Donor variation effect on red blood cell storage lesion: a multivariable, yet consistent, story

Vassilis L. Tzounakas,[1] *Hara T. Georgatzakou,*[1] *Anastasios G. Kriebardis,*[2] *Artemis I. Voulgaridou,*[3] *Konstantinos E. Stamoulis,*[4,5] *Leontini E. Foudoulaki-Paparizos,*[5]* *Marianna H. Antonelou,*[1] *and Issidora S. Papassideri*[1]

BACKGROUND: Previous studies have shown that baseline hematologic characteristics concerning or influencing red blood cell (RBC) properties might affect storage lesion development in individual donors. This study was conducted to evaluate whether variation in hemolysis, microparticle accumulation, phosphatidylserine (PS) exposure, and other storage lesion–associated variables might be a function of the prestorage hematologic and biologic profiles of the donor.

STUDY DESIGN AND METHODS: Ten eligible, regular blood donors were paired and studied before donation (fresh blood) and during storage of RBCs in standard blood banking conditions. Plasma and cellular characteristics and modifications were evaluated by standard laboratory and biochemical or biologic analyses as well as by statistical and network analysis tools.

RESULTS: Nitrate/nitrite and other bioactive factors exhibited high interdonor variability, which further increased during storage in a donor-specific manner. Storage lesion evaluators, including RBC fragility and PS exposure, fluctuated throughout the storage period in proportion to their values in fresh blood. Donors' levels of phosphatidylserine exposure and hemoglobin F correlated with stored cells' mean cell (RBC) Hb concentration, oxidative stress markers, and cellular fragility.

DISCUSSION: Storage lesion indicators change in an orderly fashion, namely, by following donor-related prestorage attributes. These correlations are illustrated for the first time in "prestorage versus storage" biologic networks, which might help determine the best candidates for in vivo biomarkers of storage quality and provide deeper insight into the apparently complex donor variation effect on the RBC storage lesion.

Red blood cells (RBCs) are the most frequently transfused blood labile product. As a result, effective ex vivo storage of RBCs is an essential

ABBREVIATIONS: GAPDH = glyceraldehyde-3-phosphate dehydrogenase; GSH = glutathione; Hsp70 = heat shock protein 70; MCF = mean corpuscular fragility; MFI = mechanical fragility index; MP(s) = microparticle(s); MP-PA = microparticle-associated procoagulant activity; NOx = nitrate/nitrite; Prx2 = peroxiredoxin-2; PS = phosphatidylserine; ROS = reactive oxygen species; TAC = total antioxidant capacity; UA = uric acid.

From the [1]Department of Cell Biology and Biophysics, Faculty of Biology, NKUA and the [2]Laboratory of Hematology and Transfusion Medicine, Department of Medical Laboratories, Faculty of Health and Caring Professions, Technological and Educational Institute of Athens, Athens, Greece; the [3]"Apostle Paul" Educational Institution, Thessaloniki, Greece; the [4]Hellenic National Blood Centre, Acharnes, Athens, Greece; and the [5]Regional Blood Transfusion Center, "Agios Panteleimon" General Hospital of Nikea, Piraeus, Greece.

Address reprint requests to: Marianna H. Antonelou, Department of Cell Biology and Biophysics, Faculty of Biology, National and Kapodistrian University of Athens (NKUA) (NKUA), Panepistimiopolis, Athens 15784, Greece; e-mail: manton@biol.uoa.gr.

*Deceased.

To the memory of beloved friend and colleague Leontini Foudoulaki-Paparizos, MD pathologist, Director of the "Agios Panteleimon" General Hospital of Nikea Blood Transfusion Center, a dedicated doctor with great contribution to the establishment of the voluntary blood donation in Greece.

This study was partly supported by the Special Account for Research Grants of the NKUA to ISP and MHA.

Received for publication November 2, 2015; revision received February 11, 2016; and accepted February 11, 2016.

doi:10.1111/trf.13582

© 2016 AABB

TRANSFUSION 2016;56;1274–1286

requirement for medical practice. However, RBC functionality and viability are progressively impaired during storage in blood banks.[1] RBCs undergo numerous structural and biochemical modifications during this period that are likely to affect their recovery and tolerance to the transfusion therapy at clinical level.[2] Although the mechanisms underlying storage lesion remain uncertain, altered metabolism, increased oxidative stress, and the so-called "donor variation effect," which refers to substantial donor-to-donor differences in blood storage quality, represent currently established contributing factors.[3] In fact, both in-bag hemolysis and 24-hour in vivo recovery, namely, the "gold quality standards" for the developed storage systems, have been associated with donor-related factors.[3,4] Interdonor variation appears to have a genetic component related to blood homeostasis.[5] In addition, cell fragility,[6] metabolites,[7] and microparticle (MP)[8] accumulation, oxidative stress sensitivity, and antioxidant capacity[9,10] have also been suggested as donor-related hallmarks of RBC storage lesion. The contribution of donor variation in hemoglobin (Hb) levels,[11] RBC metabolic rate, MP production, and other factors to the quality of stored RBCs has now been extensively examined.[12]

Hemolysis, phosphatidylserine (PS) exposure, fragility, redox homeostasis, and other properties of RBCs are exacerbated by storage[13] and, consequently, baseline differences in their levels might directly or indirectly affect the quality of RBCs in a donor-specific manner. This paired fresh-versus-stored RBC study aimed at the elucidation of the donor variation effect on storage lesion development. Specifically, it was conducted to evaluate the interdonor "variation degree" of certain storage lesion-associated variables in RBCs and their probable associations with the biologic profile of the individual donors, as tested in fresh blood. The ultimate goal was to discover RBC storage quality factors that can be evaluated before storage of the donated blood, based on their donor dependence.

MATERIALS AND METHODS

Participants

Ten regular blood donors (20-30 years old, one obese subject and one carrier of β-thalassemia minor) who met the established criteria for donation (e.g., Hb levels > 13.5 g/dL) were recruited for a paired RBC study conducted in fresh blood before RBC unit production and during storage of RBCs. Fresh blood was collected into EDTA or citrate blood collection tubes. The RBC storability was evaluated in leukoreduced (by RC high-efficiency leukoreduction filters, Haemonetics), CPD-SAGM RBCs, during 42 days of storage at 4°C.[10] Samples were analyzed after the first 2 days of storage and weekly thereafter. The study has been 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 had given written consent before their participation in the study.

