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Pathophysiological aspects of red blood cells in end-stage renal disease patients resistant to recombinant human erythropoietin therapy

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Abstract

Objective: Modified, bioreactive red blood cells (RBCs) and RBC-derived microvesicles (MVs) likely contribute to the hematological and cardiovascular complications in end-stage renal disease (ESRD). This study assesses the physiological profile of RBCs in patients with ESRD receiving standard or high doses of recombinant human erythropoietin (rhEPO).

Method: Blood samples from twenty-eight patients under sustained hemodialysis, responsive, or not to standard rhEPO administration were examined for RBC morphology, fragility, hemolysis, redox status, removal signaling, membrane protein composition, and microvesiculation before and after dialysis. Acute effects of uremic plasma on RBC features were examined in vitro through reconstitution experiments.

Results: Overall, the ESRD RBCs were characterized by pathological levels of shape distortions, surface removal signaling, and membrane exovesiculation, but reduced fragility compared to healthy RBCs. Irreversible transformation of RBCs was found to be a function of baseline Hb concentration. The more toxic uremic context in non-responsive patients compared to rhEPO responders was blunted in part by the antioxidant, antihemolytic, and anti-apoptotic effects of high rhEPO doses, and probably, of serum uric acid. A selective lower expression of RBC membrane in complement regulators (CD59, clusterin) and of CD47 "marker-of-self" was detected in non-responders and responders, respectively. Evidence for different short-term dialysis effects and probably for a different erythrocyte vesiculation mechanism in rhEPO responsive compared to non-responsive patients was also revealed.

Conclusion: Deregulation of RBC homeostasis might involve diverse molecular pathways driving erythrocyte signaling and removal in rhEPO non-responders compared to responsive patients.

KEYWORDS

end-stage renal disease, hemodialysis, recombinant human erythropoietin, red blood cells, rhEPO resistance

1 | INTRODUCTION

Reduced erythropoietin production, iron deficiency, inflammation, and shortened lifespan of red blood cells (RBCs) contribute to anemia in end-stage renal disease (ESRD). The uremic toxins along with the pathophysiology of uremia account for disturbed redox homeostasis, appearance of removal signals, and membrane proteome modifications in circulating RBCs.[1] While hemodialysis (HD) represents the main renal replacement therapy for the elimination of metabolic end products and excess of water from blood, it also exposes blood cells to oxidative, metabolic, and mechanical stresses that further affect the functionality and viability of RBCs.[2] Recombinant human erythropoietin (rhEPO) therapy leads to a significant alleviation of the anemia-associated adverse effects in ESRD.[3] There is, however, a wide variation in individual response to rhEPO. The so-called "rhEPO resistance" is defined as the failure to achieve or maintain the desired range of hemoglobin (Hb) levels despite administration of standard rhEPO doses. The underlying mechanisms are still poorly understood, yet rhEPO resistance seems to be associated with interpatient variation in oxidative stress, iron and vitamin deficiencies, and accumulation of uremic toxins or inflammatory cytokines, which are supposed to suppress erythropoiesis by blunting bone marrow response.[4] Previous studies have shown substantial variability among patients in the profile of RBC structural and functional modifications as well as in the effect of HD on them, as a result of different primary defects, comorbidities, and treatments.[2,5,6] Resistance to rhEPO probably represents another variable that adds complexity to the pathophysiological background of the anemic patient with ESRD. Apart from its erythropoietic properties, EPO is nowadays considered a pleiotropic cytoprotective factor for many cells, including mature RBCs, in which clear effects on glucose and ion transport, redox status, rheological properties, and eryptosis have been reported.[7,8] However, the physiological characteristics of RBCs in non-responsive patients with ESRD receiving high doses of rhEPO have been scarcely characterized.[5,9] The present study dealt with the variability observed in several physiological aspects of RBCs that are probably associated with the persistent anemia in ESRD, including structure, fragility, removal signaling, membrane microvesiculation, and protein composition, as a function of rhEPO dose and HD treatment.

2 | PATIENTS AND METHOD

Details about materials' providers, patients' features, and methods used are given in Data S1.

2.1 | Patients

Twenty-eight patients with ESRD on rhEPO supplementation and regular HD treatment with highly biocompatible polyamix filters, vs twelve age- and gender-matched healthy subjects exhibiting normal hematological profile and taking no medication or food supplements, were studied (Table 1). Sixteen patients were responsive (R group), and twelve patients were non-responsive (non-responders, NR group) to standard doses of rhEPO (Table 1). Resistance to rhEPO was defined as a failure to achieve target Hb levels (11-12 g/dL) with maintained doses of rhEPO>300 IU/Kg/wk of epoetin or 1.5 μg/Kg/wk of darbepoetin-α.[9] Blood samples were collected into EDTA or citrate collection tubes just before HD session and immediately after it. The study has been submitted and approved by the Research Bioethics and BioSecure Committee of the Department of Biology/NKUA. Investigations were carried out in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all blood donors that participated in this study.

2.2 | Laboratory testing and RBC fragility tests

Hematological analysis was performed using the Sysmex K-4500 automatic blood cell counter; biochemical analyses of serum factors and electrolytes were performed using the automatic analyzers Hitachi 902 and 9180 and Elecsys Systems (Roche, Indianapolis, IN). Plasma-free hemoglobin (fHb) was calculated by the method of Harboe.[10] In vitro osmotic fragility of RBCs was assessed in solutions with increasing saline concentration,[11] and the mean corpuscular fragility (MCF) index (saline concentration causing 50% of hemolysis) was calculated, before (MCF) or after incubation for 24 hours at 37°C (MCF'). The mechanical fragility index (MFI) of RBCs was evaluated as previously described,[12] in blood mixed with stainless steel beads and rocked for 1 hour on a rocker platform. All materials and common chemicals were obtained from Sigma-Aldrich (Munich, Germany), unless otherwise stated.

