JOURNAL OF PROTEOMICS 74 (2011) 2441–2452

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Oxidative stress-associated shape transformation and membrane proteome remodeling in erythrocytes of end stage renal disease patients on hemodialysis

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ARTICLE INFO

Available online 15 April 2011

Keywords:
End stage renal disease
Hemodialysis
Red blood cell
Membrane proteome
ROS production
Oxidative stress

ABSTRACT

This study was designed to evaluate the oxidative stress status of erythrocytes and its association with cellular ultrastructure and membrane proteome modifications in patients with end stage renal disease (ESRD) on hemodialysis (HD). For that purpose, we studied red blood cells' (RBCs) modifications in twelve non-diabetic ESRD patients that were responsive in erythropoietin therapy. Intracellular ROS levels were measured by fluorometry, RBCs ultra-structure was examined by electron microscopy, while the membrane proteome by electrophoresis and immunoblotting. Compared to the healthy subjects, the uremic RBCs exhibited significantly increased ROS accumulation. Dialysis partially ameliorated the basal ROS levels but triggered cellular sensitivity to exogenous oxidative stimuli. Common membrane modifications involved loss, aggregation, fragmentation and carbonylation of critical components as well as over-expression of stress markers. HD significantly contributed to membrane proteome remodeling, especially for aquaporin-1, peroxiredoxin-2 and ubiquitinated proteins. The intracellular redox status and the closely associated membrane modifications seemed to be related to membrane instability, loss of surface area through vesiculation, echinocytosis and stomatocytosis. Our data evinced a network of interactions among the uremic toxins, the RBCs membrane composition and the cellular shape modifications in ESRD, which is developed around a core of oxidative provocations and cellular responses.

1. Introduction

Anemia is a common complication in end-stage renal disease (ESRD) patients on hemodialysis (HD), often leading to higher morbidity and mortality. Inadequate production of erythropoietin, impaired response of erythroid stem cells to erythropoietin, chronic hemolysis and blood loss are leading factors of anemia in ESRD. Furthermore, the RBCs of hemodialyzed

Abbreviations: ESRD, end stage renal disease; Hb, hemoglobin; HD, hemodialysis; MW, molecular weight; PCI, proteome carbonylation index; Prx-2, peroxiredoxin-2; RBCs, red blood cells; SEM, scanning electron microscopy; TEM, transmission electron microscopy; t-BHP, tert-butyl hydroperoxide.

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doi:10.1016/j.jprot.2011.04.009

patients are mechanically stressed by the flow through the dialyzer and the peristaltic pumps as well as metabolically stressed by the accumulation of uremic toxins and the loss of glucose. Due to their reduced resistance to mechanical, metabolic and osmotic stress RBCs lifespan may be shortened [1]. Defected deformability of RBCs that are negatively affected by the HD sessions has also been reported in ESRD patients, compromising both, their rheology in the microvasculature and their survival [2]. Modifications in RBCs membrane protein composition and function may account for the diminished deformability of the ESRD RBCs. Indeed, there is strong evidence for both, damages in several proteins and also for inadequate protein repair in uremic RBCs [3]. Reactive oxygen species (ROS) released by phagocytes and platelets during the contact of blood with the dialysis membranes consist another stressful factor for RBCs in ESRD patients on HD. Disintegration of damaged RBCs and release of hemoglobin (Hb) may further aggravate the pro-oxidant status in the plasma. Moreover, depletion of antioxidants, accumulation of uremic toxins, advanced age, chronic inflammatory state, deficiency of vitamins C, E and selenium and finally, factors connected with HD trigger further accumulation of pro-oxidant compounds in the blood of chronic renal failure patients. Consequently, blood oxidative stress has been established as an intrinsic component of the uremic state [4]. Although many studies have focused on serum markers of oxidation [4,5], the oxidative defects of RBCs membrane represent a primary cause of chronic hemolysis in ESRD patients receiving HD therapy [6]. Indeed, increase in membrane fluidity and cellular osmotic fragility, disturbance of cytoskeleton protein–protein interactions [7] and increased susceptibility to complement deposition [8] have been documented in the context of oxidative stress-associated changes in ESRD erythrocytes.

The aim of the present study is the evaluation of the endogenous ROS accumulation in RBCs and its probable connection with RBCs structural and protein modifications in non-diabetic ESRD patients on HD. Our current data indicate a strong association of corpuscular oxidative and carbonyl stress with several cellular distortions in ESRD, ranging from RBCs shape modifications to membrane proteome defects and stress responses.

2. Materials and methods

2.1. Material supplies

Monoclonal antibodies against Band 3 (B9277) and actin (A5316), polyclonal antibodies against spectrin (S1515) and human IgGs (A8792) and HRP-conjugated antibodies to goat IgGs (A-5420), as well as the Protease Inhibitor Cocktail, t-butyl hydroperoxide (t-BHP) and common chemicals and buffers were all obtained from Sigma-Aldrich (Munich, Germany). Polyclonal antibodies against Hb (GR800GAP) and peroxiredoxin-2 (Prx-2, SP5464) were obtained from Europa Bioproducts (Cambridge, UK) and from Acris GmbH (Hiddenhausen, Germany), respectively. Primary antibodies against CD47 (sc-25773), HSP70 (sc-1060), Fas (Apo-1, sc-715), calpain-1 (μ-calpain, sc-7531) and Band 3 (sc-20657) were from Santa Cruz Biotechnology (Santa Cruz, CA). mAbs to ubiquitinated proteins (#3936) and aquaporin 1 (MCA2099) were obtained from Cell Signaling Technology (Beverly, MA) and AbD Serotec, respectively. mAbs against synexin (annexin VII, 610669) and flotillin-2 (610384) were obtained from BD Transduction Laboratories (San Diego, CA). 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) was from Invitrogen, Molecular Probes (C-6827). HRP-conjugated antibodies to rabbit IgGs (NA 934) and ECL Western blot detection kit were from GE Healthcare Amersham (Little Chalfont, Buckinghamshire, UK). HRP-conjugated antibodies to mouse IgGs (P0161) were from DakoCytomation (Glostrup, Denmark). The Oxyblot® detection kit (S7150) was obtained from Millipore, Chemicon (Temecula, CA). Bradford protein assay was obtained from Bio-Rad (Hercules, CA). Gel Analyzer v.1.0 image-processing system and software was obtained from Biosure (Athens, Greece). mAb against stomatin and antiserums against proteins 4.1R and pallidin (band 4.2) were kindly provided by Prof. R. Prohaska (Department of Medical Biochemistry, Medical University of Vienna, Austria) and Prof. J. Delaunay (Service d' Hématologie, Hôpital de Bicetre, Le Kremlin-Bicetre, France) respectively.

2.2. Subjects

We evaluated twelve ESRD patients (Table 1) on standard HD therapy (thrice weekly) and erythropoietin treatment to reach

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

	Controls (n=12)	ESRD patients (n=12)
Age (years)	45.0±11.5	65.8±13.9[a]
Gender (M/F)	7/5	8/4
Time on HD (months)	–	50±11
WBCs ($\times10^9$/L)	5.78±1.34	6.30±2.16
RBCs ($\times10^{12}$/L)	5.09±0.44	4.00±0.67[a]
Hb (g/dL)	15.81±0.23	11.79±0.72[a]
HCT (%)	46.90±1.26	35.32±1.35[a]
MCV (fL)	92.14±2.13	94.59±7.96
MCV (fL) post-HD	–	**94.89±7.97**
MCH (pg)	31.06±1.11	32.12±1.09
MCHC (g/dL)	33.71±2.15	30.46±4.25
RDW-CV (%)	13.41±0.39	16.32±1.48[a]
RDW-CV (%) post-HD	–	**16.48±1.45**
PLTs ($\times10^3$/μL)	258.22±23.45	236.17±48.22
Glucose (mgr/dL)	85.21±5.78	100.87±13.45
Urea (mg/dL)	29.11±3.45	162.83±34.44[a]
Urea (mg/dL) post-HD	–	**54.99±16.09**[a]
Creatinine (mg/dL)	0.74±0.21	9.56±2.31[a]
Creatinine (mg/dL) post-HD	–	**6.08±2.31**[a]
Cholesterol (mg/dL)	138.34±19.56	153.63±34.31
Uric acid (mg/dL)	4.64±1.26	6.55±2.98[a]
Triglycerides (mg/dL)	150.31±34.96	178.91±65.45
Potassium (mEq/L)	4.32±0.36	5.56±1.72[a]
Potassium (mEq/L) post-HD	–	**4.02±0.76**[b]
Iron (μg/dL)	103.34±12.31	74.11±20.57
Calcium (mg/dL)	9.46±0.21	9.38±0.24
Phosphorus (mg/dL)	3.54±1.11	5.55±1.65[a]