Laboratory testing

Hematologic analysis was performed by using the automatic blood cell counter (K-4500, Sysmex). Biochemical analysis of serum factors (including lipid and iron homeostasis factors, glucose, folic acid, total serum proteins, transferrin, uric acid [UA]) and electrolytes was performed by using automatic analyzers (902, 9180, Hitachi; and Elecsys Systems analyzer, Roche). Hb electrophoresis was performed for the estimation of HbA, HbA_2, and HbF. Quantitative determination of the glucose-6-phosphate dehydrogenase activity in blood was performed using a commercially available kit (Trinity Biotech).

Hemolysis and cellular fragility tests

Hb in cell-free plasma (plasma free Hb) was calculated by the method of Harboe.[14] Hemolysis in the supernatant was determined by the Drabkin's reagent.[15] In vitro osmotic fragility of RBCs was determined in solutions with decreasing saline concentration[16] and the mean corpuscular fragility (MCF) index (NaCl concentration causing 50% of hemolysis) was calculated. The mechanical fragility index (MFI) was evaluated as previously described[17] in blood mixed with stainless-steel beads and rocked for 1 hour on a rocker platform.

Oxidative stress variables and calcium accumulation

Total antioxidant capacity (TAC) and UA-specific antioxidant capacity of plasma were measured by the ferric reducing antioxidant power (FRAP) assay,[18] after uricase treatment, when appropriate, to estimate the UA-specific fraction of the antioxidant capacity.[19] Plasma protein carbonylation, nitrate/nitrite (NOx), and clusterin were measured by colorimetric enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions (BioCell Corp., Cusabio, and BioVendor, respectively). Reduced glutathione (GSH) determination was accomplished by following the Tietze's recycling assay method that measures the reduction of Ellman's reagent by the GSH in the presence of NADPH and glutathione reductase.[20] Intracellular accumulation of reactive oxygen species (ROS; with or without 100 μmol/L tert-Butyl hydroperoxide stimulation for 20 min at RT) and calcium (Ca^{2+}) was calculated fluorometrically (VersaFluor fluorometer system) by using the redox-sensitive probe 5-(and-6)-chloromethyl-2′,7′-dichloro-dihydro-fluorescein diacetate, acetyl ester, and the fluorescent calcium indicator Fluo-4, respectively (Invitrogen, Molecular Probes), as previously described.[10]

PS and modified CD47 exposure on RBCs: MP analysis

PS and modified CD47 exposure on RBCs was estimated by multicolor flow cytometry using phycoerythrin-annexin V apoptosis kit, fluorescein isothiocyanate (FITC)-conjugated anti-CD235 (BD Biosciences), and conformation-dependent anti-CD47 (Clone 2D3, eBioscience). Isotype-matched FITC-conjugated antibodies were used as controls. Flow cytometry analysis of circulating MPs was performed after a double 2500 $\times$ *g* spin of citrated blood at 20°C, within 15 minutes of venipuncture.[21] MPs were identified by size (<1 μm) and PS exposure (PS^+). To define the MPs gate, Megamix fluorescent beads (Biocytex, Marseille, France) were used in accordance to the International Society on Thrombosis and Hemostasis SSC Collaborative workshop recommendations.[22] TruCount bead tubes (BD Biosciences) were used to calculate the absolute MPs count. The MP-associated procoagulant activity (MP-PA) was quantified by a colorimetric ELISA kit (Zymuphen, Hyphen BioMed).

Electron microscopy

RBCs were fixed with 2% glutaraldehyde, postfixed with 1% osmium tetroxide (Serva), dehydrated in ascending ethanol series, and examined in a microscope (SEM515, Philips) after coating with gold-palladium (Samsputter-2a, Tousimis). RBC shape classification was performed as previously suggested.[23] Spherocytes and other degenerative changes in RBC shape were characterized as nonreversibly transformed RBCs.[24]

Immunoblotting of RBC membrane proteins

RBCs were isolated by the method of Beutler and colleagues.[25] After membranes were isolated, proteins were loaded in Laemmli gels, transferred to nitrocellulose membranes, and immune probed for various proteins by using horseradish peroxidase (HRP)-conjugated secondary antibodies and ECL development, as previously described.[10] The relative membrane expression of each component was estimated by scanning densitometry. Antibodies against Band 3 (B-9277) and spectrin (S-1515) as well as HRP-conjugated secondary antibodies were obtained from Sigma Aldrich. Antibodies against Hb (CR8000GAP) and flotillin-2 (610384) were from Europa Bioproducts and BD Transduction Laboratories, respectively. Primary antibodies against HSP70 (sc-1060R), calpain-1 (μ-calpain, sc-7531), and clusterin-α (secretory apolipoprotein J, CLU, sc-6419) were from Santa Cruz Biotechnology. Antibodies against peroxiredoxin-2 (Prx2, SP5464) and glucose transporter 1 (AP10084PU) were from Acris GmbH. Antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, MAB0694) was obtained from Abnova. HRP-conjugated antibodies to rabbit IgGs and ECL Western blot detection kit were from GE Healthcare. HRP-conjugated antibodies to mouse IgGs were from DakoCytomation. Chemiluminescent reagent (Western Lightning Plus ECL) was from Perkin Elmer.

Statistical and network analysis

Each volunteer donated once; all biologic measurements were run in triplicate to rule out experimental bias or some random error. For statistical analysis, statistical software (Statistical Package for Social Sciences, IBM SPSS, Version 22.0, IBM Corp.; administrated by NKUA) was used. Time-course analysis was performed by using the repeated-measures analysis of variance (ANOVA). Intergroup differences were evaluated by one-way ANOVA and general linear models. A Bonferroni correction for multiple comparisons was used where needed. Prediction outcomes were estimated by regression analysis. After testing all variables for normal distribution profile (by using the Shapiro-Wilk test), Pearson's and Spearman's correlation tests were performed to assess the relationship between parameters. Significance was accepted at a p value of less than 0.05. Hematologic entities joined by significant correlation r at all the consecutive time points of storage were topologically represented in undirected biologic networks by using the Cytoscape version 3.2.0 application.[26] The length of each edge was inversely proportional to the r value (the shortest the edge, the higher r value).

