Uric acid variation among regular blood donors is indicative of red blood cell susceptibility to storage lesion markers: a new hypothesis tested

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BACKGROUND: Oxidative stress orchestrates a significant part of the red blood cell (RBC) storage lesion. Considering the tremendous interdonor variability observed in the "storability," namely, the capacity of RBCs to sustain the storage lesion, this study aimed at the elucidation of donor-specific factors that affect the redox homeostasis during the storage of RBCs in standard systems.

STUDY DESIGN AND METHODS: The hematologic profile of regular blood donors (n = 78) was evaluated by biochemical analysis of 48 different variables, including in vivo hemolysis and plasma oxidant and antioxidant factors and statistical analysis of the results. The possible effect of the uric acid (UA) variable on RBC storability was investigated in leukoreduced CPD/SAGM RBC units (n = 8) collected from donors exhibiting high or low prestorage levels of UA, throughout the storage period.

RESULTS: Among the hematologic variables examined in vivo, cluster analysis grouped the donors according to their serum UA levels. Plasma antioxidant capacity, iron indexes, and protein carbonylation represented covariants of UA factor. RBCs prepared by low- or high-UA donors exhibited significant differences between them in spheroechinocytosis, supernatant antioxidant activity, and other RBC storage lesion–associated variables.

CONCLUSION: UA exhibits a storability biomarker potential. Intrinsic variability in plasma UA levels might be related to the interdonor variability observed in the storage capacity of RBCs. A model for the antioxidant effect of UA during the RBC storage is currently proposed.

ABBREVIATIONS: GSH = glutathione; HDL = high-density lipoprotein; LDL = low-density lipoprotein; NO = nitric oxide; ROS = reactive oxygen species; TAC = total antioxidant capacity; TIBC = total iron-binding capacity; UA = uric acid.

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*To the memory of beloved friend and partner Leontini Foudoulaki-Paparizos, MD pathologist, Director of the "Agios Panteleimon" General Hospital of Nikea Blood Transfusion Center, excellent scientist and dedicated doctor with great contribution to the establishment of voluntary blood donation in Greece. Without her interest and assistance this project would not have started. To our great regret she passed away on March 30, 2015. May she rest in peace ….

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In the context of "red blood cell (RBC) storage lesion," stored RBCs undergo a series of time-dependent physiologic alterations that are only partially reversible.[1,2] These alterations are probably related to the in vivo recovery of RBCs.[3] Growing evidence suggests a central role for oxidative stress in RBC aging in health and disease context as well as under storage in blood banks.[4] Storage lesion comprises a number of interdependent processes that are possibly orchestrated by the management of the oxidative stress ex vivo.[5] Reduction in the antioxidant activity of both stored RBCs and supernatant leads to time-dependent oxidative assaults on lipids and proteins, including hemoglobin (Hb), that are intrinsically involved in the loss of cation gradients across the membrane, microparticles release, spherocytosis, and generation of senescence neoantigens at cell surface.[5-7]

Uric acid (UA) is the final product of purine metabolism pathway. It functions as a general electron donor and scavenger of singlet oxygen, hydroxyl radicals, oxoheme oxidants, hydroperoxyl radicals, and hypochlorous acid in the plasma.[8] In fact, UA represents the most abundant plasma antioxidant with a concentration close enough to its solubility limits (120-450 μmol/L).[8] Its antioxidant activity has evolutionarily replaced ascorbate as the major water-soluble antioxidant.[9] Furthermore, UA uptake by RBCs, oxidatively stressed cells, and cancer cells suggests an antioxidant role for UA as an intracellular scavenger to prevent the accumulation of reactive oxygen species (ROS)-associated damages.[8,10]

Blood donors can exhibit a wide variability in the "storability," namely, the capacity of RBCs to sustain the storage lesion,[11] as well as in the recovery of stored RBCs after transfusion.[12] Inherent variations in the oxidative burden and antioxidant activity of the donated blood might to some extent determine the storability of RBCs and probably their posttransfusion efficacy.[13-15] This study aimed at the elucidation of donor-specific hematologic characteristics that affect the redox homeostasis and storability of RBCs.

MATERIALS AND METHODS

Material supplies

The following materials were supplied: common chemicals and anti-band 3 (B-9277, Sigma Aldrich, Munich, Germany); anti-flotillin-2 (610384, BD Transduction Laboratories, San Jose, CA); anti-calpain-1 (sc-7531, Santa Cruz Biotechnology, Santa Cruz, CA); anti-peroxiredoxin 2 (Prx2, SP5464) and glucose transporter 1 (GLUT1, AP10084PU, Acris GmbH, Herford, Germany); 5-(and-6) chloromethyl-2′,7′-dichloro-dihydro-fluorescein diacetate, acetyl ester (CMH2DCFDA) and Fluo-4 AM (Invitrogen, Molecular Probes, Eugene, OR); horseradish peroxidase (HRP)-conjugated antibodies and ECL kits (Perkin Elmer, Santa Clara, CA; and GE Healthcare, Buckinghamshire, UK); bis-sulfosuccinimidyl-suberate (BS3) cross-linker (Thermo Fisher Scientific, Rockford, IL); Bradford assay (Bio-Rad, Hercules, CA); and plasma carbonylation, nitric oxide (NO), and clusterin enzyme-linked immunosorbent assay (ELISA) kits (BioCell Corp., Papatoetoe, New Zealand; Cusabio, Wuhan, China; and BioVendor, Asheville, NC, respectively).

Blood donor preparation of RBC units

For the prestorage study, 78 young (19-24 years old) male regular blood donors were examined. Blood was collected into EDTA or citrate vacutainers. The RBC storability was evaluated in prestorage leukoreduced units prepared from eight young, nonsmoking male donors exhibiting high (n = 4) or low (n = 4) levels of serum UA in vivo. RBC units containing CPD/SAGM were stored for 42 days at 4°C. The study was submitted to and approved by the Research Bioethics and BioSecure Committee of the Faculty of Biology/NKUA. Investigations were carried out in accordance with the principles of the Declaration of Helsinki. All subjects gave a written consent before their participation in the study.

Lifestyle, hematologic, and serum biochemical analysis

The donors filled out a lifestyle questionnaire (diet, smoking, alcohol consumption, etc.). Blood cell counts and indexes (mean cell volume, mean cell Hb, mean cell Hb concentration, RBC distribution width, neutrophils, lymphocytes, monocytes, platelets [PLTs], mean PLT volume, plateletcrit, hematocrit, etc.) were estimated by an automatic blood cell counter (Sysmex K-4500, Roche, Indianapolis, IN). Twenty-five biochemical variables including iron (Fe), total iron-binding capacity (TIBC), UA, low-density lipoprotein (LDL), high-density lipoprotein (HDL), alkaline phosphatase (ALP), aspartate transaminase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase, and so forth were measured in serum samples by using an automatic analyzer (Hitachi 902, Roche). Measurement of potassium (K^+) and sodium (Na^+) was performed with an electrolyte analyzer (9180, Roche) and of ferritin with an assay (Elecsys Systems analyzer, Roche).

Plasma and supernatant analysis

Free Hb concentration and percentage of hemolysis were calculated by the methods of Harboe[16] and Drabkin,[17] respectively. The total antioxidant capacity (TAC) was measured by the ferric-reducing antioxidant power assay.[18] The contribution of UA to the TAC was determined by uricase treatment.[19] Protein carbonylation, NO, and clusterin were measured by ELISA kits according to the manufacturer's instructions.