2.3 | Redox status and calcium accumulation

Total and uric acid-dependent antioxidant capacity of plasma (TAC and UA/AC, respectively) were measured by the ferric reducing antioxidant power (FRAP) assay,[13] with/without uricase treatment (0.125 U/mL, for 20 minutes at 20°C). Intracellular accumulation of ROS (iROS) and cytosolic calcium (iCa^{2+}) levels were detected by fluorometry (VersaFluor Fluorometer, Life Science, Bio-Rad, Hertfordshire, UK) using the fluorescent probes CMH_2DCFDA and Fluo-4 AM, respectively, as previously described.[2] For reconstitution experiments, control and ESRD RBCs at 35% Hct were incubated with ESRD or control plasma from ABO-matched healthy volunteers (n=6), respectively, for 24 hours at 37°C in 5% CO_2-air. Hemolysis and ROS accumulation were measured in mixed vs unmixed samples.

2.4 | Morphological analysis of RBCs by scanning electron microscopy

Isolated RBCs were fixed with 2% glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated in ascending ethanol series and, examined in a Philips SEM515 microscope after coating with gold–palladium (Tousimis Samsputter-2a, Rockville, Maryland). RBC shape

TABLE 1 Demographic, therapy-associated, hematological, and serum biochemical characteristics of patients with ESRD and healthy subjects

	R patients (n=16)		NR patients (n=12)		
Characteristics	**pre-HD**	**post-HD**	**pre-HD**	**post-HD**	**Controls (n=12)**
Age (y)	81±7		76±8		68±9
Weight (kg)	64.8±15.1		75.4±19.8		66.3±9.8
HD treatment (mo)	6-105		5-126		-
Epo dose (IU/wk)	9350±4203		23 250±5650		-
WBC (x10^3/μL)	5.8±1.6	5.7±2.5	6.3±1.7	6.1±2.6	7.0±1.3
Neutrophils (%)	66±9[a]	59±8[b]	68±8[a]	71±8[a,b]	59±7
Lymphocytes (%)	25±8	33±9[b]	23±6	21±8[b]	29±4
Monocytes (%)	8±4	6±3	7±3	6±1	9±1
Basophils (%)	0	0	1	0	1
Eosinophils (%)	1	2	2	2	2
RBC (x10^6/μL)	3.61±0.40[a]	3.99±0.89[a]	3.87±0.73[a]	4.20±1.23[a]	4.73±0.55
Hb (gr/dL)	10.9±0.7[a]	11.7±0.9[a]	10.41±0.90[a]	11.3±1.4[a,c]	14.9±1.3
Hct (%)	33.8±1.9[a]	33.2±3.8[a]	33.5±2.2[a]	34.0±4.6[a]	41.8±4.5
MCV (fL)	95.4±9.1	84.4±8.5[a,c]	89.1±16.1	85.0±16.5[a]	93.0±6.0
MCH (pg)	30.2±3.2[a]	29.6±4.4[a]	27.8±5.9[a]	28.1±5.9[a]	31.6±2.1
MCHC (g/dL)	31.2±0.6	33.6±3.7	31.1±1.3	33.1±1.3[c]	34.0±1.2
RDW (%)	16.6±1.3[a]	15.8±2.1	16.8±1.4[a]	14.5±0.7	12.9±0.8
PLT (x10^3/μL)	173±54[a]	185±68[a]	192±55[a]	195±63[a]	348±83
PDW (%)	12.8±2.4[a]	13.7±1.8[a]	13.6±2.1[a]	14.2±2.9[a]	17.7±0.6
PTH (pg/mL)	235±129[a]	N/D	211±94[a]	N/D	42±20
Glucose (mg/dL)	100±10	N/D	98±12	N/D	77±19
Urea (mg/dL)	125±25[a,b]	37.8±8.6[c]	153±27[a,b]	46.5±10.4[a,c]	35±12
URR (%)	69.2±6.8		69.2±7.9		-
Creatinine (mg/dL)	6.7±2.0[a]	N/D	7.7±1.2[a]	N/D	0.7±0.1
Uric acid (mg/dL)	5.9±1.2[a,b]	N/D	7.5±1.0[a,b]	N/D	4.2±1.2
Cholesterol (mg/dL)	147±41	N/D	136±17	N/D	145±18
Triglycerides (mg/dL)	148±37	N/D	129±79	N/D	139±25
HDL (mg/dL)	43±11[a]	N/D	43±10[a]	N/D	80±9
Calcium (mg/dL)	9.3±0.3	N/D	9.3±0.3	N/D	9.4±0.3
Phosphorus (mg/dL)	4.7±0.9	N/D	4.9±1.1	N/D	3.7±0.5
Potassium (mmol/L)	4.90±0.48	4.20±0.36	5.12±0.65	4.05±0.65	4.21±0.30
Sodium (mmol/L)	137±2	N/D	136±2	N/D	142±2
Fe (μg/dL)	60.3±14.7[a]	N/D	59.0±24.1[a]	N/D	110.2±17.1
Ferritin (ng/mL)	825±482[a]	N/D	691±192[a]	N/D	69±23
TIBC (μg/dL)	244±39[a]	N/D	236±35[a]	N/D	383±89
Total proteins (g/dL)	7.1±0.3	N/D	7.2±0.4	N/D	7.5±0.6
Albumin (g/dL)	4.2±0.6	N/D	4.1±0.3	N/D	4.2±0.4
ALP (IU/L)	71±13	N/D	75±7	N/D	64±18
γGT (IU/L)	15±8	N/D	14±5	N/D	12±9

Values are presented as Mean±SD. [a]pathological values vs healthy subjects; [b]R vs NR; [c]pre- vs post-HD, $P<.05$. ALP, alkaline phosphatase; γGT, gamma-glutamyl transferase; Hb, hemoglobin; Hct, hematocrit; HDL, high-density lipoprotein; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PLT, platelets; PDW, platelet distribution width; PTH, parathormone; RBC, red blood cells; RDW, RBC distribution width; TIBC, total iron-binding capacity; URR, urea reduction ratio; WBC, white blood cells; ESRD, end-stage renal disease.

classification was performed using standard criteria, as previously adopted.[14] Briefly, spherocytic transformation and other shape changes (dacryocytes, elliptocytes, ovalocytes, and schistocytes) were characterized as irreversible (IRR) modifications. Shape distribution was estimated by blind assessment of at least 2000 cells from randomly chosen fields per sample.