RESULTS

Changes in numerous variables of RBCs are proportional to the duration of storage

Fresh blood analysis of the volunteers revealed a normal range variation in most of the laboratory measurements performed (Table S1, available as supporting information in the online version of this paper). Prestorage examination of cellular shape, fragilities, basal hemolysis and NOx concentration, release of extracellular vesicles, redox status, calcium homeostasis, RBC membrane proteome, and biologic markers of oxidative stress and erythrophagocytosis also revealed a normal range of variation among donors (Table 1).

Time-course evaluation of stored RBCs prepared by the corresponding individual donors revealed a progressive enrichment of the supernatant in free Hb, MPs, MPs-PA, and protein carbonyls but depletion in NOx, clusterin, and antioxidant capacity compared to basal donor levels (Fig. 1). A significant increase in the osmotic (MCF) and mechanical (MFI) fragility of stored RBCs was observed from Day 21 onward. Calcium and ROS (including those induced after tert-Butyl hydroperoxide stimulation) accumulated inside RBCs while PS externalized on cell surface, in parallel with an increase in nonreversible shape modifications (mainly spheroechinocytes) at the expense of discocytes. Accumulation of oxidative (Prx2), senescence

TABLE 1. Biologic evaluation of donors (n = 10)

Variable	Value
Plasma characteristics	
Free Hb (mg/mL)	0.17 ± 0.03
NOx (μg/mL)	2.55 ± 1.27
TAC (μmol/L Fe^{2+})	758 ± 98
UA-dep AC (μmol/L Fe^{2+})	465 ± 78
UA-ind AC (μmol/L Fe^{2+})	287 ± 42
Protein carbonylation (nmol/mg)	0.10 ± 0.05
Clusterin (μg/mL)	11,649 ± 4,084
PS^+ RBC-derived MPs (counts/μL)	6,289 ± 2,648
MP-PA (nmol/L PS)	3.78 ± 1.51
RBC characteristics	
PS^+ (%)	0.27 ± 0.11
MCF (% NaCl)	0.45 ± 0.04
MFI (%)	0.60 ± 0.16
Discocytes (%)	76.9 ± 4.0
Reversible shape modifications (%)	21.1 ± 4.1
Nonreversible shape modifications (%)	2.80 ± 0.30
ROS (RFU)	303 ± 34
tBHP-ROS (RFU)	615 ± 123
GSH (μmol/L)	670 ± 106
Calcium (RFU)	3,070 ± 320
RBC membrane proteins*	
Spectrin	1.36 ± 0.08
Adducin	1.09 ± 0.27
Band 3	5.39 ± 0.35
GAPDH	0.29 ± 0.07
Glucose transporter 1	4.09 ± 1.97
Flotillin 2	1.64 ± 0.30
Clusterin	0.54 ± 0.25
Hsp70	0.46 ± 0.25
Calpain	0.40 ± 0.17
Spectrin proteolysis (% of spectrin)	2.41 ± 0.36
Prx2 oligomers	1.44 ± 0.40
Band 3 oligomers (% of Band 3)	1.34 ± 0.25
Band 3 proteolysis (% of Band 3)	0.81 ± 0.24

* RBC membrane protein densitometry results are presented as mean ± SD after normalization to protein 4.1R values. Row data for the other variables are presented as mean ± SD.
tBHP = tert-Butyl hydroperoxide.

(spectrin-Hb complexation, Sp-Hb), metabolic (GAPDH), calcium (calpain), and proteome (heat shock protein 70 [Hsp70] and Band 3 modifications) stress markers was detected on the membrane of stored RBCs compared to nonstored cells. On the other hand we observed a trend for lower membrane expression of glucose transporter 1 and stomatin in stored RBCs, compared to fresh blood (Figs. 1 and 2).

Variables exhibiting high interdonor variation before and during storage

Plasma NOx, protein carbonylation, clusterin, and MP concentration, as well as membrane expression of calpain, GLUT-1, Hsp70, and Sp-Hb complexation, showed the widest interdonor variation in fresh blood, exhibiting wide dispersion of their values compared to the mean value (Fig. 3). In fact, interdonor variation in plasma protein carbonylation, MP concentration, and membrane expression of stomatin was higher in fresh blood than in RBCs. In contrast, variation in supernatant NOx, hemolysis, clusterin, and membrane-bound Prx2 significantly increased during storage compared to the donors' basal levels. Prx-2 and CD47 protein that has been modified to an "eat-me" signal conformation[27] and detected by flow cytometry after using a conformation-specific antibody exhibited the widest interdonor variation among membrane protein components at the end of storage (Figs. 2 and 3).

Prestorage versus storage variation relationships are evident

Despite the above-mentioned complexity, several hematologic and biologic variables changed throughout the storage period, in a manner proportional to their prestorage or Day 2 values, as evidenced by the repeatable correlations that were observed between them at all the consecutive time points of storage ("intravariable" correlations, Table 2). With the exception of PS exposure and Ca^{2+} accumulation, regression analysis using independent variables of the donors (before storage) produced significant models for the prediction of the same dependent variables during storage (adjusted R^2 range, 61%-99%; Fig. 4). Moreover, significant intervariable correlations between prestorage and storage variables were also detected. For instance, mean cell (RBC) Hb concentration (MCHC) of stored RBCs was negatively correlated with ROS accumulation and PS exposure on fresh RBCs.

The significant correlations that had been detected at all the consecutive time points of the storage period were integrated into biologic networks like the representative ones shown in Figs. 5A and 6 for Day 42 of storage. The "fresh-versus-stored" blood network (Fig. 5A) was characterized by hub nodes such as the MCHC and osmotic fragility (MCF) of the stored cells. Donor variation in plasma clusterin, RBC shape, PS exposure, HbA_2, and mainly, HbF concentration was strongly correlated with interdonor variability in numerous properties of stored RBCs and supernatant. For instance, the percentage of irreversible RBC shape modifications prestorage (NR [nonreversibly] in Fig. 5A) for each donor, was positively correlated with the percentage of PS^+-stored RBCs but negatively correlated with the osmotic fragility, the volume, and the Hb concentration of the stored RBCs throughout the whole storage period. Multivariate analysis showed a significant reduction in supernatant TAC but also in stored RBC osmotic fragility (by 25 and 17%, respectively) in units prepared from donors bearing detectable levels of HbF ($0.76 \pm 0.31\%$, n = 4; and 2.1% for the β-thalassemia minor donor) compared to the HbF-negative ones (n = 5) throughout the storage period ($p < 0.05$, power = 0.906), after including or excluding the β-thalassemia minor donor from the analysis. By regression analysis, the osmotic fragility (MCF index) of the stored cells can be predicted throughout the storage period by using the individual donors' HbF levels as an independent variant (Fig. 5B).