Characterization of RBCs

RBC morphology was evaluated by scanning electron microscopy. Purified RBCs were fixed in 2% glutaraldehyde and postfixed in 1% osmium tetroxide in 0.1 mmol/L sodium cacodylate buffer, pH 7.4. Fixed cells were successively dehydrated in ascending ethanol series and coated with gold-palladium for examination. ROS and calcium accumulation were detected by fluorometry, using the redox-sensitive probe CM-H_2DCFDA and the calcium indicator Fluo-4AM, respectively.[20] Glutathione (GSH) determination was accomplished by the recycling assay method of Tietze[21] that measures the reduction of Ellman's reagent by the GSH in the presence of NADPH and GSH reductase.

Immunoblotting analysis of RBC membrane proteins

RBCs were isolated by the method of Beutler and colleagues[22] and hemolyzed in 5 mmol/L sodium phosphate buffer (pH 8.0) containing protease inhibitors. Membrane fraction was prepared as previously described.[20] Equal amounts (12-25 μg) of membrane proteins were loaded in Laemmli gels, transferred to nitrocellulose membranes, immune-probed for RBC proteins, and detected by HRP-conjugated secondary antibodies and ECL development.[20] The relative membrane expression of each component was estimated by scanning densitometry.

Statistical analysis

All experiments were performed twice, unless otherwise stated. For statistical analysis, computer software (Statistical Package for Social Sciences, IBM SPSS, Version 22.0 for Windows, IBM Corp., Armonk, NY; administered by NKUA) was used. Donors or variables for the prestorage evaluation were categorized using the cluster and factor analysis, respectively. Time course analysis was performed by using repeated-measures analysis of variance (ANOVA). Intergroup differences were evaluated by one-way ANOVA and general linear models. Prediction outcomes were estimated by regression analysis. Pearson's and Spearman's correlation tests were performed to assess the relationship between variables. Significance was accepted at p values of less than 0.05.

RESULTS

Prestorage examination

Lifestyle, hematologic, and serum biochemical profile

Considering that the lipid, iron, and redox homeostasis in the blood might be related to the donor's lifestyle, we initially examined the interdonor variation in diet, physical exercise, smoking, and alcohol consumption. Evaluation of the questionnaire revealed a common average lifestyle for the volunteers participating in this study. Half of them were smokers and had physical activities on a weekly basis, 75% were not consuming more than one drink per week, and 27% were consuming red meat once per week (Fig. 1A). Moreover, the vast majority of the subjects displayed the anticipated normal range variation in most of the hematologic and serum and plasma biochemical variables examined (Table 1). However, donors with aberrant levels of serum cholesterol, LDL, iron, UA, AST, and clusterin were also detected. In particular, approximately 30% of the donors examined exhibited higher levels of serum UA (7.5 ± 0.4 mg/dL) compared to the normal range (<7.2 mg/dL; Fig. 1B). This finding was very interesting, since UA accounts for up to 60% of the TAC of the plasma.[18]

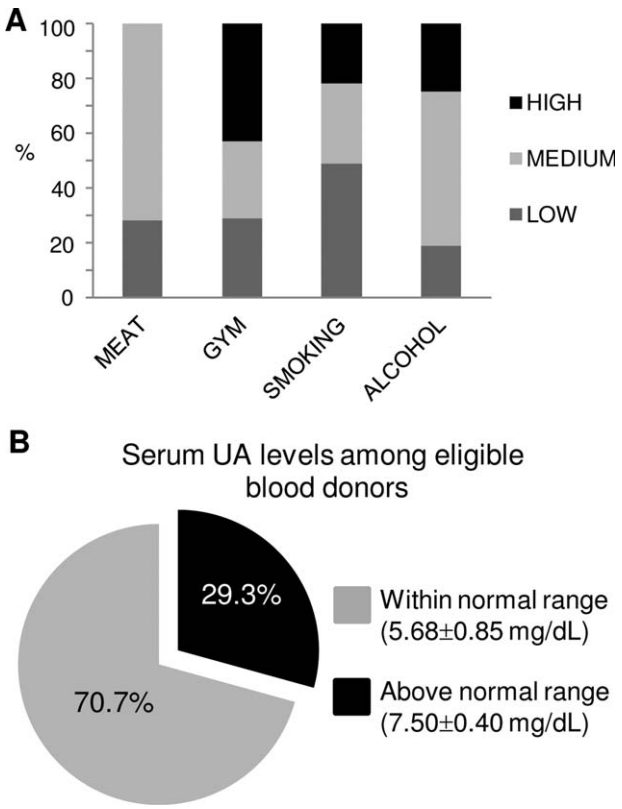


Fig. 1. Lifestyle (A) and serum UA (B) variation profile among the regular blood donors examined (n = 78). Percentage of donors exhibiting high, medium, or low frequency of red meat and alcohol consumption, physical exercise (GYM), and smoking as well as aberrant levels of UA is shown.

Classification of subjects and variables

To test for a possible classification of the donors on the basis of their variable hematologic profile (48 variables), we used cluster analysis. By this approach, all subjects were indeed grouped into three clusters according to their serum UA levels: high-UA Cluster A (7.56 ± 0.59 mg/dL), upper border–UA Cluster B (6.96 ± 0.51 mg/dL), and normal-UA Cluster C (6.01 ± 0.63 mg/dL), respectively (Fig. 2A). The

TABLE 1. Hematologic profile of the blood donors (n = 78)*

Variable	Normal range	Donors
Hb (g/dL)	12-18	15.0 ± 1.2
RBCs ($\times 10^{12}$/L)	3.8-5.3	5.0 ± 0.4
HCT (%)	36-56	44.0 ± 2.8
MCV (fL)	80-100	88 ± 7
MCH (pg)	27-32	30 ± 3
MCHC (g/dL)	32-36	34.0 ± 1.1
RDW (%)	10.0-16.5	12.5 ± 0.7
WBCs ($\times 10^9$/L)	4.0-9.0	7.0 ± 1.8
PLT ($\times 10^9$/L)	120-380	245 ± 51
Glucose (mg/dL)	65-110	89 ± 9
Cholesterol (mg/dL)	140-199	188 ± 31
LDL (mg/dL)	< 130	113 ± 27
HDL (mg/dL)	37-70	52 ± 9
HDL/LDL ratio	1.00	0.49 ± 0.15
VLDL (mg/dL)	8-40	22 ± 12
Triglycerides (mg/dL)	10-150	111 ± 29
Fe (mg/dL)	35-150	129 ± 27
TIBC (mg/dL)	260-390	328 ± 33
Ferritin (ng/mL)	18-270	96 ± 43
Transferrin (mg/dL)	200-400	306 ± 44
Transferrin saturation (%)	20-50	39 ± 6
Urea (mg/dL)	10-50	21 ± 6
Creatinine (mg/dL)	0.31-1.11	0.79 ± 0.20
UA (mg/dL)	3.5-7.2	6.9 ± 1.1
Calcium (mg/dL)	8.6-10.0	9.5 ± 0.4
Sodium (mmol/L)	135-145	140 ± 3
Potassium (mmol/L)	3.6-5.1	4.3 ± 0.4
Phosphorus (mg/dL)	2.5-4.9	3.3 ± 0.4
Total proteins	6.4-8.2	7.2 ± 0.5
Albumin (mg/dL)	3.5-5.5	4.8 ± 0.3
AST (U/L)	5-40	27 ± 18
ALT (U/L)	7-56	18 ± 10
γGT (U/L)	5-85	24 ± 12
ALP (U/L)	17-142	59 ± 11
Plasma Hb (mg/dL)	NA	41 ± 21
Plasma NO (μg/mL)	NA	3.59 ± 1.18
Plasma TAC (μmol/L Fe^{2+})	NA	984 ± 143
Plasma clusterin (μg/mL)	52.8-137.2	86.4 ± 51.9
Plasma protein carbonylation (nmol/mg)	<0.1	0.084 ± 0.063