2.5 | Flow cytometry analysis of RBCs and microvesicles

Enumeration and phenotyping of RBCs and microvesicles (MVs) were performed by multicolor flow cytometry using phycoerythrin (PE)-Annexin V apoptosis kit and FITC-conjugated anti-CD235, as previously described.[15] Plasma MVs isolation was performed after a double light spin (2500 *g*) of citrated blood at 20°C. Their identification was based on size (<1 μm), RBC origin, and annexin V binding ($AnnV^+$). Megamix fluorescent beads were used for gating in accordance with the International Society on Thrombosis and Hemostasis SSC Collaborative workshop recommendations and TruCount bead tubes for enumeration (positive events/μL).

2.6 | Immunoblotting of RBC membrane proteins

Red blood cells membranes were isolated from purified leukodepleted RBC fractions and immunoblotted against major membrane proteins as previously described.[14] In brief, equal amounts of protein (15 μg) were resolved in 10% or 5%-15% linear gradient SDS-PAGE gels and electrophoretically transferred onto nitrocellulose membranes for 45 minutes at constant voltage of 20 V. Membranes were probed with primary antibodies in 5% non-fat milk for 1 hour at RT. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:8000 or 1:10 000), the immunoreactivity was visualized by enhanced chemiluminescence. Equal loading of the gels was assessed by hybridization with a 4.1R-specific antibody. The Oxyblot detection kit was used for the detection of RBC membrane carbonylated proteins, as per manufacturer's specifications. For quantification purposes, the Proteome Carbonylation Index (PCI) was calculated.[2]

2.7 | Statistical and network analysis

For statistical analysis, the Statistical Package for Social Sciences (IBM SPSS; version 22.0 for Windows IBM Corp., Armonk, NY; administrated by NKUA) was used. Intergroup differences were evaluated by *t* test or one-way ANOVA using a Bonferroni correction. Prediction outcomes were estimated by regression analysis. Pearson's and Spearman's tests were performed to assess correlation (*r*) between parameters following or not normal distribution profiles, respectively. Significance was accepted at $P<.05$. Significant correlations among measured variables (in serum/plasma and RBCs) were topologically represented in untargeted biological networks using the Cytoscape version 3.2.0 application.[16] Length of edges was inversely proportional to the *r* value.

3 | RESULTS

3.1 | Hematological and serum/plasma features in ESRD

Patients with ESRD were anemic with hematological markers of chronic inflammatory response (disturbed iron homeostasis, RDW index, neutrophil, and HDL levels, etc.) (Table 1). The hematological and serum biochemical profiles were comparable in R and NR groups pre-HD, apart from higher urea and uric acid (UA) levels in NR patients. HD had a significant positive effect on Hb and MCHC levels mostly in non-responders but a negative effect on MCV index variation, especially in responsive patients (Table 1). Uremic plasma had pathologically increased total antioxidant capacity (TAC) and concentration of total and RBC-derived MVs (R-MVs) (Figure 1A). Plasma of R patients was further characterized by high baseline hemolysis (free Hb, fHb) both pre- (Figure 1A) and post-HD (Figure 1B). TAC and UA/AC reduced to subnormal levels post-HD. HD was associated with decreased accumulation (though no normalization) of all species of $AnnV^+$ MVs, including R-MVs, in R samples (from 3258±1375 MVs/μL pre-HD to 1214±822 MVs/μL post-HD for R-MVs); however, it did not improve accumulation of R-MVs in non-responders (2443±656 MVs/μL pre-HD vs 2619±1072 MVs/μL post-HD). Consequently, post-HD NR plasma contained significantly more $AnnV^+$ R-MVs compared to R-plasma (Figure 1B).

Reconstitution experiments showed a strong antioxidant effect of pre-HD uremic compared to control plasma in both ESRD and healthy RBCs, which was mainly attributed to uric acid as it was abolished after treatment of uremic plasma with uricase (Figure 1C). Regarding in vitro hemolysis, uremic plasma improved hemolysis of healthy RBCs in vitro in the presence of UA, as hemolysis increased at four times the baseline, preincubation levels after UA removal (Figure 1D).

3.2 | RBC characteristics

Similarly to plasma modifications, the structural and physiological characteristics of ESRD RBCs were not significantly different between responders and non-responders pre-HD (Figure 2A). Severe RBC shape distortions (irreversibly modified RBCs, IRR), increased PS exposure, and lower MCF to osmotic stress (MCF', after incubation for 24 hours at 37°C) compared to controls (0.515±0.052 vs 0.573±0.019%, respectively), were equally observed in the two groups of ESRD RBCs (Figure 2A, C). Regression analysis of data using Hb levels as an independent variable resulted in a model for the prediction of pathological RBC shape modifications levels in ESRD (Figure 2D). On average, normal levels of intracellular ROS (iROS, 445±168 vs 306±63 RFU in healthy RBCs), membrane proteome carbonylation index (PCI, 4.14±4.0 vs 3.86±0.9 In control), intracellular calcium (iCa^{2+}, 2773±1073 vs 2371± 448 RFU in healthy RBCs), and mechanical fragility index (MFI, 0.773±0.302 vs 0.804±0.194% in control) were detected in the patients pre-HD. A trend for higher percentage of IRR modifications (7.9±4.1% vs 5.3±2.9%, respectively) and Ca^{2+}