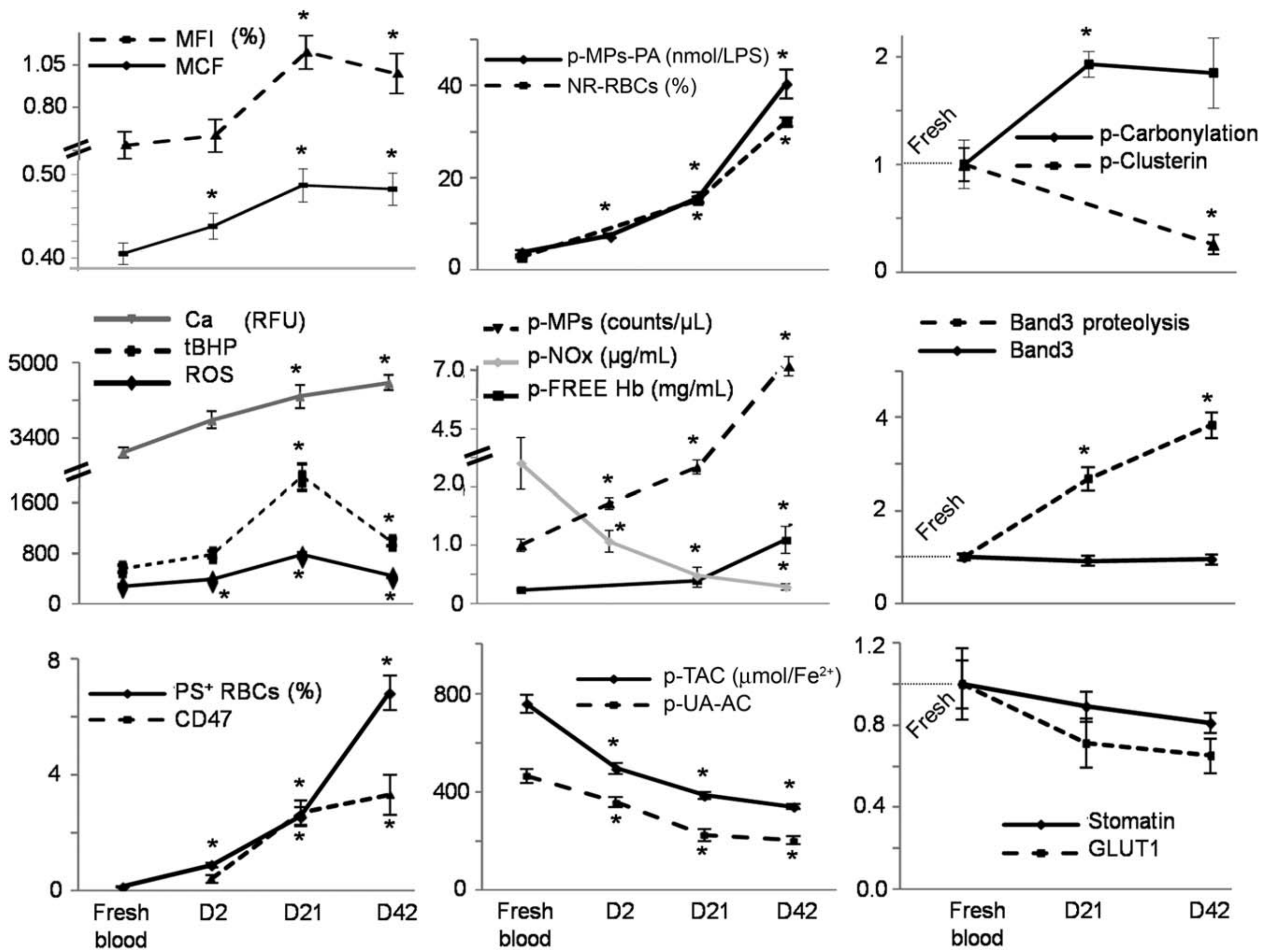


Fig. 1. Time-course fluctuation of RBC and plasma variables throughout storage compared to basal donors' levels (n = 10). Plasma (p) protein carbonylation, clusterin, and MP concentration in addition to membrane protein expression values are normalized to prestorage (basal) levels. *Storage versus fresh blood $p < 0.05$; error bars = standard error of the mean; D2-D42 = days of storage. UA-AC = UA-specific antioxidant capacity; GLUT1 = glucose transporter 1.

DISCUSSION

A tremendous variability in the storability and in vivo recovery of blood from different donors has been noticed since 1966.[4,28] Many physiologic properties of stored RBCs including in-bag hemolysis[29] and oxygen transport rate[30] exhibit strong donor dependence. Ascertainment of the correlation between RBC storage lesion and donor-related factors constitute a major problem in blood banking.[3] The introduction of unknown and highly variable coefficients in the study of RBC storage hinders its development by limiting the objective evaluation of data provided by laboratory and clinical studies. More importantly, variation in the quality of individual RBCs suggests the incidental delivery of low-quality and probably high posttransfusion risk units to multiply transfused or sustained blunt trauma patients and infants, when more appropriate units might be available. This paired fresh-versus-stored RBC study aimed at providing a deeper insight into the donor variation effect on storage lesion development and at characterizing storage lesion factors that are functionally related at a significant level, with the hematologic and biologic profiles of individual donors.