* Data are presented as mean ± SD.
γGT = γ-glutamyl transferase; MCH = mean cell Hb; MCHC = mean cell Hb concentration; MCV = mean cell volume; RDW = RBC distribution width; VLDL = very-low-density lipoprotein; NA = not available.

clusters that have arisen were also different between each other in plasma TAC, iron, ferritin, and TIBC levels (double arrows in Fig. 2A). By evaluating the cluster analysis results, we next asked whether the UA variation exhibited any significant interrelation with the cosegregated variables of TAC, TIBC, iron, and ferritin. Factor analysis revealed nine subsets of closely interrelated variables, including the anticipated RBCs, white blood cells (WBCs), PLTs, Hb, and scavenging, liver, and lipid homeostasis factors as well as the UA-specific factor that cross-talked with that of iron indexes by TIBC and plasma carbonylation variables (Fig. 2B).

Storage examination: the UA effect

To test whether UA variation in vivo correlated with the storability of RBCs, we selected eight regular blood donors exhibiting low (4.73 ± 0.25 mg/dL, normal range) or high (7.52 ± 0.5 mg/dL) plasma UA levels, to prepare and store leukoreduced RBCs in CPD/SAGM for 42 days at 4°C. High-UA donors exhibited UA values slightly above the normal range to be consistent with the cluster A of the prestorage analysis shown in Fig. 2A.

Stored RBC units prepared from high- or low-UA donors differed ($p < 0.05$) from each other in certain RBC and supernatant characteristics typically affected by the storage (Table 2). Considerably better intracellular accumulation of calcium, spheroechinocytosis percentage (nonreversible RBC shape modification), and TAC of the supernatant were found in units prepared from high-UA donors compared to the low-UA ones throughout the storage period (Figs. 3 and 4A). The distribution of those variables' levels in both groups at the middle and the end of the storage period is shown by the representative boxplots of Fig. 3A. After each variable was plotted against storage duration, linear progression graphs arose, with R^2 values high enough to allow a safe prediction of a value anywhere in the storage period on the basis of any one previous time point value, including the prestorage one (Fig. 3B). Intracellular calcium activity and spheroechinocytosis percentage (Fig. 4A) as well as protein factors like Band 3 oligomerization (Day 21) and membrane expression of flotillin-2 (Day 42; Fig. 4B) varied significantly ($p < 0.05$) from low- to high-UA units of stored cells, while they did not in vivo (Table 2, Fig. 3B). High-UA stored RBCs were characterized by normal calcium accumulation and supernatant antioxidant capacity until the last days of storage while expression of flotillin-2 was decreased at pathologic levels only in low-UA RBCs stored for a long period ex vivo.

Multivariate analysis using serum UA concentration as an independent variable and the majority of the measurements (on RBCs and supernatants) collected throughout the storage period as dependent variables indicated a significant multivariate effect on the combined dependent variables of 1) intracellular Ca^{2+}, 2) supernatant TAC, 3) percentage of spheroechinocytes, and 4) degree of protein Band 3 oligomerization (Day 21) or flottilin-2 membrane levels (Day 42) in respect to the donor UA levels in vivo with $F(4,3) = 56.119$, $p = 0.024$, partial $\eta^2 = 0.942$, and power = 0.836 for Day 21 and $F(4,3) = 89.564$, $p = 0.011$, partial $\eta^2 = 0.994$, and power = 0.990 for Day 42, respectively. Additional repeated-measures multivariate analysis showed that the above-mentioned multivariate effect was concomitantly significant at both the middle and the end of the storage period. Finally, to evaluate whether the prestorage plasma UA levels can predict the storage-associated variation profile, regression analysis using UA as the independent variable was performed. By this approach, significant prediction models for supernatant TAC, intracellular Ca^{2+}, and spheroechinocytosis were made (Fig. 4C).

Apart from the variables of the multivariate UA model that showed combined significant differences between