Post-HD measurements are presented in bold. Results are presented as mean±SD.
[a] $p<0.05$ vs. controls.
[b] $p<0.05$ vs. pre-HD.

a hematocrit >33%. All patients used highly biocompatible polysulfone (n=6) or acrylonitrile (n=6) dialyzers (Gambro-Hospal Ltd) and were clinically stable at the time of investigation. The primary causes of renal failure in the patients' group were as follows: hypertensive nephropathy (n=2), glomerulonephritis (n=1), obstructive nephropathy (n=1), polycystic kidney disease (n=1) and chronic renal failure of unknown etiology (n=7). Blood pressure was maintained under therapeutic control in 7 patients. The control group included 12 healthy subjects presenting normal hematological and serum biochemical values, with no history of renal or inflammatory diseases and as far as possible age- and gender-matched with the ESRD patients' group. Patients with diabetes mellitus, autoimmune disease, malignancy, hematological disorders and acute or chronic infection were excluded. The study has been submitted and has been approved by the Research Bioethics and BioSecure Committee of the Faculty of Biology/University of Athens. Investigations were carried out in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all blood donors participating in this study.

2.3. *Hematological and biochemical analysis and isolation of RBC membrane*

Blood samples were collected in EDTA anticoagulant before starting HD, 20 min after the start of the session and immediately after it. Differential leukocyte and RBC count, hematoctrit, Hb concentration and RBC indexes (mean cell volume, MCV; mean cell Hb, MCH; mean cell Hb concentration, MCHC; RBC distribution width, RDW) were performed 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). Purified RBCs were lysed with hypotonic (5 mM) sodium phosphate buffer (pH 8.0) containing a cocktail of protease inhibitors and membrane fractions were prepared as previously described [9]. Total protein concentration of the membrane extractions was determined using the Bradford protein assay with BSA as a standard.

2.4. *Detection of ROS in RBCs*

ROS accumulation in RBCs was detected with the membrane-permeable and redox-sensitive dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) according to the manufacturer's guidelines with minor modifications. More specifically, isolated and thoroughly washed RBCs (each sample in triplets) were incubated for 20 min in pre-warmed PBS in the absence (endogenous ROS) or in the presence (exogenously induced oxidation) of 100 μM tert-butyl hydroperoxide (t-BHP) at 25 °C. Following the removal of the oxidant (if applied), 1% RBCs suspension was loaded with 10 μM CM-H_2DCFDA in PBS buffer. After 30 min at 25 °C, the CM-H_2-DCFDA-loaded RBCs were washed once with PBS and incubated in the same buffer for a short recovery time of 10–15 min in order to render the dye responsive to oxidation. Incubation at this point of a series of samples with 100 μM t-BHP at 25 °C in the dark had proportionally similar oxidation effects to RBCs (data not shown). The production of fluorescent DCF (dichlorofluorescein) 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 protein level (~1.5–3.0 mg/ml). The following negative controls were used: (1) unstained RBCs incubated with only PBS buffer to detect auto-fluorescence, and, (2) cell-free mixtures of dye and buffers with or without the addition of the external oxidant.

2.5. *Transmission and scanning electron microscopy*

Ultrastructural evaluation of RBCs from ESRD patients was performed with a transmission electron microscope (Philips TEM300). For that purpose, peripheral blood was collected shortly before the HD session and purified RBCs were fixed with 4% paraformaldehyde (methanol-free, Polysciences, Inc., Warrington, PA), 2.5% glutaraldehyde (electron microscopy grade, Serva, Heidelberg, Germany) in 0.1 M sodium cacodylate buffer, pH 7.4, and embedded in epoxy resins according to standard procedures. For the morphologic evaluation of RBCs with a scanning electron microscope (SEM), purified RBCs were fixed with 2% glutaraldehyde (electron microscopy grade, Serva, Heidelberg, Germany) and post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4. Fixed cells were successively dehydrated in ascending ethanol series (from 30% to 100% v/v) 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).

2.6. *Immunoblotting analysis and detection of protein carbonyl groups*

Equal amounts (12–22 μg) of RBC membrane protein were loaded in Laemmli gels, blotted to nitrocellulose membranes and probed with primary and horseradish peroxidase-conjugated secondary antibodies as previously described [10]. Immunoblots were developed using an ECL reagent kit and quantified by lengthwise scanning densitometry. Individual protein levels were quantified as percentage of total and final normalization to the control, namely the healthy subjects (% of controls). For the protein carbonylation analysis, total RBC 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 being the relative percentage of the total oxyblot bands to a reference protein immunoblotted band, further normalized to the controls (100%).

2.7. *Statistical analysis*

All the presented experiments have been repeated at least twice, unless otherwise stated. Each data point (e.g. ROS analysis by fluorometry) corresponds to the mean value of the independent experiments; error bars denote standard deviation (SD). Protein expression levels were quantified against a reference membrane protein (e.g. PCI) or against the sum of the normally expressed proteins, further normalized to the controls. All quantitative measurements were expressed as mean ± SD for patients and healthy populations separately. For

statistical analysis we used the MS Excel program and the Statistical Package for Social Sciences (IBM SPSS; version 19.0 for Windows; administrated by the National and Kapodistrian University of Athens). Statistical significance was evaluated using the one-way analysis of variance (ANOVA). Comparisons between different groups were performed by independent t-test or chi-squared test. Pearson's, Spearman's and Kendall's tau correlation tests (two-sided) were used to assess the relationship between variables (correlation coefficient r). Significance was accepted at $p<0.05$. Results at $p<0.05$ and $p<0.01$ are indicated in the graphs by one or two asterisks, respectively.

3. Results and discussion

3.1. *Hematological and biochemical serum analysis*

Hematological and biochemical serum characteristics of the maintenance HD studied patients are summarized in Table 1. ESRD patients were anemic with increased uremic markers. A statistical significant increase in RDW was also observed which probably indicated incidence of reticulocytosis and anisocytosis due to morphological changes in RBCs.

3.2. *RBCs endogenous ROS accumulation and susceptibility to exogenous oxidant*

Previous studies have reported that the RBCs enzymatic antioxidant defense mechanisms are suppressed in chronic renal failure patients particularly in those on HD treatment [11]. In order to examine the oxidative status of patients' RBCs, we estimated the endogenous intracellular ROS levels by fluorometry. By evaluating the patients before and after the HD session, we assessed whether or not they respond similarly to the HD process.

Compared to an equal number of apparently healthy subjects (controls), the patients exhibited a significantly increased ROS accumulation in RBCs before and after the HD session (Fig. 1 gray bars). That finding suggested the effect of renal pathology and chronic HD on the oxidative status of RBCs. It should be noted that only one patient, the youngest one (40 years old), depicted normal ROS levels before the HD, probably signifying the aggravating role of organism aging on RBCs ROS production and management, as previously suggested [12]. Our data indicate an average increase of ~140% in the ROS content of the patients' RBCs before the HD, which was slightly reduced (by 8%) after the HD session (Fig. 1 gray bars). Usually, the post-HD ROS levels were lower compared to the pre-HD levels (by 5–47%), although in 3 patients HD exhibited an opposite effect (increase of 20–100%). Thus, despite the fact that HD therapy may relieve the dangerous ROS overload in RBCs, it does not restore the normal oxidation status nor induce gross changes in RBCs endogenous ROS content. These findings are in accordance with previous studies on uremic RBCs lipoperoxidation which suggested that the oxidative stress is not a result of HD but an intrinsic state in uremia [13], failed to be normalized by HD [14]. In the same context, the pathologically increased ROS accumulation in ESRD lymphocytes has not been found significantly different before and after the HD procedure [15]. Notably, HD also seems to have no effect on the cellular deformability [16].