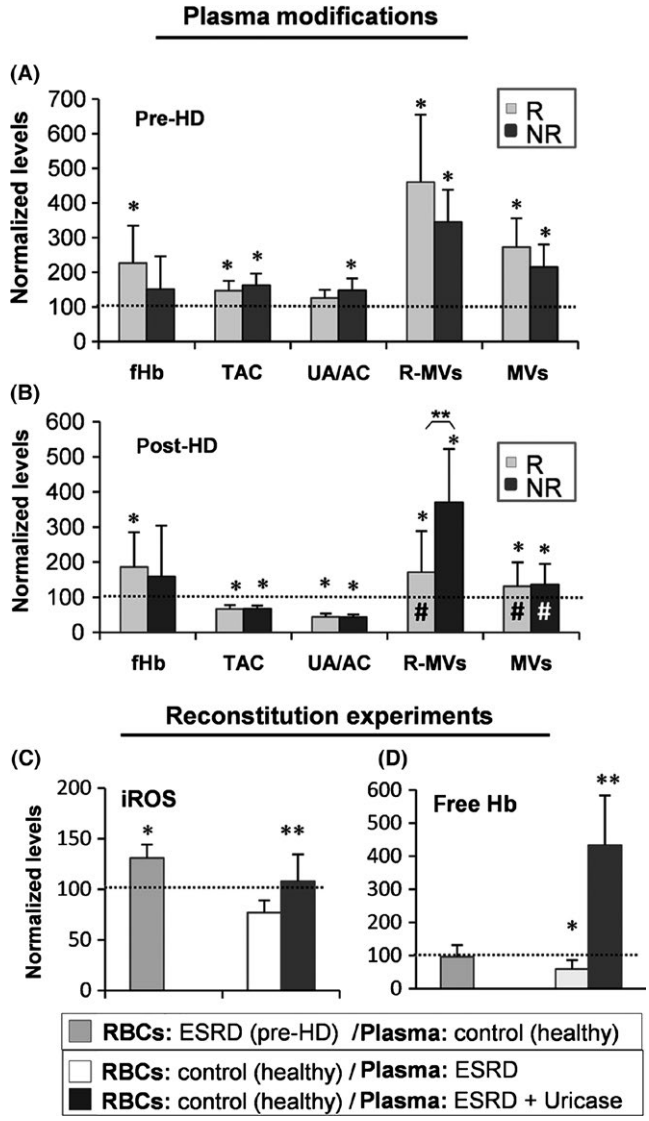

FIGURE 1 Variation in soluble plasma components in responders (R) and non-responders (NR) pre- (A) and post-HD (B) after normalization to controls (100%, dotted line). fHb, free Hb; TAC, total antioxidant capacity; UA/AC, uric acid-dependent antioxidant capacity; R-MVs, RBC-derived MVs; MVs, total MVs. **P*<.05 vs control; ***P*=.006 R vs NR; #*P*<.05 pre- vs post-HD. Bars: Mean±SD. (C) Reconstitution experiments showing the antioxidant and antihemolytic effects of uremic plasma and uric acid. iROS, intracellular ROS. **P*<.05 vs baseline (prereconstitution) levels. ***P*<.05 before vs after uricase treatment. Bars: Mean±SD

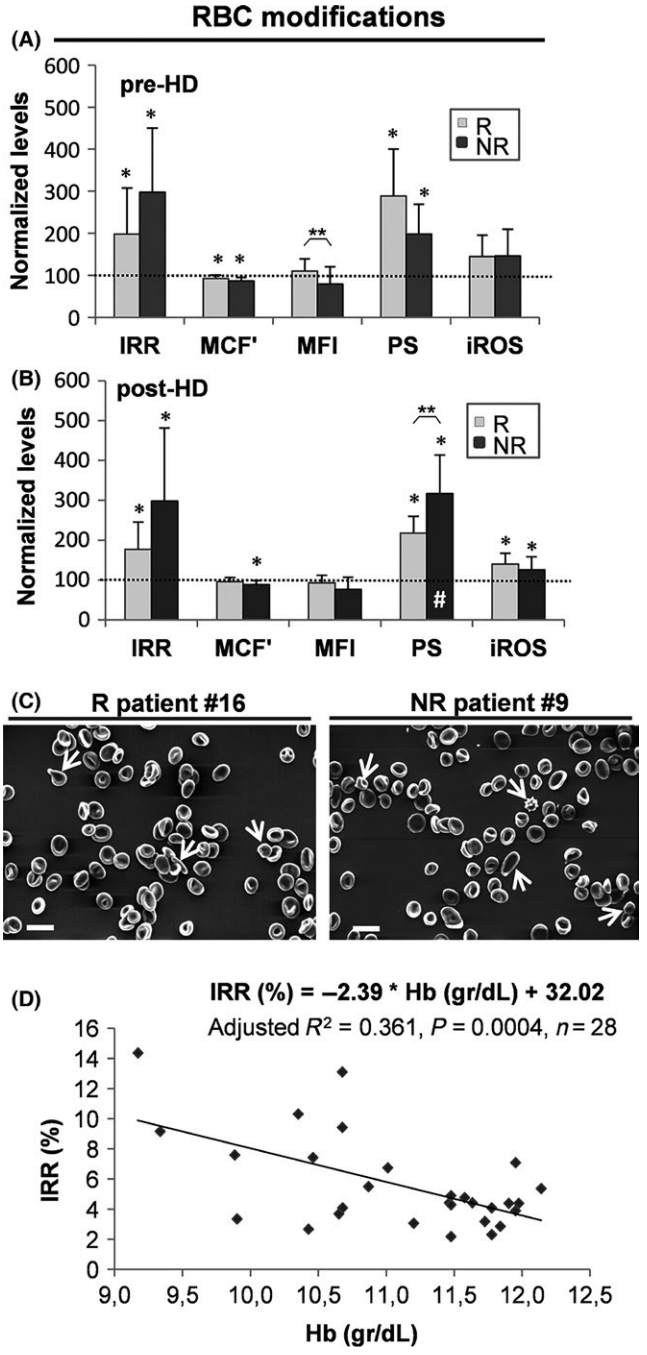

FIGURE 2 Variation in Red blood cell(RBC) parameters in responders (R) and non-responders (NR) pre- (A) and post-HD (B) after normalization to controls (100%, dotted line). IRR, irreversible RBC shape modifications; MCF', mean corpuscular fragility (following incubation at 37°C for 24 hours); MFI, mechanical fragility index; PS, PS exposure; iROS, intracellular ROS. **P*<.05 vs control; ***P*<.050 R vs NR (*P*=.006 for MFI in A; *P*=.024 for PS in B); #*P*<.05 pre- vs post-HD. Bars: Mean±SD. (C) Representative scanning electron micrographs of RBCs from responder (R) or non-responder (NR) patients before the HD session. Arrows show irreversible RBCs shape modifications (IRR), such as spheroechinocytes, dacryocytes, elliptocytes, spherocytes, and ovalocytes. Scale bars: 10 μm. (D) Regression analysis of cellular Hb levels (gr/dL) as an independent variable produced a model that predicts percentage of IRR shape modifications in patients with end-stage renal disease (ESRD)