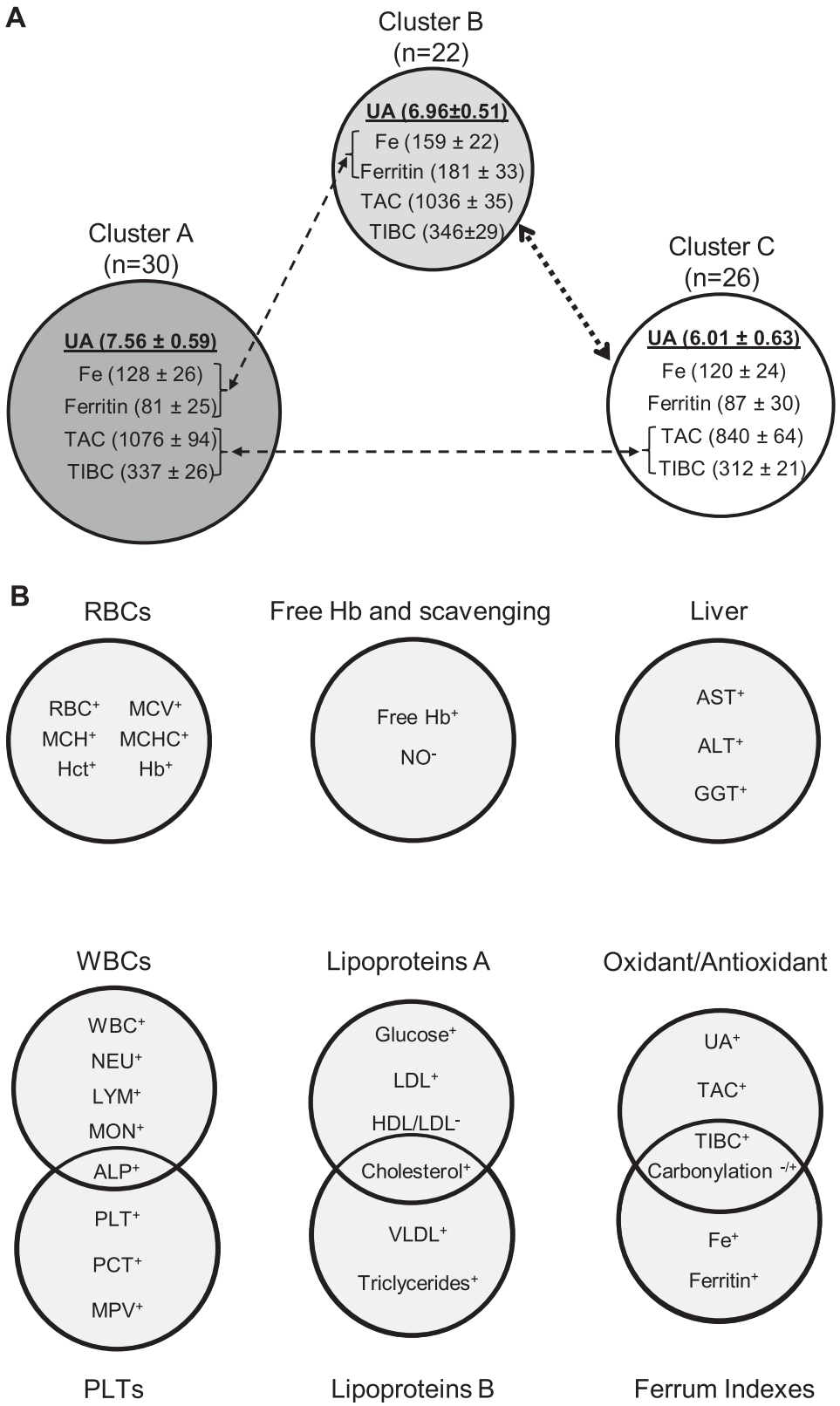


Fig. 2. Multiparametric statistical analysis (n = 78). (A) Cluster analysis using 48 different hematologic, serum biochemical, and RBC variables grouped the donors to three clusters according to their serum UA levels (mg/dL). TAC (μmol/L Fe^{2+}), TIBC (mg/dL), Fe (mg/dL), and ferritin (ng/mL) were significantly ($p < 0.05$) different between clusters (double arrows). Cluster B differed from Cluster C ($p < 0.05$) in all five variables included (bold-lined double arrow). Data are shown as mean ± SD. (B) Factor analysis aggregated the same variables ensemble in nine subsets of closely interrelated (positively + or negatively −) factors specific for blood cells, free Hb, liver, iron, lipid, and oxidant and antioxidant homeostasis. ALP, cholesterol, TIBC, and carbonylation were present in more than one subset. (−/+ superscript indicates that carbonylation is negatively correlated with the oxidant or antioxidant factor but positively with the ferrum factor.) GGT = γ-glutamyl transferase; LYM = lymphocytes; MCH = mean cell Hb; MCHC = mean cell Hb concentration; MCV = mean cell volume; MON = monocytes; MPV = mean PLT volume; NEU = neutrophils; PCT = plateletcrit; RDW = RBC distribution width; VLDL = very-low-density lipoprotein.

TABLE 2. Time course evaluation of the variability observed in selected RBC and supernatant characteristics among the high- and low-UA donors during the storage period*

		Time point			
Variable	Group (n = 8)	NS	Day 2	Day 21	Day 42
pTAC (μmol/L Fe^{2+})	Low	690 ± 64†	469 ± 44†‡	364 ± 14†‡§‖	317 ± 23†‡‖
	High	866 ± 96	618 ± 107‡	463 ± 52‡	440 ± 68‡
UA-dep AC (μmol/L Fe^{2+})	Low	377 ± 34†	337 ± 38†	184 ± 24†‡§‖	174 ± 47†‡‖
	High	483 ± 64	497 ± 103	292 ± 33‡§	278 ± 36‡
UA-ind AC (μmol/L Fe^{2+})	Low	313 ± 40	132 ± 14‡	180 ± 20‡§	143 ± 27‡
	High	383 ± 44	121 ± 13‡	171 ± 21‡§	162 ± 31‡
Calcium (RFU)	Low	3,813 ± 218	ND	4,353 ± 131†‡‖	5,331 ± 327†‡§‖
	High	3,365 ± 493	ND	3,834 ± 320‡	4,413 ± 353‡‖
NR (%)	Low	2.37 ± 0.90	ND	14.68 ± 1.04†‡‖	32.55 ± 1.45†‡§‖
	High	1.90 ± 0.23	ND	11.95 ± 1.42‡‖	27.26 ± 1.15‡§‖
Band 3 oligomers	Low	1.00 ± 0.14	ND	4.35 ± 0.81†‡‖	4.48 ± 0.98‡‖
	High	1.26 ± 0.19	ND	2.96 ± 0.52‡‖	4.27 ± 1.12‡‖
Flotillin-2	Low	1.00 ± 0.18	ND	0.99 ± 0.14	0.66 ± 0.07†‡§‖
	High	1.00 ± 0.24	ND	0.98 ± 0.11	0.80 ± 0.06
ROS (RFU)	Low	233 ± 25	321 ± 12†‡‖	563 ± 16‡‖	284 ± 16§
	High	222 ± 23	288 ± 21‡	551 ± 32‡§‖	265 ± 40§
pFREE Hb (mg/mL)	Low	0.246 ± 0.029	ND	0.269 ± 0.028	0.897 ± 0.177‡§
	High	0.215 ± 0.046	ND	0.476 ± 0.363	1.090 ± 0.816
GSH (μmol/L)	Low	628 ± 16	ND	558 ± 73	434 ± 102‡‖
	High	712 ± 163	ND	520 ± 84	385 ± 72‡‖
p-Carbonylation (nmol/mg)	Low	0.108 ± 0.058‖	ND	0.173 ± 0.024‖	0.195 ± 0.066‖
	High	0.057 ± 0.052	ND	0.151 ± 0.042‖	0.132 ± 0.076‖
Band 3 proteolysis	Low	1.00 ± 0.22	ND	2.80 ± 0.18‡‖	4.18 ± 0.75‡§‖
	High	1.16 ± 0.46	ND	2.13 ± 0.14‡‖	3.49 ± 0.70‡§‖
Calpain	Low	1.00 ± 0.53	ND	2.12 ± 0.82‡‖	4.47 ± 2.12‡‖
	High	1.06 ± 0.41	ND	1.59 ± 0.47	3.24 ± 1.82‡‖
Prx2	Low	1.00 ± 0.20	ND	2.5 ± 1.3‡‖	15.7 ± 10.7‡§‖
	High	0.80 ± 0.30	ND	1.8 ± 0.9	10.8 ± 6.9‡§‖
pClusterin (μg/mL)	Low	59,169 ± 44,401	ND	ND	10,920 ± 9,915‡‖
	High	78,195 ± 10,536	ND	ND	23,292 ± 15,760‡‖
Stomatin	Low	1.00 ± 0.37	ND	0.97 ± 0.27	0.91 ± 0.28
	High	1.15 ± 0.26	ND	1.00 ± 0.15	1.02 ± 0.18
GLUT1	Low	1.00 ± 0.34	ND	0.51 ± 0.35	0.61 ± 0.19
	High	0.69 ± 0.32	ND	0.51 ± 0.15	0.61 ± 0.17

* Row data are presented as mean ± SD except for RBC membrane densitometry results that are further normalized to the nonstored values of low-UA donors.
† $p < 0.05$ low- versus high-UA donors.
‡ $p < 0.05$ stored versus nonstored samples.
§ $p < 0.05$ versus the previous time point of storage.
‖ Abnormal values.
ND = not determined; NR = nonreversible; RBC shape modifications; NS = nonstored.