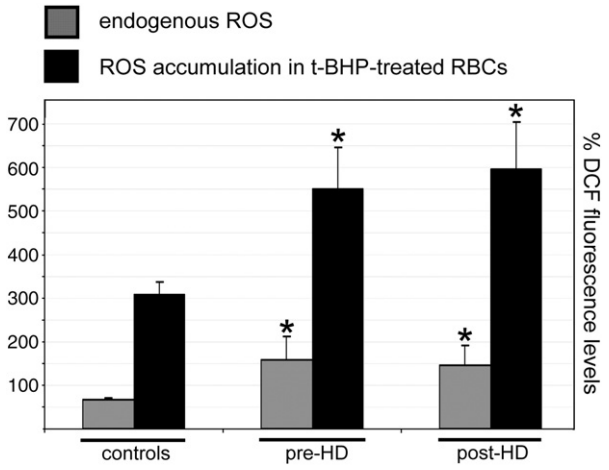

Fig. 1 – Bar graph showing the collective intracellular ROS accumulation in ESRD patients compared to the controls. Data represents the mean ± SD (error bars) of DCF fluorescence levels estimated by two independent experiments (done in triplicate) following normalization to protein levels in untreated cells (gray bars) or t-BHP-treated RBCs (black bars). (*) $p<0.01$ vs. controls.

On the contrary, HD may occasionally (in 3 patients) stimulate the endogenous ROS accumulation in ESRD RBCs and moreover, it generally renders cells more susceptible to exogenous oxidative stimuli. As shown in Fig. 1 (black bars), the t-BHP-treated RBCs of ESRD patients, especially those collected after the HD session, exhibited a further increase (by ~70–100%) in ROS production compared to the induced ROS production in the control RBCs. Similar results have been reported regarding the membrane fluidity analysis [7] and the lipid peroxidation sensitivity [14] of in vitro oxidized uremic RBCs. Loss of antioxidant plasma substances during the HD session [4] might trigger the post-HD intracellular ROS accumulation. In our patients, after normalization to the endogenous ROS levels, the t-BHP-induced ROS accumulation exhibited an average of ~4.8- to 4.9-fold increase in control RBCs and post-HD RBCs vs. the ~4.1-fold increase in pre-HD samples. This finding suggests a triggered antioxidant defense of pre-HD RBCs as an adaptive mechanism to the increased in vivo oxidative stress or a saturated state in intracellular ROS accumulation, as it has been previously implied [7,14]. In conclusion, the RBCs in ESRD on HD were under sustained increased oxidative stress. Dialysis seemed to be associated with a rather individual response in ROS production, although in the majority of the cases it is associated with a reduction in the net ROS cellular content but also an increase in cellular susceptibility to exogenous oxidative stimuli. Finally, a strong correlation between the endogenous ROS production and the RDW index was observed (Table 2).

3.3. *Morphological analysis of pre-HD RBCs–ROS correlations*

Ultrastructural study of pre-HD erythrocytes through TEM (Fig. 2A–G) gave evidence of membrane instability (inset in Fig. 2G) and release of membrane exovesicles (Fig. 2B). RBCs with echinocytic indentation of the membrane (Fig. 2A) as well

as cup-shaped, stomatocyte-like cells (Fig. 2C and D) with membrane-coated vacuoles within the cytoplasm (Fig. 2E–G), similar to those previously described [17], were also observed. Reticulocytes were abundant (inset in Fig. 2F). SEM morphological analysis revealed variations from normal discocyte shape to intent anisocytosis and poikilocytosis (Fig. 2H–L). Compared to healthy subjects (inset in Fig. 2L), significantly elevated numbers of circulating echinocytes (2.68±0.77%, in addition to a higher percentage of discocytes presenting with minor echinocytic tendencies), target cells (codocytes, 2.4±0.73%), as well as stomatocytes (3.86±1.37%) and knizocytes (3.96±2.05%), were present in the majority of the patients (Fig. 2H–L). Classification of echinocytes and stomatocytes was achieved by using the standard criteria [17]. Reticulocytes (6.11±1.44%, vs. 0.5–2.0% in controls) were also significantly increased, signifying the RBCs rejuvenation under the influence of erythropoietin. Low but detectable presentation of spheroechinocytes (0.2–0.6%) was evident in the blood of 4 patients. Occasionally, dysmorphic cells such as dacryocytes, ovalocytes, elliptocytes and schistocytes were also noticed (Fig. 2J). Their formation is consistent with the degree of anemia (Table 1) and the abnormal RBCs membrane composition (see below). Using the Pearson's linear regression analysis, a significant direct relation between the stomatocyte and the knizocyte percentages was observed (Table 2), in consistency with the notion that the latter represents a deeper alteration of the stomatocyte. Interestingly, the pre-HD ROS intracellular levels were positively correlated with the echinocyte abundance and inversely correlated with the elevated numbers of stomatocytes and knizocytes (Table 2). The variation in the echinocytic percentage was also following that of the RDW index (Table 2).

Surface homeostasis in response to various environmental and corpuscular conditions leads to the transformation of

Table 2 – Statistically significant correlations among the morphological and biochemical variables tested in the serum and erythrocytes of ESRD patients in relation to the dialysis session.

	Pre-HD			After 20 min of HD			Post-HD		
Variation-1	Variation-2	r value	p value	Variation-2	r value	p value	Variation-2	r value	p value
PCI	Urea	0.636(a)	0.048	CD47	−0.665	0.018	Calpain-1	0.612	0.035
	Creatinine	0.699(a)	0.024	IgGs	0.660	0.020	Urea	0.537[b]	0.031
	Calpain-1	0.851	0.001*						
ROS	Echinocytes	0.943[a]	0.005*				4.1R	−0.812	0.004*
	Stomatocytes	−0.825	0.043				Ub-proteins	−0.868	0.001*
	Knizocytes	−0.827	0.042						
	RDW	0.813	0.004*						
	Ub-proteins	−0.711	0.021						
	B3 proteolysis	0.762[a]	0.028						
Echinocytes	Reticulocytes	−0.838	0.019						
	RDW	0.918	0.004*						
	ROS	0.943(a)	0.005*						
	Sp-fragments	0.857[a]	0.014						
Stomatocytes	Knizocytes	0.939	0.002*						
	ROS	−0.825	0.043						
	Ub-proteins	0.937	0.002*						
	Prx-2	0.932	0.002*						
	Stomatin	0.857[a]	0.014						
Knizocytes	Stomatocytes	0.939	0.002*						
	ROS	−0.827	0.042						
	Prx-2	0.956	0.001*						
	Ub-proteins	0.837	0.019						
Prx-2	Stomatocytes	0.932	0.002*	Calpain	0.641[a]	0.025	Sp–Hb	0.608[a]	0.036
	Knizocytes	0.956	0.001*				Calpain-1	0.697[a]	0.012
	Creatinine	0.635[a]	0.027						
	Calpain-1	0.713[a]	0.009*						
HSP70	MCH	−0.631	0.028	Sp-fragments	0.774	0.003*	Sp–Hb	0.711	0.010
	MCHC	−0.837	0.001*				Urea	0.739	0.006*
Calpain-1	PCI	0.851	0.001*	Band 3	−0.693	0.012	PCI	0.612	0.035
	Prx-2	0.713[a]	0.009*	Prx-2	0.641[a]	0.025	Prx-2	0.697[a]	0.012
Sp-fragments	Echinocytes	0.857[a]	0.014	HSP70	0.774	0.003*	Time on HD	0.630	0.028
	Target cells	0.867	0.011	Ub-proteins	−0.579	0.049			
	Fas	−0.599	0.040						
Ub-proteins	Stomatocytes	0.937	0.002*	4.1R	0.730	0.007*	ROS	−0.868	0.001*
	Knizocytes	0.837	0.019	Pallidin (4.2)	0.679	0.015	4.1R	0.685	0.014
	ROS	−0.711	0.021	Sp-fragments	−0.579	0.049	Aquaporin-1	0.608(a)	0.036

r value represents linear correlation coefficient according to Pearson's two-tailed test, except otherwise noted.
[a] Spearman.
[b] Kendall's tau.
* $p<0.010$.