levels (2914±1269 vs 2122±782RFU, respectively) was observed in NR- compared to R-RBCs, however, at no statistical significance level, owing to extreme intragroup variations. In a similar way, R-RBCs were more susceptible to hemolysis under mechanical stress than NR-RBCs (MFI=0.884±0.235% vs 0.634±0.332, respectively). HD triggered intracellular ROS formation in R- and NR-RBCs but had no significant effect on RBC shape profile (Figure 2B). It aggravated, however, PS exposure on NR-RBCs (1.19±0.42% pre-HD to 1.90±0.58% post-HD), as opposed to its neutral effect on R-RBCs (Figure 2B).

TABLE 2 Variation in the expression and carbonylation index of RBC membrane proteins in responder (R) and non-responder (NR) patients pre- and post-HD

	R patients		NR patients	
Proteins	**pre-HD**	**post-HD**	**pre-HD**	**post-HD**
Adducin	80±28	82±24	103±74	100±60
Aquaporin 1	91±46	58±24[a,c]	81±62	92±52
Band 3	107±26	72±41[c]	74±46	94±40
Band 3 oligomers	622±332[a]	158±130[b,c]	946±610[a]	5386±4568[b]
Calpain	96±73	90±80	75±39	72±53
CD47	39±15[a,b]	21±20[a,b,c]	75±40[b]	71±34[a,b]
CD59	123±40[b]	140±63[b]	79±20[b]	68±18[a,b]
Flotillin 2	117±24[b]	85±20[c]	87±21[b]	100±13
GAPDH	110±40	69±36[c]	101±36	117±78
Glucose transporter 1	222±120[a]	116±58[c]	161±120	107±58[c]
Hsp70	190±121	173±78	160±114	178±125
IgGs	754±670[a,b]	397±269[a,c]	240±27[a,b]	203±104
Oxidized Hb	82±60[b]	37±32[b]	24±19[b]	101±56[b,c]
Peroxiredoxin 2	95±32[b]	71±16[a,b,c]	129±35[b]	103±38[b]
sCLU (clusterin)	111±43[b]	88±39[b]	53±25[a,b]	54±15[a,b]
Spectrin	115±28	83±32[c]	96±37	104±47
Spectrin proteolysis	103±63	84±40	96±35	138±74
Stomatin	118±32	72±28[c]	90±22	105±66
Synexin	110±58	101±43[b]	155±70	169±86[b]
Ubiquitin	52±21[a]	60±27[a]	60±38[a]	62±48
PCI	124±113	98±30[b]	101±95	247±165[b]

[a]significantly different values vs healthy subjects; [b]R vs NR; [c]pre- vs post-HD, $P<.05$. PCI, Protein Carbonylation Index; RBC, Red blood cells. Values are presented as Mean±SD, after normalization to control (100%).

The protein composition of the RBC membrane differed significantly in patients with ESRD compared to control (Table 2 and Figure S1), and overall, the ESRD RBCs were characterized by excess of band 3 oligomers (B3-O) and membrane-bound IgGs but lower expression of ubiquitinylated proteins compared to healthy counterparts. But in contrast to the plasma and cellular modifications mentioned above, the profile of membrane proteome modifications in R-RBCs was totally different from that observed in NR-RBCs. In fact, excess of membrane-bound IgGs but severe deficiency in CD47 protein were mostly (IgGs) or exclusively (CD47) detected in the membrane of R- compared to the NR-RBCs. On the other side, NR-RBC membrane selectively exhibited lower expression of CD59 and sCLU proteins but more peroxiredoxin 2 (Prx2) compared to R- and/or healthy RBCs. Notably, HD had a sharp effect on the membrane expression of numerous proteins in R- as opposed to the NR-RBCs, including the MVs-associated proteins stomatin, flotillin 2, band 3, IgGs, CD47, GAPDH, Prx2, and aquaporin-1 (Table 2). Statistically significant differences in the membrane expression of certain components including band 3-oligomers, CD47, CD59, Prx2, sCLU, synexin, oxidized/denatured Hb and carbonylated components were observed between R- and NR-cells post-HD.

3.3 | Sorting the data using statistical and bioinformatics tools

Seven subsets of closely interrelated hematological, biochemical, and HD-related parameters in ESRD arose by factor analysis (Figure S2). Variation in rhEPO dose responded similarly to variation in baseline hemolysis (and the closely associated RBC mechanical fragility index). Free Hb, which is the sum of extracellular Hb species (including those enclosed in circulating R-MVs), is a joint component of the Hb/Hct and plasma antioxidant capacity factors. Similar patterns of responses were observed for certain uremic toxins and RBC-related variables, reflecting underlying associations between them, while RBC indexes and shape variables co-segregated along with osmotic fragility one.

Statistically significant correlations ($P<.05$) between hematological, physiological, and proteome factors in responders and non-responders were integrated into biologic networks (Figure S3 and Figure 3, respectively). As clearly shown in these constructs, the same parameter was connected with different variables in the two groups of patients. For instance, rhEPO dose was negatively correlated with PS exposure in R-RBCs (Figure S3) but with sCLU expression in NR-RBCs (Figure 3). R-MVs concentration was negatively correlated with serum UA levels in responders, but with serum urea reduction ratio (URR) and