high- and low-UA units throughout the whole time of storage (graphically shown in the core of Fig. 5), the UA-specific antioxidant capacity was also better in unit supernatants prepared from high-UA donors compared to the low-UA ones and was not affected by the storage until Day 21 (Fig. 5). In contrast, the UA-independent antioxidant capacity of the supernatant exhibited a sharp decrease (by 30%-40%) soon after the preparation of the RBC units compared to that of the plasma in vivo in both groups. At the end of the storage both activities were reduced to approximately half the in vivo values (Fig. 5). An increase of the UA-independent activity was noticed on Day 21 supernatants (Table 2). Additional storage-associated factors, including in-bag hemolysis (Table 2) and intracellular GSH (Fig. 5), were also examined in both low- and high-UA units but they were not statistically different from each other. However, a trend for better variation in redox and calcium homeostasis markers was evident in high-UA units compared to the low-UA ones (Fig. 5). Among them, lower plasma protein carbonylation, Band 3 proteolysis, and membrane binding of calpain (calcium stress marker) and Prx-2 (oxidative stress marker), along with greater plasma clusterin levels in the high-UA units than in the low-UA units, were included. A significant increase in the intracellular ROS levels was observed in low-UA units soon after the beginning of the storage. Abnormal binding of calpain and Prx-2 to the membrane of stored RBCs was observed earlier during the storage in the low-UA RBC units compared to the high-UA ones (Table 2). Finally, although no significant change in the membrane expression of stomatin and GLUT1 was detected in stored cells, the low-UA donors exhibited a trend for higher levels of GLUT1 in vivo. The same was also observed for the plasma carbonylation measurement (Fig. 5).

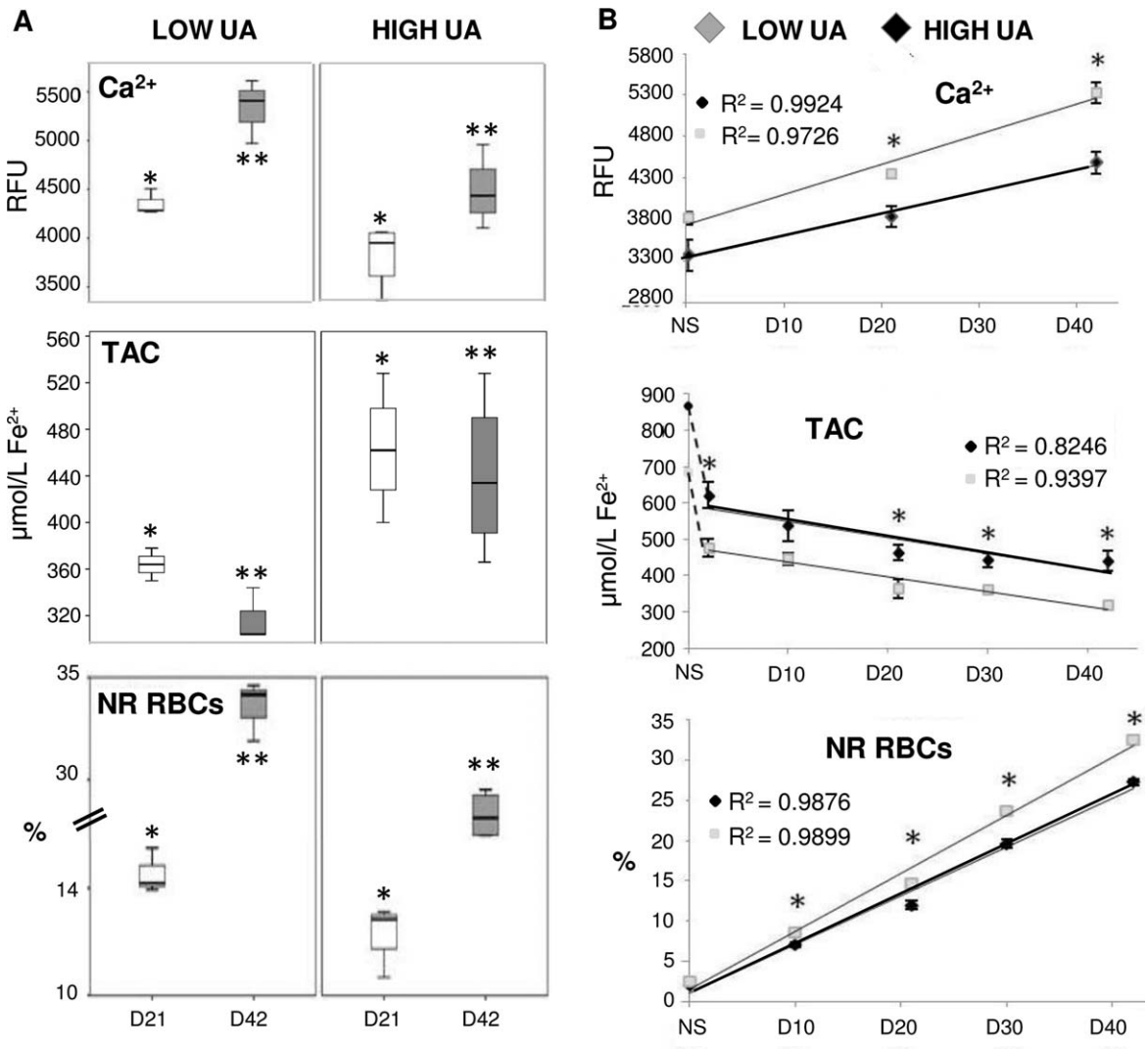


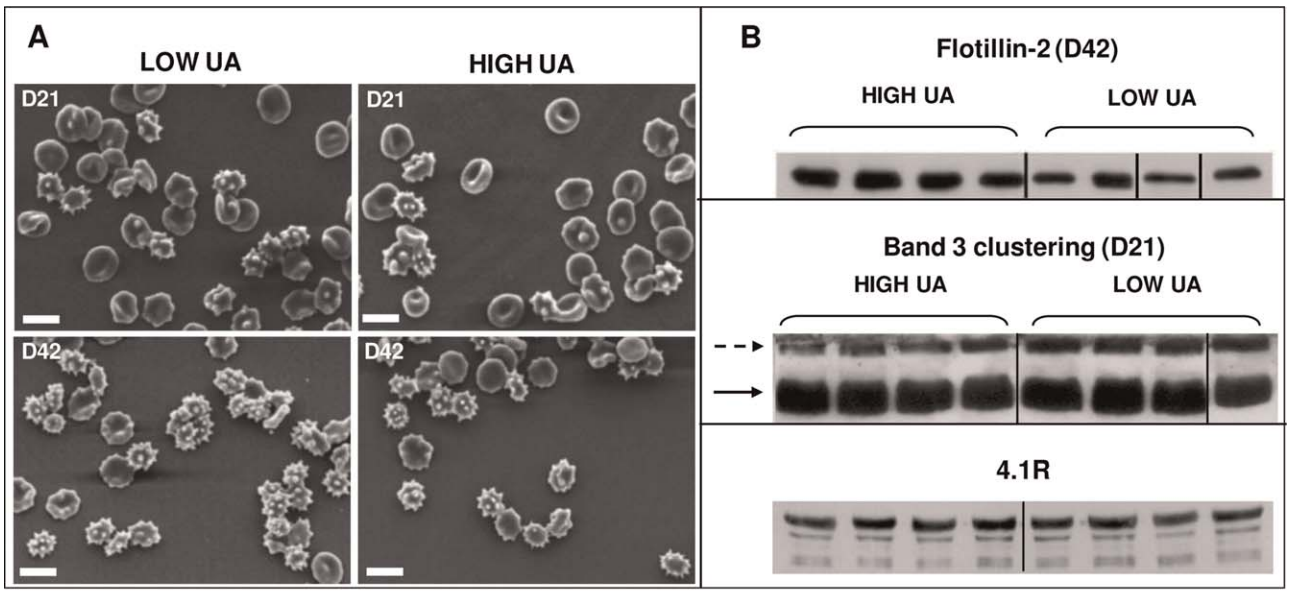
Fig. 3. In vivo UA levels as an evaluator of donor-specific RBCs storability (n = 8). (A) Representative boxplots showing the distribution of intracellular calcium (Ca^{2+}), TAC, and nonreversible RBC shape modification (NR RBCs) levels in low- (4.73 ± 0.25 mg/dL) and high-UA (7.53 ± 0.51 mg/dL) donors at the middle (white boxplots) and the end (gray boxplots) of the storage period. *Low vs. high donors on Day 21; **low vs. high donors on Day 42; $p < 0.010$. (B) Time course variation of the same variables throughout the storage period compared to the nonstored (NS, in vivo) levels. The differences shown are significant (*$p < 0.05$, low vs. high UA), linearly progressed (R^2 values), and predictable for any time point of storage based on any previous time point value, including the in vivo measurements. Discontinuous lines in the TAC scatter plot indicate the sharp gap in TAC between plasma in vivo and stored units' supernatants. Error bars = standard error of the mean.