Regular blood donors represent a highly heterogeneous source of RBCs, mainly due to intrinsic differences in blood homeostasis, which influence RBC properties. Inherent differences among donors might affect the progression of the RBC storage lesion in individual units[31] as well as the posttransfusion recovery,[32] in the case of endpoint modifications, such as PS exposure. Hemolysis, RBC shape, fragility, aging-related variables, metabolism, and redox homeostasis are severely affected by the storage system, as shown by the current and numerous studies in the past.[2,10,13,33-36] Indeed, the RBC units under examination exhibited considerable fluctuation in several storage

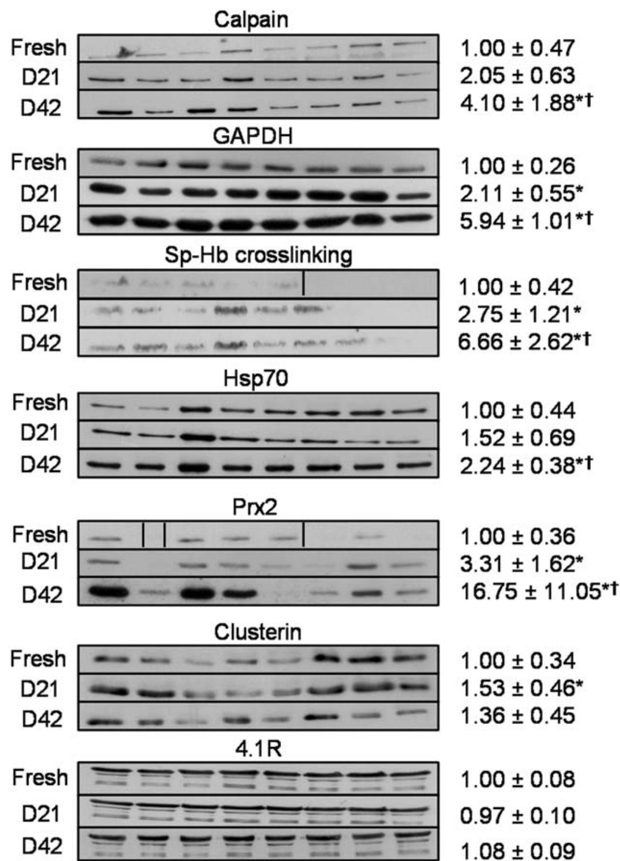


Fig. 2. Representative immunoblots and scanning densitometry results for membrane expression of proteins exhibiting high interdonor variability (n = 8 donors shown). Vertical lines represent results from different blots. $p < 0.05$ storage vs. fresh blood (*) or vs. a previous time point of the storage period (†); D21-D42 = days of storage.

lesion hallmarks in response to the duration of storage, suggesting that stored RBCs degrade over time through more than one mechanism of cellular injury.[10] The antioxidant effectiveness of the units (evaluated by TAC, carbonylation, ROS, etc.), the NO-scavenging potential (through free Hb and MPs[37]), spheroechinocytic shape modification, and the expression of senescent and removal signals (PS, Band 3, and CD47 [27] modifications, etc.) are significantly affected by storage in the examined donors.

In several cases, the variation profile is affected either by storage per se or by the duration of storage. To explain these findings, we must consider that RBC homeostasis exhibited substantial differences in vivo compared to the ex vivo state. Cellular aging represents the most characteristic example of this fact.[1,2] As a result, plasma NOx, clusterin, and membrane-bound Prx2[9,10] varied more among donors in RBCs than in fresh blood, suggesting substantial donor-to-donor differences in hemolytic, oxidative, and cellular aging effects of storage. In contrast, circulating PS^+ MPs varied significantly among donors, in agreement with previous reports,[38] but less during storage, while in-bag hemolysis, another storage time–dependent factor, exhibited higher donor-to-donor variation after prolonged storage. Osmotic and mechanical fragility showed the anticipated storage time and donor dependence;[17,34] however, the variation of MFI was threefold higher than the variation of MCF before and during storage. These data suggest that more factors and/or molecular pathways are involved in withstanding shear stress compared to those involved in the response to osmotic stress. Regarding the membrane protein reorganization, the membrane-bound cytosolic components (e.g., calpain, Prx2, Hsp70) exhibited high interdonor variation in contrast to the major structural components (e.g., Band 3, spectrin), suggesting that donors mainly differ from each other in the "management" of storage-associated stress. Notably, the sensitivity of RBCs to oxidation and calcium-associated stressors has been found considerably variable among donors and/or transfusion units.[39]

A significant finding of this study was that several basal interdonor differences are impressively maintained during storage, as previously reported for total Hb concentration[11] and GSH/GSSG content.[40] In our donors, not only the classic RBC indexes (mean cell [RBC] volume, MCH, MCHC, RBC distribution width), but also osmotic fragility, PS exposure, and intracellular accumulation of calcium, in addition to supernatant NOx, clusterin, and UA–dependent antioxidant capacity levels fluctuated in RBCs in proportion to their values in fresh blood. Storage affects these factors by a stable factor of magnitude, preserving thus the interdonor differences observed in their basal, prestorage levels. In contrast, MP accumulation in the supernatant was a function of Day 2 levels, suggesting that unknown donor-related factors affect the RBC susceptibility to release vesicles during the RBC unit preparation steps.

As mentioned, fragility, hemolysis, and other measurable storage lesion hallmarks represent the cumulative result of modifications in many functionally associated factors. Dinkla and colleagues[41] have recently showed a positive correlation between the percentage of PS-exposing RBCs and the plasma Hb concentration of the donor. In other studies,[9] donors exhibiting increased membrane binding of Prx2 in fresh RBCs showed high membrane lipoperoxidation levels in stored RBCs, suggesting that RBC oxidation markers in circulating blood may be predictive of the RBC susceptibility to oxidative storage lesions. In this study, we observed similar intervariable correlations, including that between PS exposure on fresh RBCs and MCHC of stored cells. Although these (not previously reported) correlations do not imply causation, they are physiologically relevant. For example, correlation between the percentage of spheroechinocytosis and