biconcave discocytes to echinocytes and stomatocytes, which represent the two extreme shape transformations of RBCs. As already known, the degree of both modifications may influence blood viscosity, membrane deformability and rheological properties of RBCs [17,18]. Through the direct modification of cell geometry (surface area/volume ratio) and the closely associated alterations of the membrane skeleton, those transformations may contribute to the shortened survival of RBCs in ESRD. Moreover, the redox status of RBCs appears linked to shape control. Echinocytosis is generally thought as the morphological sign of increased oxidative stress and aging. Indeed, in vitro oxidation of RBCs leads to progressive echinocyte formation with apparent membrane rigidity defects, increased erythrophagocytosis and accumulation of protein oxidative signs in the membrane [19]. The echinocytic transformation is common in the blood of ESRD patients on HD, especially in those exhibiting secondary complications [20], and seems to be dictated by a uremic factor through the elevated Ca^{2+} control [20,21]. The occurrence of specific membrane protein modifications in our samples (see Section 3.5) supports the contribution of impaired redox status, aging and probably of Ca^{2+}-driven processes in the echinocytic transformation of pre-HD RBCs. HD seems to be a significant contributor to a transient increase in the echinocyte percentage [22], in relation to the intracellular ROS and calcium levels [23]. Nevertheless, the pre-HD levels of echinocytes have been found significantly increased compared to the observed post-HD echinocytosis [23]. Our results documented a strong correlation between ROS intracellular accumulation and spectrin fragmentation with the degree of pre-HD echinocytosis (Table 2) suggesting that the oxidative stress of RBCs before the HD session (Fig. 1) might be another factor associated with the pathologically increased number of circulating echinocytes. Notably, previous studies have reported fragmentation of Band 3 protein in renal failure patients, exhibiting an increased number of echinocytes [24].

Since the mechanical properties of the membrane are mirrored in the RBCs shape, the co-existence of echinocytes with stomatocytes in ESRD patients was a surprising finding. Previous studies have reported significant differences in the mechanical properties and rheological behavior of RBCs as a consequence of erythropoietin treatment [25]. In our study, the response to erythropoietin was well reflected in the hematological profile and the relatively high value of the MCV index (Table 1). Furthermore, the vast majority of echinocytes and stomatocytes presently observed seemed to be reversible distortions, since they were not carried out to the spherocyte stage. Following the retraction of an echinocytogenic stimulus, RBCs can recover from the echinocyte transformation. In some cases, these recovering cells develop a redox-related morphological instability and instead of maintaining the discoid shape, they further proceed to form stomatocytes, in accordance to the degree of the initial crenation [26]. Consequently, it might

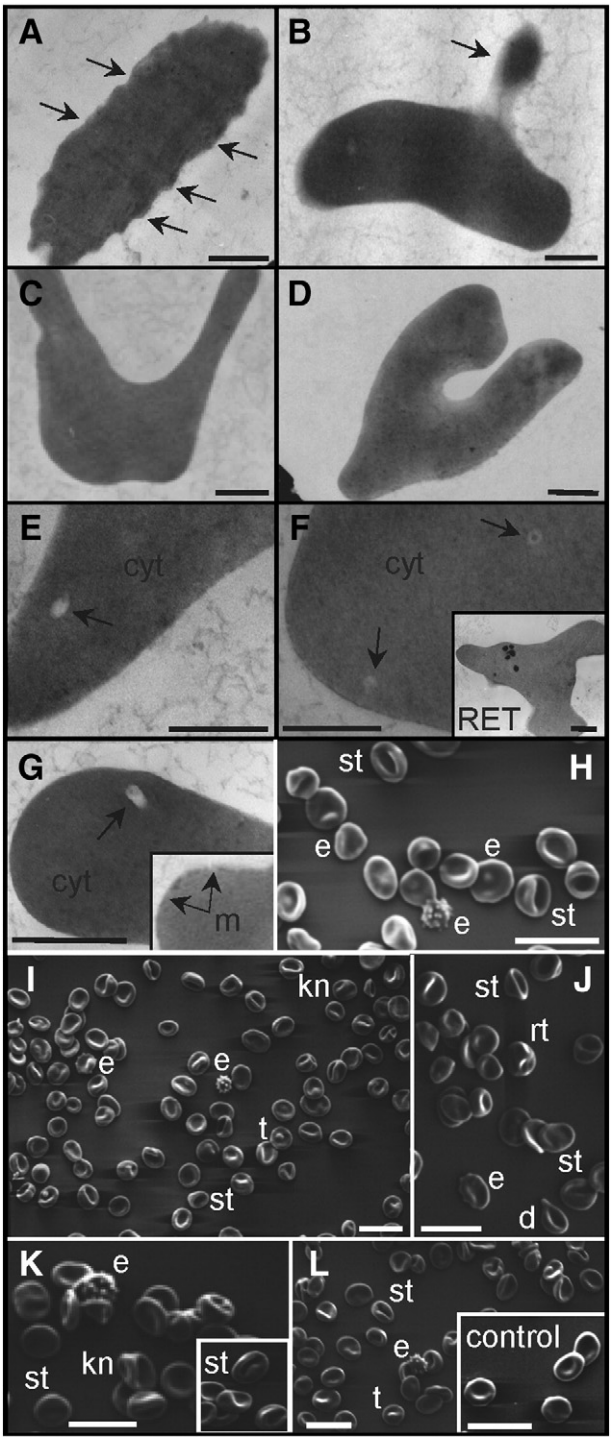

Fig. 2 – Electron micrographs showing morphological and ultrastructural details of ESRD RBCs. TEM micrographs (A–G) of thin sections of resin-embedded RBCs from different ESRD patients. Evident were the indentation of the cell periphery in the echinocytes (A) and the release of vesicles (B). Cup-shaped cells (C, D) and membrane-coated vacuole-like structures (arrows) within the cytosol (cyt, E–G) were also observed. In some cases, the membrane exhibited signs of discontinuity (inset in G). Reticulocytes were abundant (inset in F). SEM images of erythrocytes from ESRD patients and healthy subjects (H–L), illustrating the increased susceptibility of ESRD RBCs to both echinocytic (e) and stomatocytic (st) shape changes. Triconcave knizocytes (kn), target cells (t), dacryocytes (d) and reticulocytes (rt) with pits on their surface were also present. Control RBCs were biconcave discocytes (inset in L). Bars 1 μm for A–G; 10 μm for H–L.

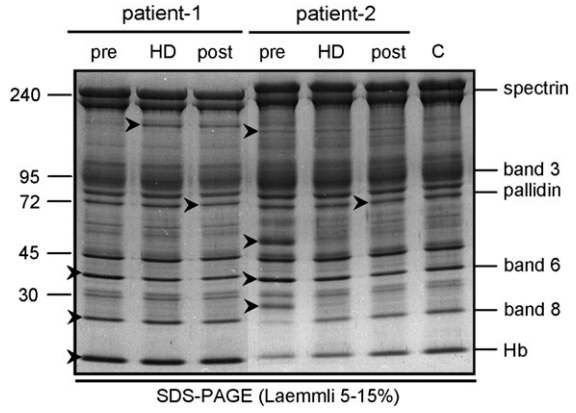

Fig. 3 – Representative SDS-PAGE analysis of membrane proteins isolated from ESRD RBCs. Two patients' samples were presented from RBCs collected before (pre), during (HD) and after (post) the HD session compared to a healthy subject (C). Arrowheads indicate the aberrant bands and the fluctuation in the membrane expression of proteins pallidin, band 6, band 8 and Hb. Molecular weight markers (in kDa) are indicated to the left of the gels.

be speculated that the stomatocytes seen in the ESRD patients on HD represent recovering echinocytes in response to the fluctuation of the plasma echinocytogenic factors. Considering that the echinocytes loose membrane by shedding vesicles, the recovering echinocytes are expected to have a decreased outer-to-inner membrane balance that favors the stomatocytic transformation. Probably for the same reason, a negative correlation between the stomatocyte–knizocyte percentages and the ROS levels in ESRD patients was observed (Table 2). In lower oxidative stress conditions, the membrane preservation is better and consequently more echinocytes can recover back to the discocyte–stomatocyte shapes. Our findings remind the transformations adopted by RBCs being under the combinational influence of both oxidant and antioxidant factors [27]. In that particular case, while echinocytes were considered as morphological signs of oxidative stress, the stomatocytes were described as "anti-oxidative" stress signs. It should be noticed here that "classic" stomatocytosis is associated with redox imbalance and cytoskeleton modifications [28], while the hereditary stomatocytoses are often connected to membrane deficiency and modifications in stomatin and Band 3 proteins [29]. In our samples, the contribution of the secondary stomatin depletion (see Section 3.5) and probably of other corpuscular alterations in the stomatocytic transformation cannot be excluded. On the other hand, the positive correlation of stomatocytosis with the membrane expression of stomatin (Table 2), signified that those variables were not interconnected, at least in the pre-HD blood. Reversely, increased antioxidant activity has been associated with the stomatocytic transformation of RBCs [30], in accordance to our data which show increased membrane binding of Prx2 in the stomatocyte-enriched samples (Table 2).