DISCUSSION

Regular blood donors, though meeting the established criteria for blood donation, do not represent a hematologically homogeneous population. More importantly, a part of this variation in vivo seemed not to be functionally neutral regarding RBC storability but rather associated in part with the capability of RBCs to cope with the oxidative stimuli imposed by the storage system. It is worth saying at this point that the currently selected storage strategy (leukoreduced CPD/SAGM units of RBCs) is superior compared to similar alternatives (e.g., nonleukoreduced units in CPDA) by exhibiting longer and better biopreservation as well as milder oxidative lesions.[13]

Donors' classification according to the prestorage levels of serum UA

Cluster analysis with an input of 48 different hematologic variables in vivo grouped the donors according to UA levels. Iron and redox homeostasis indexes followed the UA-based classification of both donors and profiles according to the



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$Ca^{2+} = 6141 - (336 \cdot \mathbf{UA})$ Adjusted $R^2 = 0.542$ $P = .039$	$TAC = 182 + (38 \cdot \mathbf{UA})$ Adjusted $R^2 = 0.963$ $P = .0001$	$NR = 26.4 - (2.1 \cdot \mathbf{UA})$ Adjusted $R^2 = 0.591$ $P = .026$
$Ca^{2+} = 6332 - (282 \cdot \mathbf{UA})$ Adjusted $R^2 = 0.636$ $P = .023$	$TAC = 92 + (46 \cdot \mathbf{UA})$ Adjusted $R^2 = 0.912$ $P = .0005$	$NR = 39.5 - (1.6 \cdot \mathbf{UA})$ Adjusted $R^2 = 0.742$ $P = .009$

Day 21

Day 42

Fig. 4. (A) Representative scanning electron micrographs (Philips SEM515) of stored RBCs from donors exhibiting low or high UA levels in vivo, showing the prevalence of spheroechinocytosis in the units of low-UA donors at both the middle and the end of the storage period (scale bars = 10 μm). (B) Representative immunoblot analysis showing the lower membrane expression of flotillin-2 as well as the higher proportion of Band 3 oligomerization in stored RBCs prepared from low-UA donors compared to the high-UA preparations. Band 3 and oligomer bands are indicated by solid and discontinuous arrows, respectively. (C) Regression analysis using in vivo UA levels as an independent variable produced several significant models (equations) to predict the levels of the Fig. 3 variables throughout the storage period.

cluster and factor analysis, respectively, suggesting that normal-range UA variation in vivo may reveal intrinsic interdonor variation in the basal redox status. The UA factor[18] seemed to cross-talk with that of the iron indexes[23] through plasma protein carbonylation and TIBC. As for the first one, its negative correlation with UA might be associated with the well-established antioxidant activity of urate in the plasma.[9] Although a direct association of iron levels with plasma carbonyls has not been previously reported, plasma protein carbonylation has been found elevated in hemodialysis patients treated with intravenous iron.[24] TIBC was also found proportionate to the serum UA levels in line with the previously reported correlation of iron homeostasis with urate production.[25] It has been suggested that UA inhibits the Fenton reaction and thus the ROS generation through its ion chelator activity and formation of stable coordination complexes with iron ions.[26]

In vivo levels of UA and stored RBC susceptibility to storage lesion markers

Our findings suggested that intrinsic interdonor variability in serum UA levels is associated and might reveal the RBCs susceptibility to certain storage lesion hallmarks. Indeed, regression analysis of the data collected by integrated analysis of the stored RBCs showed that 91% of the supernatant TAC, 74% of the spheroechinocytosis, and 64% of the intracellular calcium level variability observed in the units examined might be associated with the inherent variability in UA concentration. These variables represent key factors of the storage quality: supernatant TAC is a holistic meter of the antioxidant status of the blood bag, spheroechinocytosis[27,28] undermines the posttransfusion viability of the stored cells,[29] while intracellular calcium can directly induce or indirectly promote a cascade of events leading to cellular dehydration, echinocytosis,