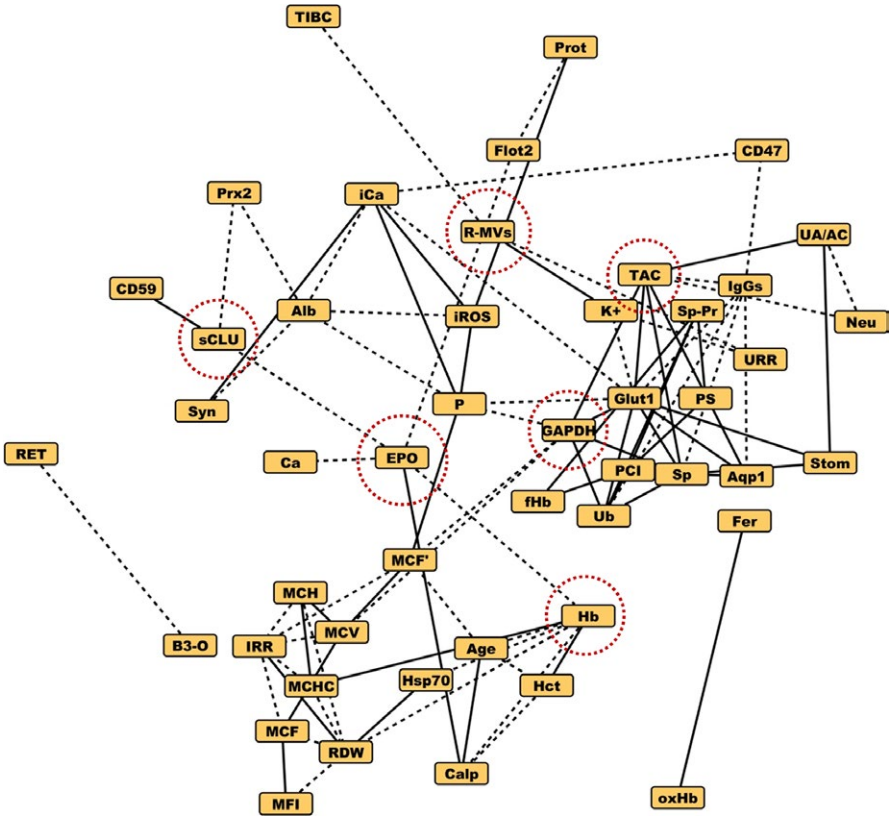

FIGURE 3 Network analysis connecting hematological and biological parameters in non-responders. The edges represent statistically significant ($P<.05$) positive (continuous lines) and negative (dashed lines) correlations among factors (the shortest the edge, the higher r value). Dashed circles: hub nodes (parameters of high connectivity). Abbreviations: Alb, albumin; Aqp1, aquaporin1; B3, Band3; Ca, serum calcium; Calp, calpain1; EPO, rhEPO dose; Fer, ferritin; fHb, free Hb; Flot2, flotillin 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Glut1, glucose transporter-1; Hb, hemoglobin; Hct, hematocrit; HDL, high-density lipoprotein; Hsp70, heat shock protein 70; iCa, intracellular calcium; IRR, irreversibly modified RBCs; iROS, intracellular ROS; K, serum potassium; MCF, mean corpuscular fragility; MCF', mean corpuscular fragility after incubation at 37°C; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MFI, mechanical fragility index; Neu, neutrophils; oxHb, oxidized/denatured Hb; P, serum phosphorus; PCI, protein carbonylation index; Prot, total serum proteins; Prx2, peroxiredoxin-2; PS, phosphatidylserine; RDW, RBC distribution width; RET, reticulocytes; REV, reversibly modified RBCs; R-MVs, RBC-derived microvesicles; sCLU secretory clusterin; Sp, spectrin; Sp-Pr, spectrin proteolysis; Stom, stomatin; Syn, synexin; TAC, total antioxidant capacity; TIBC, total iron-binding capacity; UA/AC, uric acid-dependent plasma antioxidant capacity; Ub, ubiquitinylated proteins; URR, urea reduction ratio. [Color figure can be viewed at wileyonlinelibrary.com]

total iron-binding capacity (TIBC) in non-responders. Baseline hemolysis in vivo was correlated with duration of HD and RBC oxidative stress markers in responders, while RBC fragility was found reversibly related to severe shape distortions in non-responders (r=-.871, P=.011, n=12). The antioxidant capacity of the plasma and RBC redox status were correlated with variation in numerous membrane proteins and removal signaling in NR-cells. In fact, TAC, RBC shape-related parameters (i.e, RDW index) and nodes of proteins involved in energy metabolism (i.e, GAPDH, Glut1) were hub points in the biological network of non-responders (Figure 3).

4 | DISCUSSION

The present study provided insight into the mechanisms underlying RBC lifespan in ESRD and assessed RBC variables that are associated with resistance to erythropoiesis-stimulating agents and HD treatment.

4.1 | Overall modifications in ESRD plasma and RBCs

End-stage renal disease RBCs undergo certain pathophysiological modifications in structure, composition, and signaling potential. Severe shape distortions (IRR), which render RBCs susceptible to clearance, reflect a systemic inflammatory profile in almost all pathologies characterized by oxidative stress, including ESRD.[17,18] We currently show for the first time that the percentage of irreversibly modified RBCs is a function of baseline Hb concentration, highlighting the close relationship between severe RBC shape distortions, inflammation, and anemia in ESRD. Uremic toxins may induce PS exposure on RBCs, accelerating therefore their clearance and pathophysiological interactions with immune cells, platelets, and endothelium,[19] through molecular pathways involving or not Ca^{2+} influx,[20] as highlighted by the segregation pattern of those variables following a factor analysis as well.

Moreover, the current study verified the strong relation between PS exposure and R-MVs formation,[21] in response to HD and rhEPO dose. Some indolic uremic solutes can induce vesiculation along with PS

exposure on healthy RBCs.[22] Loss of phospholipid asymmetry and additional ESRD-associated pathologies, including shape distortion, mechanical stress, chronic inflammation, oxidative stress, cellular activation, and endothelial dysfunction, also affect vesiculation of endothelial and blood cells, including RBCs.[23-25] The majority of previous studies have focused on MVs derived from platelets and endothelial cells for their probable prothrombotic and pro-inflammatory roles.[25] Nevertheless, circulating, PS+ RBC-derived MVs modulate NO-redox homeostasis and contribute to several hypercoagulable states in human.[26] Membrane vesiculation is an integral part of RBC aging. The majority of the RBC-derived MVs expose PS while their protein composition resembles that of the oldest RBCs.[27] In consistence with previous studies,[2,14] the proteomic profile of the ESRD RBC membrane showed elevated levels of Band 3-based RBC aging model modifications (modified band 3 protein, membrane-bound IgGs, etc.) but deficiency in the microvesicles-associated[27] ubiquitinylated components. Considering that patients with ESRD are characterized by a younger RBC population compared to healthy controls, those markers imply RBC injury and/or premature aging.