3.4. *Electrophoretic profile of RBC membrane proteins in relation to the HD*

Previous studies have reported modifications in the electrophoretic profile of RBCs membrane in uremic patients on HD [31,32], while others did not detect any significant variation in

Table 3 – RBCs membrane proteome analysis in ESRD patients and dialysis effect.

Protein	Controls (N=12)	Pre-HD (N=12)		20 min of HD (N=12)		Post-HD (N=12)		HD effect		
	Relative %	Relative %	Pat #	Relative %	Pat #	Relative %	Pat #	Positive (pat #)	Negative (pat #)	During HD (pat #)
Spectrin	100±6	84±20	6	81±15*	10	89±22	8	6	5	2
Band 3	100±7	85±15*	7	83±15*	7	86±17*	8	2	7	4
Pallidin	100±15	77±24	4	75±28	6	61±30*	6	1	6	1
Actin	100±14	65±40*	7	63±33*	6	62±34*	5	4	4	1
Stomatin	100±12	88±13	3	87±19	5	82±14	7	2	7	3
CD47	100±20	79±26	3	77±22	5	77±23	6	2	3	2
Aquaporin-1 (+/–)	100±17	156±92*	7	110±90	8	98±83	9	4	7	3
Ub-proteins (+/–)	100±15	208±194	11	146±54*	10	131±47*	9	7	3	3
Prx-2	100±18	264±60	6	314±67*	8	367±70*	7	4	5	2
HSP70	100±14	164±138	6	155±119	6	153±107	6	4	3	3
Calpain-1	100±19	198±174	7	176±152	5	188±177	5	4	3	1
Spectrin-Hb	100±40	638±470*	10	677±423*	11	546±400*	12	6	6	4
IgGs	100±8	349±477	4	302±370	5	237±235	6	4	2	1
Band 3 fragments	100±28	136±52	6	120±52	6	136±96	5	2	5	2
Spectrin fragments	100±9	173±116*	7	179±120*	7	160±102	9	5	4	4
Band 8[a]	100±26	170±83*	7	152±83	6	156±87*	7	4	3	2
Hemoglobin	100±17	113±53	6	104±49	4	126±55	7	2	4	1
Aberrant bands[a]	100±7	127±29*	10	119±26*	8	114±22	8	7	2	3

Data represent averaged (N=12) relative membrane protein expression±SD after normalization to the controls (100%, immunoblotting data). (+/–): protein excess or deficiency among the samples. Pat #: subgroup of patients presented with statistically significant protein modifications compared to the controls. Positive HD effect: trend towards normal values. Negative HD effect: the opposite trend. During HD: protein distortion observed or aggravated during the HD session.

* $p<0.05$ vs. controls (N=12).

[a] SDS-PAGE data.

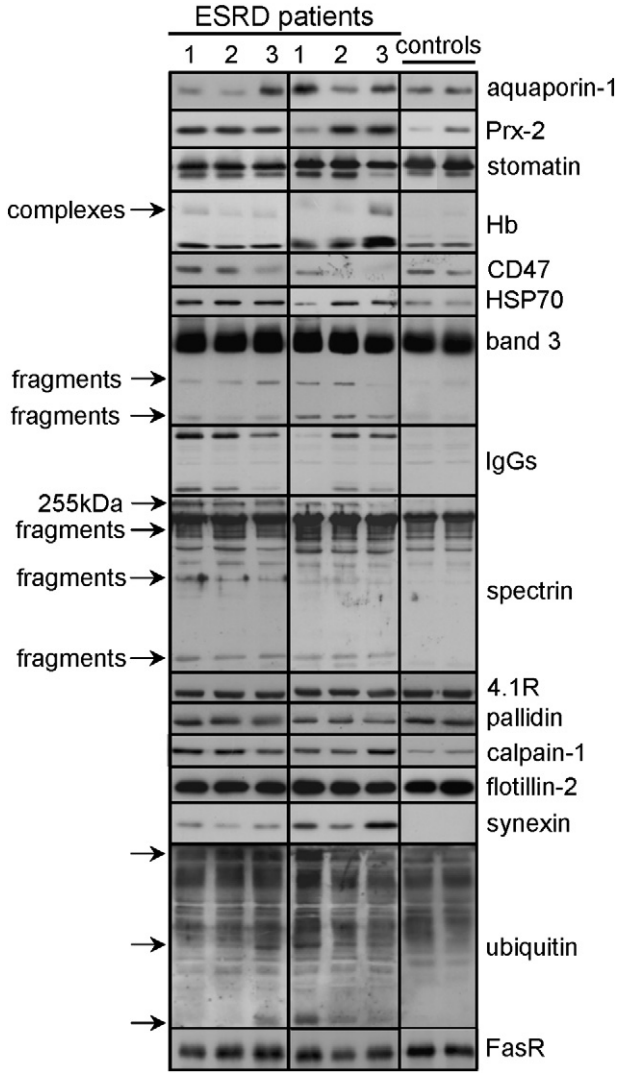

Fig. 4 – Representative immunoblot analyses of RBCs membrane proteins from ESRD patients vs. healthy controls. The ESRD samples were prepared from RBCs collected before (1), during (2) or after (3) the HD session and probed with polyclonal and mAbs against the proteins indicated to the right of the blots. Arrows indicate the aberrant immunoblotted bands of spectrin- and Band 3-associated proteolytic fragments (fragments), non-reducible Hb-immunopositive crosslinkings (Hb complexes and spectrin-Hb band of 255 kDa) and fluctuated ubiquitinated components.

the protein composition of the membrane [3]. The present study shows substantial alterations in RBCs membrane's electrophoretic profile in ESRD patients in relation to the HD procedure. Prominent characteristic of the membrane protein separation by SDS-PAGE was the presence of several aberrant bands of MW between 220–100 kDa, 72–42 kDa and lower than 35 kDa (Fig. 3, Table 3). That finding suggested either enhanced proteolysis of higher MW membrane components or pathologically increased binding of cytosolic or plasma components to the membrane. Gel densitometry analysis further revealed a statistically important reduction in the pre-HD membrane expression of spectrin, 4.1R and pallidin (band 4.2) proteins with a concomitant increase in the expression of band 8 and Hb bands (Fig. 3, Table 3). Some cases were further characterized by pathological expression of Band 3 (deficiency) and band 6 (excess), as previously observed by others [31,32]. Those modifications were indicative of altered membrane protein interactions as already reported [16]. The aberrant electrophoretic bands and the percentage of over-expressed membrane proteins were gradually reduced in relation to the HD procedure (Fig. 3, Table 3), with the minimum amount observed at those samples collected after the HD session. We therefore supposed that the removal of the uremic toxins has an ameliorative effect on the membrane structural integrity, which when combined to the normalization of serum osmolarity might underlie the previously reported improved osmotic fragility of the post-HD RBCs [16,33]. Furthermore, the increased rate of erythrophagocytosis [34] and/or mechanical removal of the most damaged RBCs during HD [1] might underlie the observed improved membrane expression of some proteins (e.g. spectrin) in the post-HD samples.

3.5. *RBCs membrane proteome in relation to the HD procedure*

The electrophoretic profile of the RBCs membrane proteins in ESRD patients suggested substantial membrane remodeling compared to the healthy subjects but also in relation to the HD event. However, many significant membrane components were not evident by PAGE. In addition, the presence of aberrant bands in the gels rendered impossible the accurate quantification of protein expression. Therefore, we investigated the membrane proteome in ESRD by immunoblotting detection of twenty different protein bands, in triplicate assays per patient, before, during and after the completion of the HD session (Fig. 4).