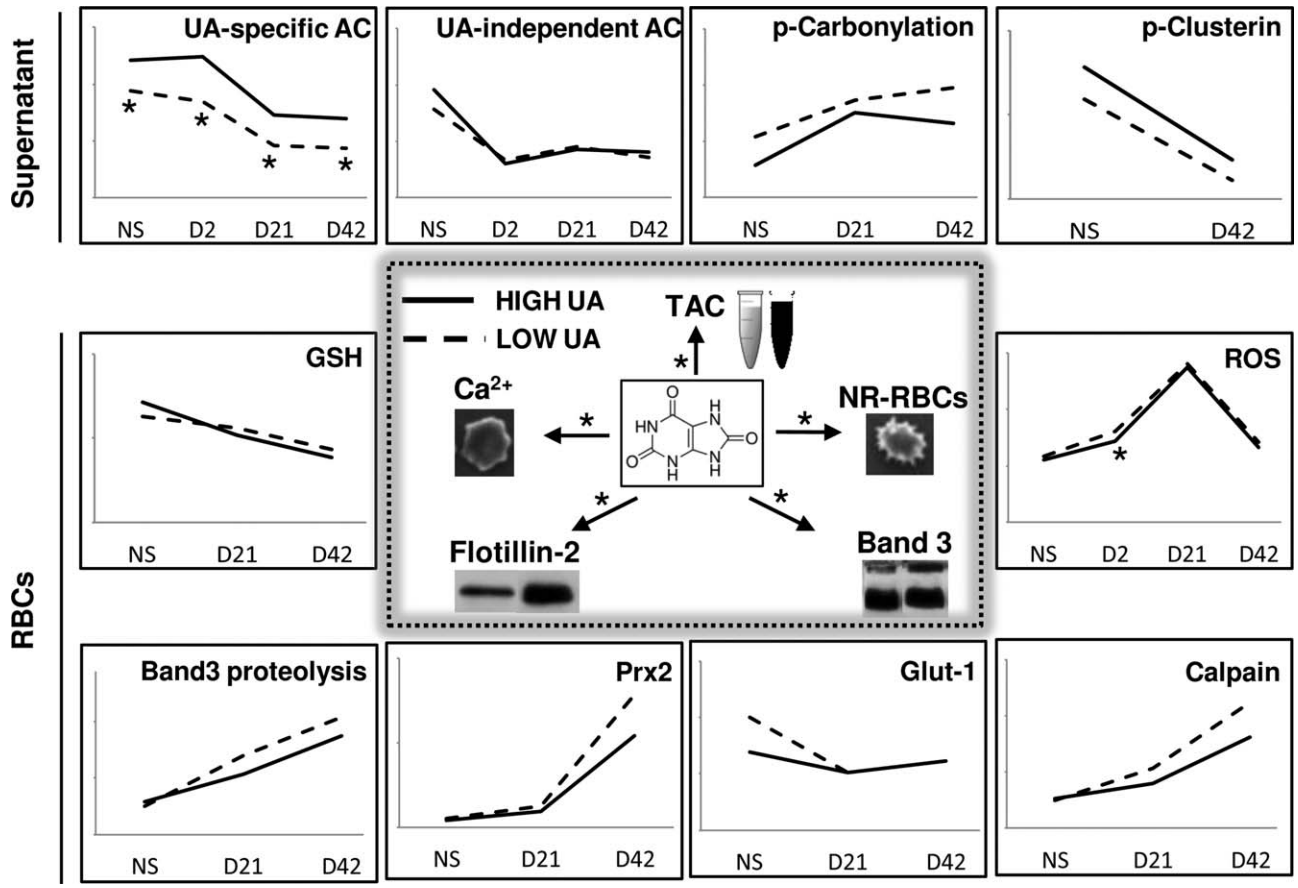

Fig. 5. Variation in measurable biologic metrics that are no components of the UA multivariate model (core panel). Plasma or supernatant (p) as well as RBC variables are shown in stored units versus nonstored (NS) samples. A trend for better variation in redox and calcium homeostasis markers is evident in high-UA units compared to the low-UA ones. (*$p < 0.05$, low vs. high UA).

protein degradation, recognition signaling, and eryptosis inside the blood bag.[13,30,31]

Donor UA present in the residual plasma of RBCs unit might reduce the ROS and reactive nitrogen species accumulation in situ leading to less oxidative hits to protein and lipid targets in the soluble component and the RBC surface. According to cluster analysis, UA variation was negatively correlated with protein carbonylation and, notably, low-UA donors were characterized by abnormal mean levels of plasma protein carbonylation in vivo. At storage system, RBC units produced by high-UA donors exhibited better supernatant TAC compared to the low-UA ones throughout the storage period owing to the UA antioxidant effect and not to that of the coexisting plasma compounds (ascorbate, albumin, α-tocopherol, carotenes, etc.). Those antioxidants seemed severely affected by the unit preparation steps exhibiting a sharp decline during the first days of storage compared to the plasma in vivo, unlike the UA-dependent activity. Better supernatant TAC may alleviate the oxidative attacks to the Ca^{2+}-permeable nonselective cation channels and Ca^{2+} transporter extracellularly, ensuring better ion transport regulation in stored RBCs.[31] In turn, better regulation of Ca^{2+} influx and efflux can minimize the activation of Gardos channel and, as a result, the calcium-dependent K^+ loss, cell shrinkage,[31] membrane blebbing, and spheroechinocytic transformation of stored RBCs.[7] Our results, showing lower calcium accumulation, late appearance of the calcium-driven membrane binding of calpain and Prx2, better preservation of the lipid raft–associated flotillin-2,[32] and lower level of spheroechinocytic transformation in the high-UA units, were in agreement with that hypothesis.

Further on, the UA might also act intracellularly. It has been reported that serum UA enters oxidatively stressed and cancer cells to prevent wide spreading of oxidative assaults.[10] Regarding RBCs, plasma UA can enter them either by passive membrane diffusion or through ATP-dependent transportation probably involving UA-specific protein carriers.[33] RBC membrane permeability to UA is increased under low-pH conditions[33] like those present in RBCs.[34] In line with that, an increased influx:efflux ratio of UA has been shown in stored RBCs in

proportion to the duration of storage.[35] In our study, the significant decrease in UA activity in Day 21 supernatants coincided with the sharp increase in intracellular ROS and the previously reported opposite decrease in pH,[36] conditions that jointly promote UA influx to RBCs.

As a result of the high iron and oxygen content, the generation of hydroxyls represents the primary mechanism of oxidative injury in RBCs.[37] Considering the protective role of chelators against the products of Fenton and Haber-Weiss reactions carried out in RBCs, the most important role of UA in RBCs might be the formation of a coordination complex with Fe^{3+} ions and thus the inhibition of iron-catalyzed oxidations.[38] Not surprisingly, the peroxidation of plasma and RBC membrane lipids, the peroxide-induced hemolysis,[8] and the nitrite-induced oxidation of oxyhemoglobin[39] can be stopped or delayed by UA. Inside RBCs, UA could restrain Hb from oxidation and denaturation into methemoglobin and hemichromes as previously reported[39] and consequently the Band 3 clustering on the membrane,[32,40-42] currently shown in high-UA donors. Interestingly, high-UA uremic plasma from renal disease patients can effectively mitigate the intracellular ROS accumulation in CPD/SAGM-stored RBCs in vitro (unpublished results), providing additional evidence for the antioxidant effect of UA influx into RBCs.

Urate radical and allantoin represent the ordinary products of UA oxidation. Urate radical can react with ascorbic acid (if present), regenerating urate and producing the more innocuous ascorbate radical.[8] Membrane GLUT-1 transporter in collaboration with stomatin protein transports dehydroascorbate to RBCs where it is reduced to ascorbic acid.[43] In light of our data showing a trend for higher GLUT1 expression in low-UA donors in vivo, a redox homeostasis cross-talk between ascorbic acid and UA cannot be excluded. To support, it has been reported that oxidation of ascorbic acid to dehydroascorbic proceeds much slower in high UA states, like uremia.[44] Certainly this reaction might be bidirectional in that UA, as most antioxidants can also exert a prooxidant effect. Dehydroascorbic acid can be reduced by UA inside RBCs leading to generation of UA radical and ascorbate that can be subsequently released extracellularly. In our samples, the transient increase in the UA-independent (and probably ascorbic acid–dependent) antioxidant capacity of the supernatant on Day 21 might be associated with that UA effect and its increased influx inside RBCs. Apparently, the steady state of those redox reactions and pathways operating inside the closed system of stored RBC units needs further investigation; even more regarding UA that is not reactive against oxidants involved in RBC homeostasis, such as superoxide.