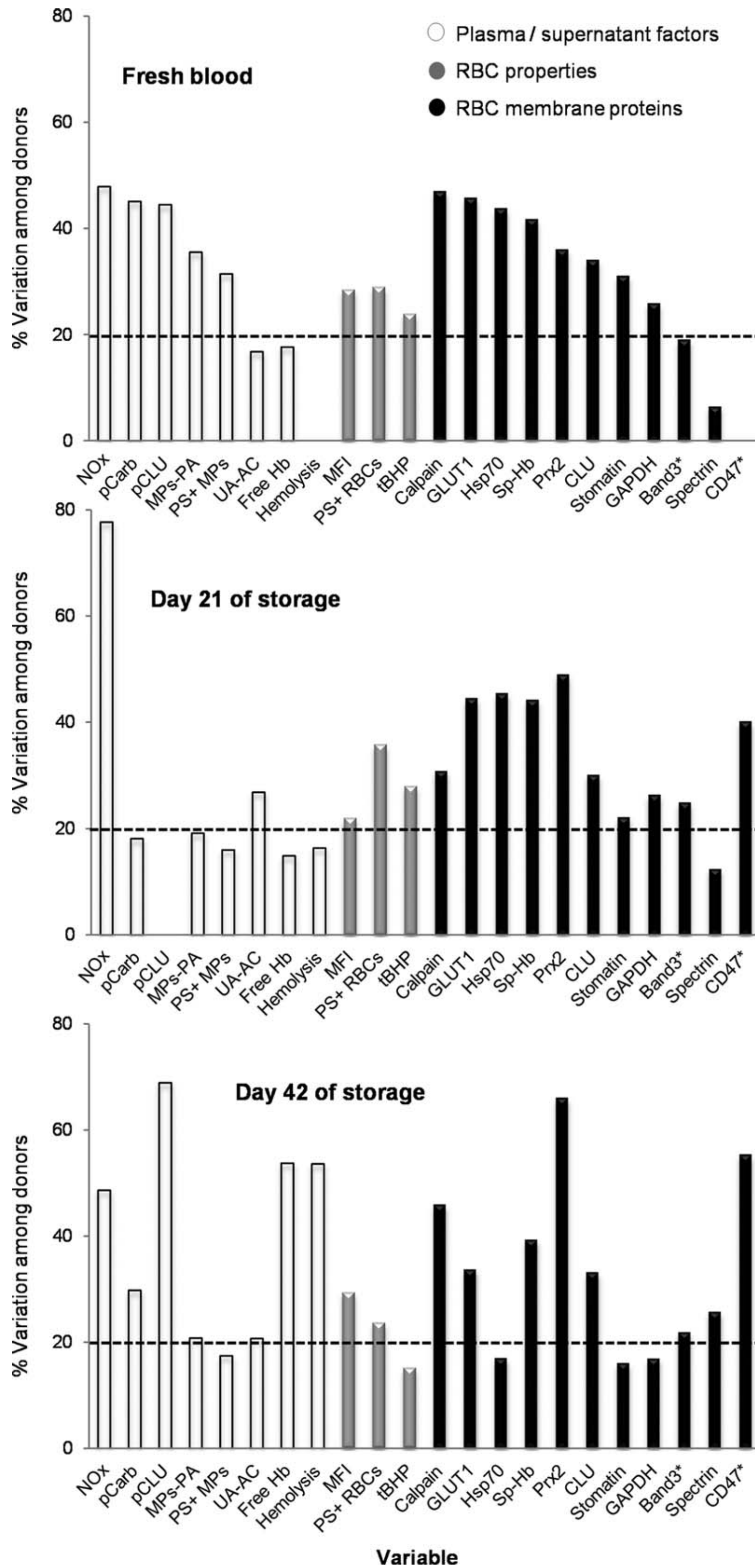


Fig. 3. RBC and plasma variables exhibiting high variation degree (>20% above or below the mean value, dashed horizontal line) among the regular blood donors under examination (n = 10). CD47* = structural conformation of CD47 protein present on senescent and oxidatively damaged RBCs; Band3* = modifications of Band 3 protein.

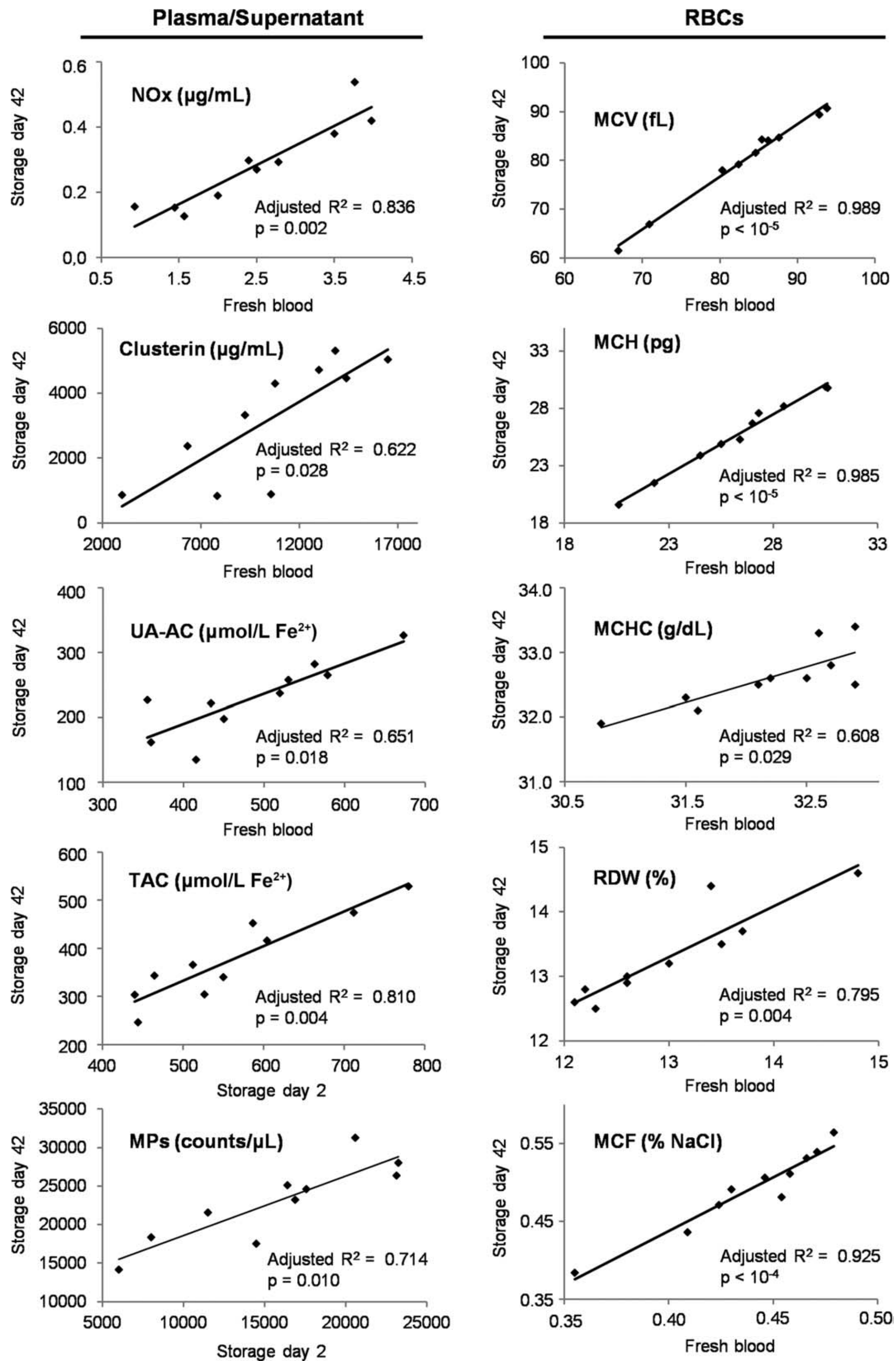


Fig. 4. Scatter plots representing significant regression models for the prediction of variables shown in Table 2, which exhibit intravariable correlations between fresh and stored blood. Although graphs for the last day of storage are shown, the same regression models (with slightly different r) exist between donors' basal levels and all the consecutive time points of storage, as shown in Table 2.