The observed differences in the baseline status, the underlying primary nephropathy lesion and the reticulocytes abundance were consistent with the absence of a common protein expression profile among the examined patients. Indeed, in anemic patients the presence of reticulocytes can influence the protein profile because their protein content differs considerably from that of mature RBCs [35]. Noticeably, there was no common HD effect on a protein fluctuation among the patients, in consistency with early studies reporting a similar variable HD effect on the morphology of RBCs [22]. Table 3 presents the averaged variation in the membrane protein expression of the most common modifications in respect to the HD, along with the beneficial or deteriorative HD effect on them. The membrane remodeling in ESRD concerns normal components of the membrane as well as molecular markers of cellular, including oxidative, stress. According to the quantification results, the membrane in ESRD was not only deficient in spectrin and Band 3 as previously reported [24,31,32], but also in pallidin, actin and stomatin (Table 3, Fig. 4). Moreover, half of the post-HD samples were also deficient in CD47 (Table 3), a "marker of self" for RBCs preventing their phagocytosis [36]. On the other hand, the membrane-bound Prx-2, HSP70, calpain-1 and IgGs were notably increased, in association with the prominent presence of the spectrin–Hb complex (band of 255 kDa) and the Band 3- and spectrin-related degradation products. The uremic factors urea and creatinine probably inflict sensible proteome stress in the membrane, as documented by their direct correlation with

the membrane binding of HSP70 and Prx-2 markers (Table 2). In less than half of the patients who were examined, the membrane was characterized by subnormal levels of 4.1R, flotillin-2 and Fas receptor and by excess of synexin and oxidized/denatured Hb (Fig. 4).

According to the above findings, the emerging membrane remodeling in ESRD involved common oxidative protein modifications and adaptive responses to the redox status as well as cell senescence marks. The formation of spectrin–Hb complex (band of 255 kDa) [19] and the triggered binding of cytosolic HSP70 and Prx-2 to the membrane (Table 3, Fig. 4), as previously shown in uremic lymphocytes [37], were typical examples. The Band 3-based aging model (consisting of Band 3 structural alterations in combination with the membrane binding of oxidized/denatured Hb and IgGs) (Fig. 4) was only detected in a minority of the examined samples (Table 3). This fact is probably connected to both the low averaged RBCs age in ESRD patients [7], as well as the echinocytosis (Fig. 2) which favors the removal of damaged membrane patches and senescence signals through exovesiculation [38]. Nevertheless, half of the patients presented with pathologically increased proteolysis of Band 3 (Table 3), which has been already reported in renal failure cases [24]. Furthermore, some cases presented with numerous RBCs senescence and removal biomarkers, including the CD47 deficiency, while the most prominent and generalized modification among the patients examined was the early RBC aging biomarker [9] of spectrin–Hb complex formation. Those findings suggested that a disturbance of the normal cellular aging might indeed be taking place in ESRD RBCs, probably in response to the increased levels of intracellular ROS and echinocytosis [19]. In support, in our samples the degree of Band 3 proteolysis positively correlates with the intracellular ROS levels ($r=0.762$, $p=0.028$, Table 2).

Defected calcium homeostasis in ESRD RBCs [20,21] is probably another factor contributing to the RBCs membrane remodeling. Although we did not measure the intracellular calcium levels in our samples, we observed increased membrane binding of the cytosolic proteins synexin and calpain-1 in ESRD RBCs (Fig. 4) that is a Ca^{2+}-dependent procedure [39,40]. Elevation of intracellular Ca2+ levels triggers the release of vesicles [39] and the calpain-mediated degradation of a variety of membrane proteins, like the spectrin and Band 3. The calpain-mediated cleavage of spectrin is nowadays used as a marker of disease state in kidney malfunction experimental models [41]. Interestingly, in our study spectrin proteolysis (Fig. 4) was mostly evident in the echinocytes-enriched samples (Table 2).

Overall membrane protein loss [24] and increased circulating levels of RBCs-derived vesicles [42] have been previously reported in renal failure. In the present study we documented substantial data regarding the triggered exovesiculation of the membrane in ESRD. Apart from the morphological modifications (see Fig. 2B), the lower expression and proteolysis of cytoskeletal components (Fig. 4, Table 3) might lead to membrane vesiculation by weakening the cohesion of the skeleton to the lipid bilayer. Additionally, the currently presented progressive loss of numerous vesicle-associated [43,44] components from the membrane, like the Band 3, actin, stomatin, flotillin, Fas receptor and CD47, further supports the notion of an active membrane vesiculation mechanism in ESRD and its relation to the HD event. On the other hand, loss of spectrin seemed to be a result of proteolytic events, in full agreement with previous studies showing significant changes in the conformational state of cytoskeletal proteins in patients on HD [7]. Pearson's linear regression analysis showed significant relations among the levels of the various RBCs membrane proteome stress markers (e.g. Prx-2/calpain-1, Prx-2/255 kDa) (Table 2). The increased expression of membrane proteome stress markers was also related to the loss (e.g. Band 3) or the modification (e.g. spectrin) of critical membrane components (Table 2), signifying a probably common underlying effect.

Regarding the HD impact on protein variations, the most impressive transition concerned the pre- and after-HD levels of aquaporin-1, pallidin, CD47, Prx-2, spectrin–Hb complex, IgGs and ubiquitinylated proteins. The impressive disturbance in RBCs membrane protein ubiquitination pattern is reported for the first time in ESRD patients and might reflect the challenges and the concomitant changes in membrane protein conformation imposed by the uremic state. Furthermore, considering that protein ubiquitination decreases during cellular senescence in association with a deformability defect [45], it is reasonable to observe more ubiquitinated proteins in ESRD patients who possess younger and more flexible cells [7]. Irrespective of the basal (increased or decreased) pre-HD levels, the HD usually resulted in significant decreased levels of protein ubiquitination. That finding might be understood considering the previously reported modulation of protein ubiquitination by the cellular redox status [46]. Additionally, in our samples there was a strong negative correlation between the levels of membrane protein ubiquitinylation and the intracellular ROS levels (Table 2). Alternatively, the lower post-HD levels of ubiquitinated proteins might also be associated with the HD-induced increase in membrane vesiculation, as previously reported in stored RBCs [9].

Aquaporin-1 is the major water transporter in erythrocytes and is responsible for the rapid response of cellular volume to various changes in plasma tonicity. In a previous study [47], the membrane expression of aquaporin-1 has been found significantly lower before HD but increased afterwards, following the

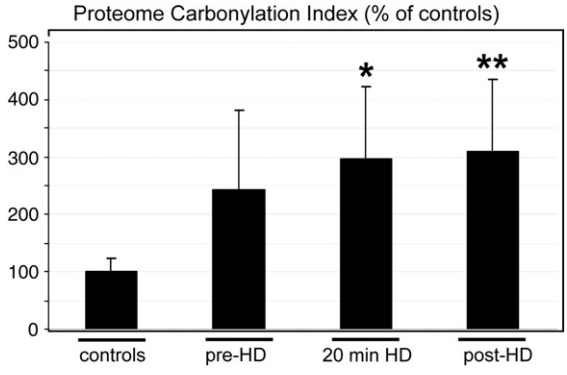

Fig. 5 – Bar graph showing the collective densitometric analysis of proteome carbonylation index (PCI) levels in RBCs membrane preparations from ESRD patients in relation to the HD session. Results were normalized against the PCI of the controls (100%). Error bars indicate SD (N=12); (*) $p<0.05$ vs. controls; (**) $p<0.01$ vs. controls.

variation of MCV index. Although we observed a similar deficiency in two pre-HD samples (Fig. 4 left panel of aquaporin-1 blots), the protein response in HD was irrespective of the MCV. On the contrary, 5 patients exhibited significantly increased pre-HD aquaporin levels that decreased in respect to the HD (Fig. 4 right panel of aquaporin-1 blots; Table 3). During the maturation of reticulocytes, the aquaporin-1 is differentially released via the exosomal pathway, under the influence of both, cellular ubiquitination and extracellular osmotic conditions [10]. It is intriguing to assume that the hyper-osmotic plasma in ESRD [47] might block the release of aquaporin from the numerous circulating immature RBCs, in order to meet the need for reinforced cell volume regulation. Following HD, the plasma tonicity is gradually restored and the aquaporin-restraint signal disappears. Other HD-associating factors, such as protein ubiquitination, membrane remodeling, echinocytosis–exovesiculation and ROS production might also signal the loss of aquaporin from the RBCs membrane. Notably, the Spearman's correlation test revealed a strong positive correlation between the levels of protein ubiquitination and aquaporin-1 in the membrane of post-HD samples (Table 2). Comparative analysis of the above biochemical and morphological data, with respect to the different biocompatible dialyzer (polysulfone or acrylonitrile) did not reveal any statistically significant difference (data not shown).