The currently presented data in association with the previously reported UA kinetics and functions discussed were integrated into the model proposed in Fig. 6. Following the shown series of events, a part of the interdonor variability in the RBCs susceptibility to certain storage lesions can be associated with donor-related variation in the UA molecules inherently present in the residual plasma of the RBCs units and its well-established antioxidant effects. In support of this, UA-related antioxidant capacity was constantly higher in supernatant of units produced from high-UA donors compared to the low-UA ones throughout the storage period. However, the donor-dependent implication of UA in the storage quality of RBCs might be indirect and not causal. UA variation within normal range might be indicative of the donor's basal antioxidant capacity that inherently characterizes the stored RBC units. By following this reasoning, low-UA donors' RBCs probably correspond with worse starting level of antioxidant competence and redox homeostasis on Day 0 of storage, due to the chronic contact with a UA-poor natural environment in vivo. To support this view, low-UA donors were not only characterized by lower plasma TAC compared to the high-UA ones, but also by pathologic levels of plasma protein carbonylation in vivo. A trend for worse levels of plasma clusterin, intracellular GSH, and Prx2 variation was also evident. In fact, pathologic levels of the secreted chaperone clusterin were only measured in low-UA donors in vivo. Clusterin is a marker of oxidative stress and injury in many cells including RBCs.[45] It is involved in several physiologic processes including apoptosis, inflammation, cellular senescence, membrane vesiculation, and in vivo aging.[46,47] Notably, its plasma levels have been positively correlated with UA in healthy adults.[48]

In any case, namely, either through direct antioxidant function or as an indicator of the basal redox status and homeostasis, UA variation among donors seemed to be associated with the RBCs storage lesion profile. To our knowledge, the new hypothesis that has been introduced and tested by this study reported for the first time the possibility of predicting storage lesion components based on a simple and accessible serum factor that can be measured accurately before, at the time, or after blood donation for producing RBCs transfusion units. Both statistical significance and power (=0.990) of the multivariate model currently reported indicate that the sample size used to test our hypothesis at storage level was sufficient to support our results. Apparently, the typing of UA as a RBC storability biomarker needs further investigation in large-scale studies examining the storage lesion profile in groups of eligible blood donors associated by default with normal range UA variability, including women and ulcer patients. Based on our pre-storage and storage analysis we strongly suggest UA for evaluation in storage quality biomarkers identification studies. Finally, whether UA variation in vivo is also indicative of stored RBC performance after transfusion is a much harder—yet necessary—target to hit. Although recovery is expected to be better for a low-spheroechinocytosis unit compared to a higher one,

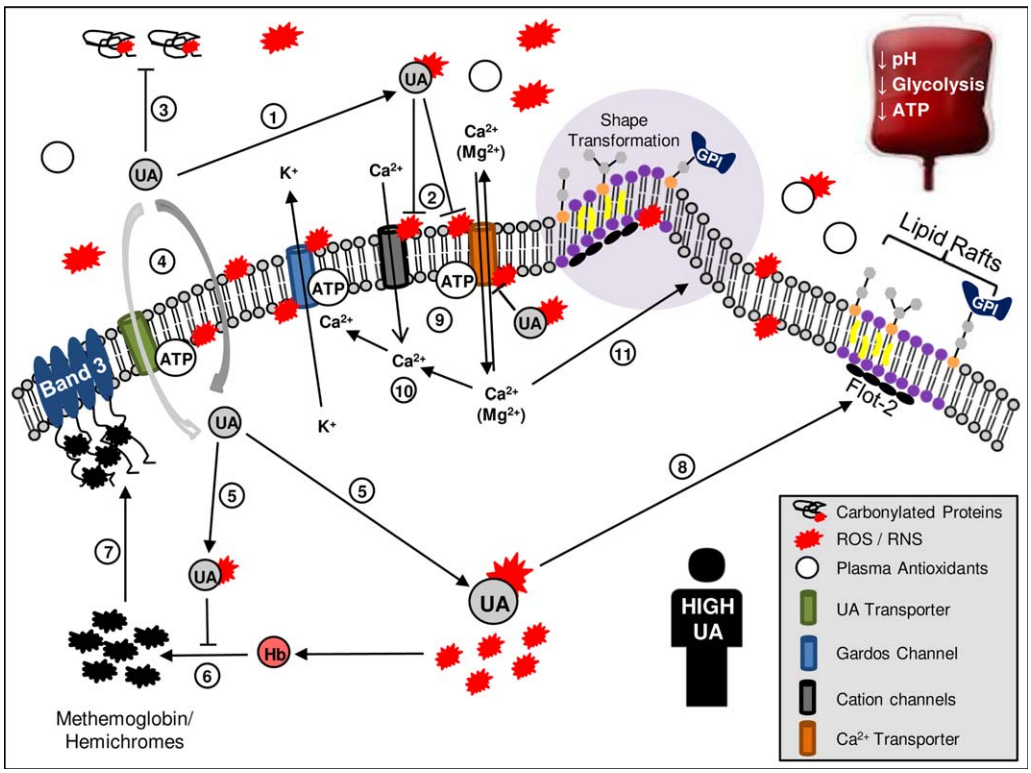

Fig. 6. Proposed model for a direct antioxidant effect of inherent donors' UA on supernatant and stored RBCs during the storage period. As an effective electron donor and ROS scavenger,[9] UA exhibits antioxidant activity extracellularly by lowering the oxidative attacks to RBC membrane channels and transporters (1). This effect on Ca^{2+}-permeable nonselective cation channels and Ca^{2+} transporter ensures better ion transport regulation in stored RBCs[31] (2). Moreover, UA-based ROS scavenging can partly inhibit supernatant protein carbonylation (3). UA might enter RBC through UA-specific transporters or by membrane diffusion[33] (4). The progressive lowering of the pH of the supernatant during the preservation period causes an increase in the influx rate of UA in RBCs that is proportional to the storage time itself[35] (4). Inside RBCs, UA similarly alleviates the oxidative burden in situ[10] (5), thus restraining Hb from oxidation or denaturation into methemoglobin and hemichromes[39] (6) and consequently the Band 3 clustering on the membrane (7). Another benefit might be the better preservation of the lipid rafts (showing by the high flotillin-2 levels; 8) as well as of the membrane channels activity (the selective cation channels and the Ca^{2+}-transporter are indicated in the graph), ensuring better calcium influx and efflux rate and thus low levels of calcium accumulation in RBCs (9). Low Ca^{2+} activity intracellularly minimizes the activation of Gardos channel (10) and as a result, the calcium-dependent K+ loss, cell shrinkage,[31] membrane blebbing, and spheroechinocytic transformation of stored RBCs[7] (11).

posttransfusion studies will lead to the identification of RBC biomarkers or assays to safely predict RBC recovery and function inside the recipient.

Concluding remarks

Storage time is only a relative quality criterion for the evaluation of the RBC storage lesion since donor-related factors are probably more effective in determining its profile. RBC storage lesion depends on the ability of stored units to cope with a range of oxidative stimuli and defects. Intrinsic variability in UA levels in vivo seems to be associated with RBCs storage lesion hallmarks like the degree of spheroechinocytosis. Although it is currently not known whether this association arises by direct antioxidant function of UA inside the RBCs unit or by its capability to reveal the redox homeostasis of the individual donor, normal-range variation in UA might be used to evaluate the susceptibility of blood labile product to storage lesions. The ability to evaluate storage lesion profiles by using easily accessible donor factors is probably the first step for the effective management of the donated blood (storage strategies, additive solutions, leukoreduction, appropriate storage period, etc.) by the blood bank services. Although we cannot claim the discovery of a storability biomarker, we strongly recommend UA for consideration and evaluation in future large-scale studies on donor variation effects, on storage quality biomarkers, and on individualized therapies applicable in transfusion medicine.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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