TABLE 2. Intravariable correlations between donors' variables (in fresh blood) and RBC data (n = 10)

Fresh blood	Day 2	Day 10	Day 21	Day 30	Day 42
Plasma/supernatant					
NOx	0.974†	ND	0.812*	ND	0.928†
Clusterin	ND	ND	ND	ND	0.880*
UA-AC	0.950†	0.844*	0.938†	0.830*	0.807*
TAC		0.913†	0.947†	0.826*	0.808*
MPs		ND	0.962†	0.812*	0.916†
RBC					
MCV	1.000†	0.999†	0.999†	0.995†	0.995†
MCH	0.993†	0.997†	0.996†	0.994†	0.994†
MCHC	0.845*	0.957†	0.885†	0.808*	0.808*
RDW	0.817*	0.894†	0.959†	0.907†	0.907†
MCF	0.979†	0.990†	0.985†	0.997†	0.969†
PS^+ RBCs	0.763*	0.807*	0.758*	ND	0.821*
Calcium	0.517	0.836*	0.838*	0.807*	0.827*

* $p < 0.050$.
† $p < 0.010$.
MCV = mean cell (RBC) volume; ND = not determined; RDW = RBC distribution width; UA-AC = UA-specific antioxidant capacity.

PS-exposing RBCs is compatible with the hypothesis that the same profile of membrane reorganization favors the exposure of PS and the generation of vesicles from the tips of the spheroechinocyte surface.[42] Circulating clusterin correlation with stored RBC indexes and PS exposure signify the role of this protein in RBC homeostasis.[43] Additionally, donors' HbF percentage (which varies by more than 10-fold in normal adults[44]) correlates with oxidative stress factors and cellular fragility. This finding is in accordance with the previously reported osmotic resistance[45] and susceptibility of HbF-enriched RBCs to eryptosis after oxidative stress.[46] Compared to HbA, HbF exhibits high rate of iron release, which might subsequently play a key role in the oxidative stress–driven signaling in stored RBCs.[47] Finally, the correlation observed among donors' serum UA and storage lesion hallmarks like spheroechinocytosis verified recent studies showing that UA could be a storability biomarker, either through a direct antioxidant function inside the stored unit and/or as an indicator of the donor's basal redox status.[31] Networks have long been used in biology to show relationships between biologically relevant elements.[48] Illustration of these correlations into a biologic network helps to unravel the complexity and reveal the "consistency" of the multivariable storage system in respect to the donor-specific blood profile. Apparently, the donor variation effect may be associated with donor-to-donor differences directed by genetic and/or lifestyle factors. Evaluation of similar correlations in concrete donor groups (e.g., β-thalassemia or glucose-6-phosphate dehydrogenase–deficient carriers,[5] smokers) might help us understand how RBC storage lesion proceeds as a function of those donor-related factors.

This study has certain limitations, mainly related to the relatively small size of the cohort involved (n = 10) and the lack of posttransfusion data. As for the first one, although higher number of blood units is always preferable, the size of this study was limited by the experimental design, which included paired measurements of fresh versus stored blood. Despite this limitation, it has been considered adequate to provide data of strong statistical power and high true discovery rate. To support this, the large effect size and power of the HbF model (along with the p value) verified the sufficiency of the sample size and the strong effect of HbF on the target variables that were tested. Moreover, the regression models between MCF and HbF exhibit p values of less than 0.005. Finally, only a subgroup of intra- and intervariable correlations were used to construct biologic networks, namely, those existing between fresh blood and all the consecutive time points of the storage period and, notably, the majority of these "connections" exhibited significance below a p value of 0.010. As for the second limitation, this hypothesis-generating study focuses on the effect of donor variation on RBC storage lesion and not on the effect of the storage attributes on clinically relevant outcomes. However, many of the studied variables, like hemolysis, PS exposure, or spheroechinocytosis, are strongly related to the survival or removal of stored RBCs after transfusion.

CONCLUDING REMARKS

Substantial donor-to-donor differences in how RBCs respond to storage and how many RBCs survive after transfusion may be associated with the hematologic profile of individual donors. Although complexity was introduced by donor variability in many storage lesion factors, RBC indexes, osmotic fragility, PS exposure, and other variables fluctuated over time in RBCs in proportion to their basal, prestorage values. Another group of storage lesion hallmarks correlated with donor characteristics such as HbF concentration. Construction of fresh-versus-stored blood biologic networks might provide deeper insight into the donor variation effect as a determinant of storage lesion, in combination with the duration of storage. Studying donor-related biomarkers of RBC storability in fresh blood may allow the selection of the most appropriate storage (e.g., additive solutions, leukoreduction) or transfusion strategy (early delivery to the patient, delivery after rejuvenation or wash, avoidance of specific patient groups, etc.) for the donated blood at the time of donation. Identifying donor-to-donor differences in RBC storability may enable the individualization of blood processing and transfusion by blood bank services to gain maximum benefit from each donation and offer optimum care to each patient.[49]