3.6. *RBCs membrane proteome carbonylation index — The HD effect*

Protein carbonylation is an irreversible and non-reparable reaction, caused by the introduction of carbonyl derivatives into proteins, either through direct oxidation processes or through secondary protein reactions with reactive carbonyl compounds. Considering its effect in protein and cellular dysfunction, a causative role for protein carbonylation in many diseases' onset or progression has been suggested [48]. Uremia is considered to be a state of carbonyl stress [5]. Increased levels of reactive carbonyl compounds and plasma protein carbonyls have been identified in uremic patients undergoing HD [49].

In order to examine the RBCs membrane proteome carbonylation assaults in ESRD patients in vivo, we estimated the proteome carbonylation index (PCI) through immunoblotting assays before, during and after the HD procedure. As shown in Fig. 5 we found an increase in the pre-HD PCI of an average value ~150%, compared to healthy subjects, which was further raised by the HD session. Since some patients exhibited non-significantly elevated pre-HD PCI levels, the average PCI before the HD was not significantly increased compared to the controls. On the contrary, that was changed during and after the dialysis (Fig. 5) signifying the HD impact on the proteome carbonylation defect. Indeed, HD appeared to have a transient or permanent aggravating impact on membrane PCI in the majority of the patients examined, similar to the study of Brazscsynska et al., (2008) [7], which showed severe modifications in membrane integrity during the HD session. Nevertheless in three cases the HD session ameliorated the pre-HD PCI, manifesting, once again, that ESRD patients did not respond in the same manner to the HD process. Although dialysis filters might lessen the blood carbonyl overload by removing both low MW reactive carbonyl compounds and carbonylated proteins from the plasma, increased plasma carbonyl groups have been previously detected after the HD session [50].

The statistical analysis of the results showed no correlation between PCI and the intracellular ROS accumulation (Table 2), since some patients exhibited membrane protein carbonyl balance in spite of the abnormal ROS accumulation. That finding suggested either that the protein carbonyl stress in ESRD patients results from non-oxidative pathways, as previously suggested [49] or that an effective proteome response (chaperoning, proteasomal activity etc.) counteracts the intracellular pro-oxidant factors before the creation of permanent carbonyl defects. Alternative explanations might include the successful detoxification of reactive carbonyl compounds by specific enzymatic pathways or the clearance of carbonylated proteins through vesiculation, as previously suggested for the ex vivo stored RBCs [43,44]. On the other hand, in our study the PCI exhibited a significant correlation to both the RBCs membrane-bound calpain-1 and the serum levels of urea and creatinine (Table 2), suggesting a probable contribution of calpain-mediated proteolysis and uremic factors to protein carbonylation defects. The intra-HD increase in the PCI was further associated with erythrophagocytosis through the loss of CD47 protein (Table 2) [36].

4. Conclusions

The present study showed significant modifications in RBCs structure and membrane proteome in ESRD patients in the context of increased ROS accumulation. Although no common variation profile among the examined patients exists, the results are consistent with the notion that oxidative stress is an intrinsic factor in uremia. All patients did not respond same wise as the HD procedure. In most cases, HD ameliorated ROS intracellular burden but failed to eliminate it and moreover, it seemed to trigger the sensitivity of RBCs to oxidative stimuli. The membrane proteome undergoes an impressive remodeling that was significantly influenced by the HD procedure, at least regarding aquaporin-1, Prx-2 and ubiquitinated components. The major membrane modifications involved loss of membrane components, over-expression of cellular stress markers, increased membrane binding of cytosolic and plasma factors, protein aggregation, fragmentation and carbonylation. Those changes are indicative of cellular aging and defense responses to increased oxidative and/or carbonyl stress. The intracellular redox status and the closely associated membrane modifications seemed to be related to membrane and cytoskeleton instability, loss of surface area through vesiculation, echinocytosis and stomatocytosis, thus, the two extreme shape transformations of normal discocytes. Although the linear regression analyses presented here do not establish a "cause-and-effect" relationship between the connected variables, they certainly outlined a network of interactions among the uremic toxins, the RBCs membrane protein composition and the cellular shape distortions in ESRD, developed around a core of oxidative provocations and responses. The observed changes might contribute to the untimely death of young RBCs in uremia and to the progression of anemia, as well as the other complications often observed in this certain, apparently complex, disease.

Acknowledgments

The authors wish to thank the Assistant Prof. I.P. Trougakos (Dept. of Cell Biology & Biophysics, Faculty of Biology, Univ. of Athens) for the kind disposal of fluorometer device and appropriate filters we used in the fluorometry experiments; Mrs H. Sardeli (Chronic Hemodialysis Centre "Ionion") for her contribution in blood sampling; the pre-graduate students Georgatzakou H., Triantafillou E., and Kontouri K. (Dept. of Cell Biology & Biophysics, Faculty of Biology, Univ. of Athens) for their assistance in the performance of a part of the verification immunoblotting experiments; and finally, Dr Vassiliki Mpakou (Dept. of Cell Biology & Biophysics, Faculty of Biology, Univ. of Athens) for the critical evaluation and helpful suggestions on the manuscript. This study was partly supported by the "Special Account for Research Grants of the University of Athens" to Associate Prof. I.S. Papassideri.

