]. GLUT1 overexpression in relation to the duration of ESRD is reported for the first time. It might be related to the improved RBC morphology and vesiculation, since GLUT1 is effectively exocytosed [62]. Most probably it represents a compensative response to the prolonged osmotic [63], mechanical, metabolic and oxidative stress imposed by the uremic milieu on RBCs. Increased GLUT1 expression could mean increased glucose uptake for metabolism purposes. Indeed, it has been established that glucose deprivation and hypoxia promote the membrane expression of GLUT1 [64] and that plasma levels of glucose are reduced in ESRD [7]. Moreover, it is known that GLUT1 accomplishes a different, structural role in RBCs, the adhesion of skeletal components to the membrane [56]. The amplification of this linkage in the mechanically stressed ESRD RBCs might be compensatory for the currently and previously observed [8,43] loss of the main structural membrane component, the band 3 protein, that also mediates the adhesion of cytoskeleton to the membrane. The same context of protein interactions could probably define the parallel adducin variation in the majority of our patients, similar to other studies [65]. Apart from these functions, the abundant molecules of GLUT1 in the RBC membrane might insure plasma ascorbic regeneration in ESRD. Ascorbic is a critical antioxidant factor, protecting the vitamin E of plasma lipoproteins as well as the RBC membrane components from oxidative defects, a mechanism that is especially important in atherosclerosis [66]. As an effective ROS scavenger, plasma ascorbic acid is oxidized to dehydroascorbic (DHA) that enters RBCs through GLUT1 [67]. Inside RBCs, DHA is quickly reduced to ascorbic and then it slowly diffuses back to plasma. HD has been associated with increased conversion of ascorbic to DHA [9]. Notably, the physical association of GLUT1 with stomatin [68] favors DHA transport [69] at the expense of glucose transport activity [70]. In the light of this evidence, the currently observed common variation profile of GLUT1, stomatin and urate-independent plasma TAC might be well interpreted in our patients.

Apart from GLUT1 regulation, stomatin accomplishes critical regulatory interactions with many ionic channels, membrane transporters and proteins including urea transporter, calcium pump, CD47, pallidin, flotillins and aquaporin 1 [71]. Dehydrated hereditary stomatocytosis for instance, has been found to be functionally connected with mechanotransduction pathways mediated by “mechanosensor” proteins and stomatin family members [72,73]. Notably, this disease is characterized by RBC dehydration, increased MCHC, resistance to osmotic lysis, mild stomatocytosis and peripheral edema. In a striking similarity, the mechanically stressed RBCs in ESRD present alike distortions regarding intracellular Hb concentration (present study), stomatocytic transformation [8] and resistance to osmotic lysis [43]. Mechanosensor proteins hold critical role in the RBC volume homeostasis [74] through pathways implicating calcium flux.

Although a mechanosensory feedback mechanism in ESRD RBCs is needed and reasonably expected, it has not been studied yet. However, to this respect, it is worth noting that in our study the duration of HD parallels the overexpression of stomatin at the expense of the stomatocytic transformation of RBCs.

4.3. Conclusions

In conclusion, this study demonstrates novel blood modifications probably associated with the duration of Epo/HD treatment, the disease progression and the cardiovascular mortality in ESRD. The duration of HD is associated with increased intracellular Hb concentration and membrane expression of GLUT1, stomatin and Aqp1. High mortality risk seems to be related to increased levels of uremic toxins, structurally altered and oxidatively damaged erythrocytes probably as a result of poor response to the HD therapy. Contrary, the smooth disease progression in ESRD patients is presented with maintainable or even improved uremic and erythrocyte stress markers. Both erythrocytes and plasma in prolonged HD show compensatory responses to the challenges of uremic milieu. Although the currently studied target groups are rather small, the volume of the hematologic and cellular parameters examined adds credibility to our results. The currently presented hematological and RBC protein variability adds new pieces to the erythrocyte pathophysiology puzzle in ESRD and suggests novel factors for consideration in future large scale studies on cardiovascular morbidity and mortality biomarkers in uremic patients.

Competing interests

The authors declare that no competing interests exist.

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