On the other side, ESRD RBCs were benefited to some extent by the cytoprotective effects of EPO and probably, of uric acid. ESRD RBCs were found resistant to osmotic lysis as a result of EPO's strengthening effect,[6] although RBC shape distortions may also account for this resistance, as currently (Figure 3) and previously[15] suggested. In the same wavelength, and despite being subjected to numerous oxidative provocations, ESRD RBCs contained normal basal levels of ROS, probably owning to the ROS scavenging activity of EPO[28] and the antioxidant capacity of uric acid (UA)[29,30] and other natural antioxidants that are eliminated by HD. In accordance with this assumption, in our study, subnormal antioxidant capacity of plasma post-HD appeared along with over-accumulation of intracellular ROS, suggesting uptake of plasma UA by ESRD RBCs, as previously shown in oxidatively stressed and cancer cells.[31,32] Being on average younger than healthy RBCs, ESRD RBCs are expected to contain more EPO binding sites[33] and thus to be more susceptible to EPO effects.

4.2 | Hypo-responsiveness to EPO is associated with a modified homeostatic context in ESRD RBCs

Despite the aforementioned profile of baseline blood modifications, the current and previous studies in the field of ESRD have highlighted a huge interpatient variability in measures of RBC homeostasis. Differences in primary causes of renal failure, age, comorbidities, time on HD, and therapeutic schemes among patients may account for it. To examine the probable contribution of rhEPO resistance to this phenomenon, we compared the profiles of equally anemic responsive (R) and non-responsive (NR) patients.

With the striking exception of the protein composition of the RBC membrane, the structural and physiological characteristics of ESRD RBCs did not differ significantly between groups before HD. At the peak of the uremic stress that is imposed to patients' RBCs just before the dialysis session, subtle differences in the homeostatic context between the two groups can only be imprinted in a highly dynamic and responsive cellular structure, as the RBC membrane is.

Indeed, low expression of the complement inhibitors CD59 and clusterin was detected in non-responsive patients, suggesting RBC susceptibility to complement-mediated injury (Figure 4). Clusterin

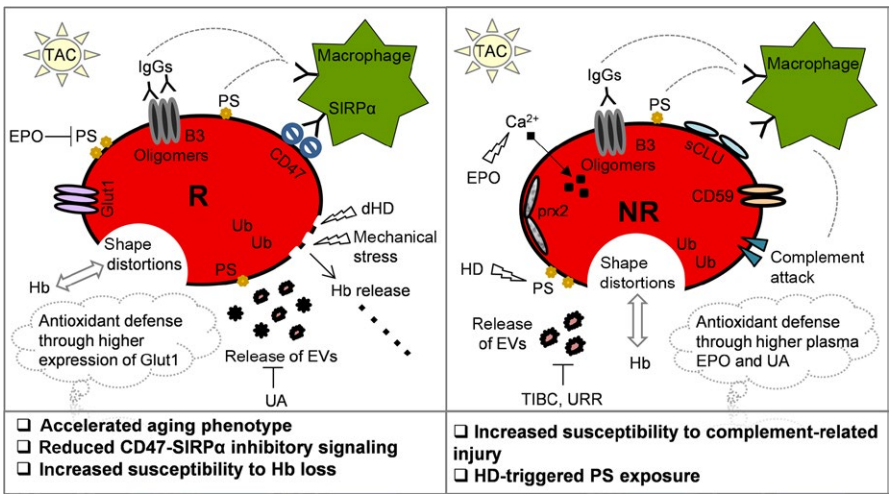

FIGURE 4 Schematic presentation of structural and functional modifications detected in RBCs of patients responsive (R, left panel) or not (NR, right panel) to standard range of rhEPO dose and their probable association with anemia in end-stage renal disease (ESRD). Generally, ESRD erythrocytes are characterized by augmented exovesiculation of the membrane, phosphatidylserine (PS) exposure, band 3 oligomerization, IgGs binding, decreased ubiquitinylated (Ub) components, and severe shape changes. The percentage of shape distortions can be predicted in part by the baseline Hb concentration. R-RBCs further show susceptibility to erythrophagocytosis (through IgGs- and CD47-related changes) in addition to mechanical stress and hemolysis, which seems to be triggered by longer duration under HD treatment (dHD). PS exposure and membrane vesiculation are ameliorated by rhEPO and UA levels, respectively, in R-RBCs. In contrast, complement-mediated injury promoted by low expression of CD59 and sCLU seems to underlie anemia in rhEPO resistance (NR). Hemodialysis (HD) and high EPO dose deteriorate PS exposure and calcium influx, respectively, but TIBC and URR inhibit vesiculation in EPO resistance. Plasma (UA and EPO) and membrane-associated components (Glut1 in R-RBCs and Prx2 in NR-RBCs) probably strengthen the antioxidant defenses in ESRD RBCs. [Color figure can be viewed at wileyonlinelibrary.com]

represents a biomarker of redox stress and senescence for RBCs[34] and a novel biomarker of kidney injury.[35] Decreased levels of clusterin have been found (in general) in ESRD RBCs[14] and in the plasma of long-term ESRD survivors.[36] EPO can not only reduce clusterin's rise in plasma as previous studies shown,[37] but it can also negatively affect its association with RBCs in NR patients in vivo, as currently reported.

On the other side, although previous studies have reported an accelerated aging phenotype in ESRD RBCs,[2,14] the current study made clear that this is the case for patients responsive to standard EPO doses, as excess of aging markers and deficiency of RBC membrane in the marker of "self" protein CD47[38] were both detected in R-RBCs. In addition to that, R-RBCs were susceptible to hemolysis pre- and post-HD and more fragile to mechanical stress compared to NR-RBCs. It is tempting therefore to speculate that anemia in responders might be in part the result of increased susceptibility to Hb loss, increased opsonization, and reduced CD47-SIRPα inhibitory signaling to splenic macrophages (Figure 4).