REFERENCES

[1] Eckardt KU. Pathophysiology of renal anemia. Clin Nephrol 2000;53:S2–8.
[2] Sotirakopoulos N, Tsitsios T, Stambolidou M, Athanasiou G, Peiou M, Kokkinou V, et al. The red blood cell deformability in patients suffering from end stage renal failure on hemodialysis or continuous ambulatory peritoneal dialysis. Ren Fail 2004;26:179–83.
[3] Perna AF, Ingrosso D, Zappia V, Galletti P, Capasso G, De Santo NG. Enzymatic methyl esterification of erythrocyte membrane proteins is impaired in chronic renal failure. Evidence for high levels of the natural inhibitor S-adenosylhomocysteine. J Clin Invest 1993;91:2497–503.
[4] Goi G, Massaccesi L, Baquero Herrera CJ, Musetti C, Ciurlino D, Cusi D, et al. Oxidative stress in elderly chronic renal failure patients: effects of renal replacement therapies on cell membrane fluidity. J Nephrol 2009;22:630–6.
[5] Antolini F, Valente F, Ricciardi D, Baroni M, Fagugli RM. Principal component analysis of some oxidative stress parameters and their relationships in hemodialytic and transplanted patients. Clin Chim Acta 2005;358:87–94.
[6] Hashimoto H, Mio T, Sumino K. Lipid abnormalities of erythrocyte membranes in hemodialysis patients with chronic renal failure. Clin Chim Acta 1996;252:137–45.
[7] Brzeszczynska J, Luciak M, Gwozdzinski K. Alterations of erythrocyte structure and cellular susceptibility in patients with chronic renal failure: effect of haemodialysis and oxidative stress. Free Radic Res 2008;42:40–8.
[8] Himmelfarb J, McMonagle E, Holbrook D, Hakim R. Increased susceptibility to erythrocyte C5b-9 deposition and complement-mediated lysis in chronic renal failure. Kidney Int 1999;55:659–66.
[9] Antonelou MH, Kriebardis AG, Stamoulis KE, Economou-Petersen E, Margaritis LH, Papassideri IS. Red blood cell aging markers during storage in citrate-phosphate-dextrose–saline–adenine–glucose–mannitol. Transfusion 2010;50:376–89.
[10] Blanc L, Liu J, Vidal M, Chasis JA, An X, Mohandas N. The water channel aquaporin-1 partitions into exosomes during reticulocyte maturation: implication for the regulation of cell volume. Blood 2009;114:3928–34.
[11] Durak I, Akyol O, Basesme E, Canbolat O, Kavutcu M. Reduced erythrocyte defense mechanisms against free radical toxicity in patients with chronic renal failure. Nephron 1994;66:76–80.
[12] Glass GA, Gershon D. Decreased enzymic protection and increased sensitivity to oxidative damage in erythrocytes as a function of cell and donor aging. Biochem J 1984;218:531–7.
[13] Hirayama A, Nagase S, Gotoh M, Takemura K, Tomida C, Ueda A, et al. Hemodialysis does not influence the peroxidative state already present in uremia. Nephron 2000;86:436–40.
[14] Lucchi L, Bergamini S, Iannone A, Perrone S, Stipo L, Olmeda F, et al. Erythrocyte susceptibility to oxidative stress in chronic renal failure patients under different substitutive treatments. Artif Organs 2005;29:67–72.
[15] Tepel M, Echelmeyer M, Orie NN, Zidek W. Increased intracellular reactive oxygen species in patients with end-stage renal failure: effect of hemodialysis. Kidney Int 2000;58:867–72.
[16] Peuchant E, Salles C, Vallot C, Wone C, Jensen R. Increase of erythrocyte resistance to hemolysis and modification of membrane lipids induced by hemodialysis. Clin Chim Acta 1988;178:271–82.
[17] Reinhart WH, Chien S. Red cell rheology in stomatocyte–echinocyte transformation: roles of cell geometry and cell shape. Blood 1986;67:1110–8.
[18] Brimble KS, McFarlane A, Winegard N, Crowther M, Churchill DN. Effect of chronic kidney disease on red blood cell rheology. Clin Hemorheol Microcirc 2006;34:411–20.
[19] Snyder LM, Fortier NL, Trainor J, Jacobs J, Leb L, Lubin B, et al. Effect of hydrogen peroxide exposure on normal human erythrocyte deformability, morphology, surface characteristics, and spectrin-hemoglobin cross-linking. J Clin Invest 1985;76:1971–7.
[20] Sakthivel R, Farooq SM, Kalaiselvi P, Varalakshmi P. Investigation on the early events of apoptosis in senescent erythrocytes with special emphasis on intracellular free calcium and loss of phospholipid asymmetry in chronic renal failure. Clin Chim Acta 2007;382:1–7.
[21] Gafter U, Malachi T, Barak H, Djaldetti M, Levi J. Red blood cell calcium homeostasis in patients with end-stage renal disease. J Lab Clin Med 1989;114:222–31.
[22] Hasler CR, Owen GR, Brunner W, Reinhart WH. Echinocytosis induced by haemodialysis. Nephrol Dial Transplant 1998;13:3132–7.
[23] Agroyannis B, Kopelias I, Fourtounas C, Paraskevopoulos A, Tzanatos H, Dalamangas A, et al. Relation between echinocytosis and erythrocyte calcium content in hemodialyzed uremic patients. Artif Organs 2001;25:486–90.
[24] Saradhadevi V, Sakthivel R, Vedamoorthy S, Selvam R, Parinandi N. Alterations in band 3 protein and anion exchange in red blood cells of renal failure patients. Mol Cell Biochem 2005;273:11–24.
[25] Bor-Kucukatay M, Yalcin O, Meiselman HJ, Baskurt OK. Erythropoietin-induced rheological changes of rat erythrocytes. Br J Haematol 2000;110:82–8.
[26] Truong HT, Daleke DL, Huestis WH. Human erythrocyte shape regulation: interaction of metabolic and redox status. Biochim Biophys Acta 1993;1150:51–6.
[27] Battistelli M, De Sanctis R, De Bellis R, Cucchiarini L, Dacha M, Gobbi P. *Rhodiola rosea* as antioxidant in red blood cells: ultrastructural and hemolytic behaviour. Eur J Histochem 2005;49:243–54.
[28] Malorni W, Straface E, Pagano G, Monti D, Zatterale A, Del Principe D, et al. Cytoskeleton alterations of erythrocytes from patients with Fanconi's anemia. FEBS Lett 2000;468:125–8.
[29] Flatt JF, Bruce LJ. The hereditary stomatocytoses. Haematologica 2009;94:1039–41.
[30] Richards RS, Wang L, Jelinek H. Erythrocyte oxidative damage in chronic fatigue syndrome. Arch Med Res 2007;38:94–8.
[31] Costa E, Rocha S, Rocha-Pereira P, Castro E, Miranda V, do Sameiro Faria M, et al. Altered erythrocyte membrane protein composition in chronic kidney disease stage 5 patients under

haemodialysis and recombinant human erythropoietin therapy. Blood Purif 2008;26:267–73.
[32] Costa E, Rocha S, Rocha-Pereira P, Castro E, Miranda V, Faria Mdo S, et al. Changes in red blood cells membrane protein composition during hemodialysis procedure. Ren Fail 2008;30:971–5.
[33] Wu SG, Jeng FR, Wei SY, Su CZ, Chung TC, Chang WJ, et al. Red blood cell osmotic fragility in chronically hemodialyzed patients. Nephron 1998;78:28–32.
[34] Bonomini M, Sirolli V, Settefrati N, Dottori S, Di Liberato L, Arduini A. Increased erythrocyte phosphatidylserine exposure in chronic renal failure. J Am Soc Nephrol 1999;10:1982–90.
[35] Liu J, Guo X, Mohandas N, Chasis JA, An X. Membrane remodeling during reticulocyte maturation. Blood 2010;115:2021–7.
[36] Oldenborg PA, Zheleznyak A, Fang YF, Lagenaur CF, Gresham HD, Lindberg FP. Role of CD47 as a marker of self on red blood cells. Science 2000;288:2051–4.
[37] Calabrese V, Mancuso C, Sapienza M, Puleo E, Calafato S, Cornelius C, et al. Oxidative stress and cellular stress response in diabetic nephropathy. Cell Stress Chaperones 2007;12:299–306.
[38] Willekens FL, Werre JM, Groenen-Dopp YA, Roerdinkholder-Stoelwinder B, de Pauw B, Bosman GJ. Erythrocyte vesiculation: a self-protective mechanism? Br J Haematol 2008;141:549–56.
[39] Salzer U, Hinterdorfer P, Hunger U, Borken C, Prohaska R. Ca (++)-dependent vesicle release from erythrocytes involves stomatin-specific lipid rafts, synexin (annexin VII), and sorcin. Blood 2002;99:2569–77.
[40] Glaser T, Schwarz-Benmeir N, Barnoy S, Barak S, Eshhar Z, Kosower NS. Calpain (Ca(2+)-dependent thiol protease) in erythrocytes of young and old individuals. Proc Natl Acad Sci U S A 1994;91:7879–83.
[41] Vanderklish PW, Bahr BA. The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states. Int J Exp Pathol 2000;81:323–39.
[42] Amabile N, Guerin AP, Leroyer A, Mallat Z, Nguyen C, Boddaert J, et al. Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure. J Am Soc Nephrol 2005;16:3381–8.
[43] Bosman GJ, Lasonder E, Luten M, Roerdinkholder-Stoelwinder B, Novotny VM, Bos H, et al. The proteome of red cell membranes and vesicles during storage in blood bank conditions. Transfusion 2008;48:827–35.
[44] Kriebardis AG, Antonelou MH, Stamoulis KE, Economou-Petersen E, Margaritis LH, Papassideri IS. RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components. Transfusion 2008;48: 1943–53.
[45] Corsi D, Paiardini M, Crinelli R, Bucchini A, Magnani M. Alteration of alpha-spectrin ubiquitination due to age-dependent changes in the erythrocyte membrane. Eur J Biochem 1999;261:775–83.
[46] Jahngen-Hodge J, Obin MS, Gong X, Shang F, Nowell Jr TR, Gong J, et al. Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. J Biol Chem 1997;272: 28218–26.
[47] Buemi M, Floccari F, Di Pasquale G, Cutroneo G, Sturiale A, Aloisi C, et al. AQP1 in red blood cells of uremic patients during hemodialytic treatment. Nephron 2002;92:846–52.
[48] Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. J Cell Mol Med 2006;10:389–406.
[49] Miyata T, van Ypersele de Strihou C, Kurokawa K, Baynes JW. Alterations in nonenzymatic biochemistry in uremia: origin and significance of “carbonyl stress” in long-term uremic complications. Kidney Int 1999;55:389–99.
[50] Pavone B, Sirolli V, Bucci S, Libardi F, Felaco P, Amoroso L, et al. Adsorption and carbonylation of plasma proteins by dialyser membrane material: in vitro and in vivo proteomics investigations. Blood Transfus 2010;8(Suppl 3):s113–9.