In light of higher urea and uric acid levels found in the NR- compared to the R-plasma, one would expect more protein oxidative defects in NR-RBCs. The higher membrane expression of peroxiredoxin 2, typically induced by oxidative stress,[39] in NR- than in R-cells reflects this kind of provocation. Oxidative stress might be alleviated by the antioxidant effects of both rhEPO and serum uric acid on mature RBCs, as the current reconstitution experiments and previous studies shown in healthy subjects and in thalassemic or uremic patients.[7,8,40,41] In contrast, the oxidative provocations imposed by the uremia on R-RBCs seemed to be rather repelled by overexpression of Glut 1 transporter (Figure 4). This modification that was previously reported in unclassified groups of hemodialysis patients,[14,42] is indicative of the hypermetabolic state of ESRD RBCs,[43,44] and is expected to promote the preservation of intracellular ascorbate that is lost during HD.[42]

4.3 | Evidence for different short-term HD effects and probably for a different vesiculation mechanism in R- vs NR-RBCs

By eliminating uremic toxins, HD ameliorated anemia and accumulation of MVs,[22,23,45] equally in responders and non-responders. On the other side, HD did not affect the concentration of RBC-derived MVs or the protein composition of the RBC membrane in non-responders, and moreover, it aggravated PS exposure on NR-RBCs. In striking contrast, HD had a beneficial effect on responsive patients by leading to a selectively, sharp decrease in the accumulation of RBC-derived MVs. Notably however, that amelioration was not consistent with the extensive remodeling of the RBC membrane in respect to the decreased expression of numerous MVs-associated components (stomatin, flotillin 2, IgGs, band 3 oligomers, CD47, GAPDH, Prx 2, aquaporin, etc.) post-HD, neither with the selective sharp decrease in MCV following HD in responsive patients. These findings are rather consistent with the release of small and/or PS-negative MVs and exosomes that are undetectable by the flow cytometry approach used. Indeed, conventional cytometry cannot detect vesicles <300 nm, the area which harbors the majority of extracellular vesicles in human blood.[46] Of note, MVs in ESRD appear to be less procoagulant compared to other diseases,[24] while RBCs are capable of releasing vesicles under conditions of well maintained lipid asymmetry.[47] Through the potential release of those small and/or AnnV-negative extracellular vesicles, the protein composition of the R-RBC membrane might be appropriately remodeled post-HD in consistence with the proteome analysis findings. Our results suggest generation of different species of extracellular vesicles in responders compared to non-responder patients with ESRD. Cells produce highly dynamic and versatile populations of heterogeneous extracellular vesicles in relation to cell age, stress, and activating stimuli. Considering that extracellular vesicles are functionally associated with coagulation and inflammation[48] and that endothelium damage or cardiovascular disease is more frequently appeared in rhEPO resistance,[49] our data deserve further study through methods more suited to assess issues of small particle biology.

4.4 | The different homeostatic contexts in responsive and non-responsive patients can be charted in biological networks

The physiological properties and the protein composition of the RBCs in responsive and non-responsive patients with ESRD revealed variability in molecular lesions and probably in pathways driving erythrocyte signaling and removal. Networks have long been used in biology to show relationships between biologically relevant elements. The currently reported biological networks that integrated statistically significant correlations between hematological and biological parameters in ESRD clearly denoted a different context of interactions in responsive vs non-responsive patients. According to these constructions, EPO dose-range used in responders alleviates PS exposure on RBCs while higher EPO dose in non-responders is associated with clusterin and intracellular Ca^{2+}-driven protein variations. Notably, it has been reported that rhEPO induces Ca^{2+} influx in spite of a partial decrease in PS exposure on human mature RBCs in vitro.[40] Our results suggested that the first effect is mainly observed in non-responders while the second one in responder patients, while the positive correlation between sCLU and CD59 variation verified the previously reported physical interaction between the two proteins in mature human RBCs.[50] In addition, duration of HD seemed to negatively affect hemolysis and intracellular ROS accumulation in responders while iROS was correlated with plasma soluble factors (albumin, P, total proteins) in non-responders. Plasma factors were correlated with the exovesiculation of RBC membrane in both groups, however, while TIBC and URR were negatively correlated with R-MVs levels in non-responders, UA levels seemed to play a similar role in responders. Of note, UA and TAC levels were correlated with variation in numerous RBC-associated variables supporting a cytoprotective activity of uric acid in human RBCs in vivo. Construction of similar networks enriched in omics data would help to unravel the complexity of RBC homeostasis in the highly multivariable system of ESRD.

5 | CONCLUSIONS

End-stage renal disease RBCs were characterized by severe shape distortions, membrane exovesiculation, and senescence/death signaling

but reduced fragility compared to healthy RBCs. The profile of RBC modifications is the overall result of the negative effect of the uremic toxic environment and of the cytoprotective activity of rhEPO and uric acid. Baseline Hb concentration contributes in part to the extent of irreversible RBC transformation. RBCs in non-responsive patients are characterized by increased susceptibility to complement-mediated injury and by HD-triggered PS exposure. On the other side, anemia in responders might be in part the result of increased susceptibility to Hb loss, increased opsonization, and erythrophagocytosis, and is probably associated with a different erythrocyte vesiculation mechanism compared to non-responders. Deregulation of RBC homeostasis might involve different molecular pathways driving erythrocyte signaling and removal in rhEPO non-responders compared to responsive patients. The high interpatient variability in many hematological and physiological factors, reported by this and numerous other studies in the field, calls for a more individualized approach of this apparently complex disease. In light of the recently adopted consideration for RBCs as vital indicators of health at systemic level, and of the latest NIH guidelines suggesting focusing on precision medicine,[17] the structural and biochemical modifications in RBCs might be used during ESRD diagnosis and following of HD and rhEPO treatments in order to establish not only the anemic but also the inflammatory and oxidative profiles of each individual patient.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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