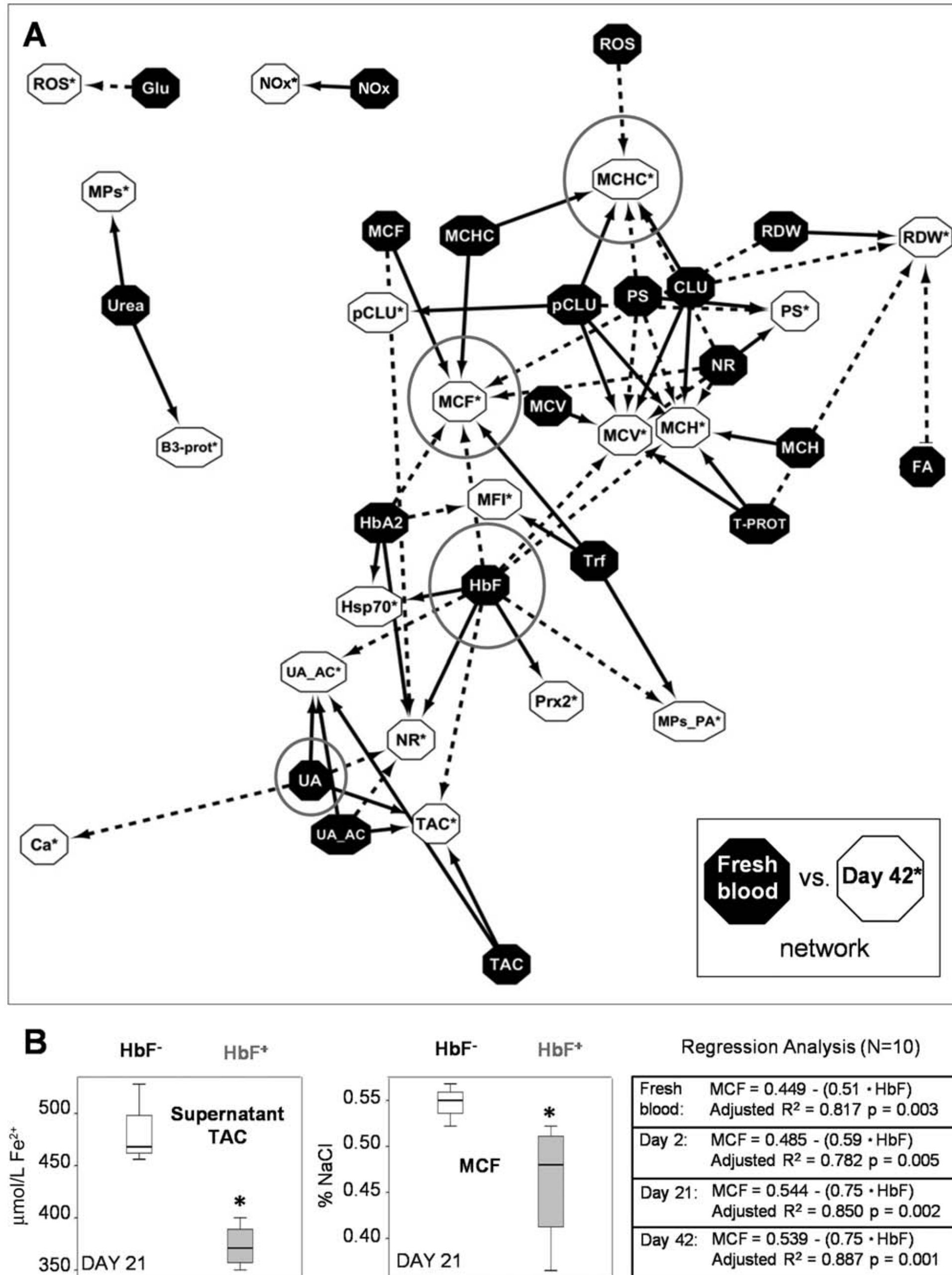


Fig. 5. (A) Network analysis connecting hematologic and biologic variables of regular blood donors (n = 10) in fresh blood (black hexagons) and RBCs (white hexagons*). The arrows represent significant ($p < 0.05$; $p < 0.01$ for approximately half of the connections) positive (continuous lines) and negative (dashed lines) correlations among factors (the shorter the edge, the higher r value). Although Day 42 measurements are shown, the same connections (with slightly different r) exist between donors' basal levels and all the consecutive time points of storage (Days 2, 10, 21, 30, and 42). Gray circles = variables of high connectivity. (B) Representative box plots showing the distribution of TAC (supernatant TAC) and RBC osmotic fragility (MCF) values in RBCs prepared by donors with detectable (HbF^{+}) or no detectable (HbF^{-}) levels of HbF, at the middle of the storage period. Regression analysis using individual donor's HbF percentage in fresh blood as an independent variable produced several significant models (equations) to predict the levels of osmotic fragility (MCF) of fresh and stored RBCs throughout the storage period.

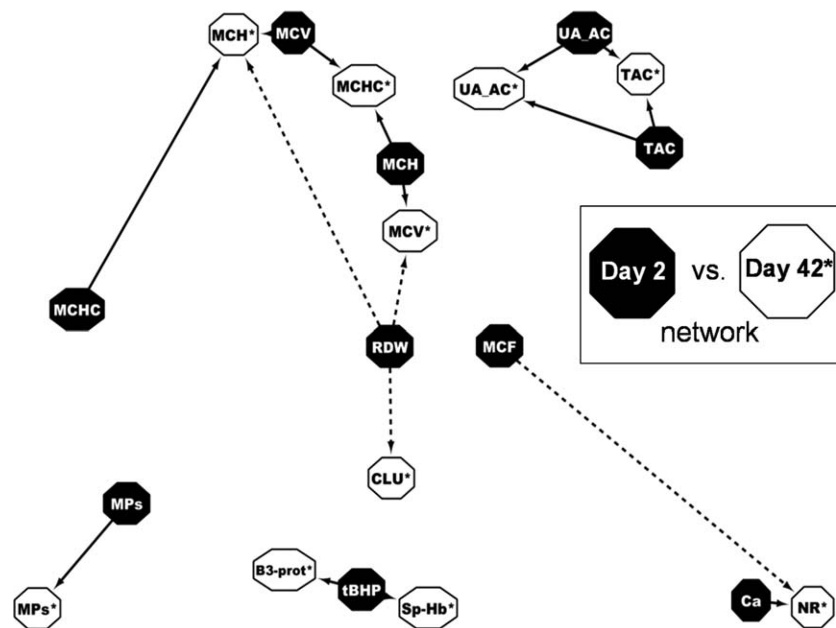


Fig. 6. RBC network showing the topologic arrangement of the significant correlations ($p < 0.05$, $p < 0.01$ for 58% of the connections) between hematologic and biologic variables measured on Day 2 (black hexagons) and Days 42 (white hexagons) of storage in $n = 10$ regular blood donors. The nodes shown are pairwise interconnected according to the correlation coefficient r value (the shorter the edge, the higher r value). The connections are repeatable and significant for each time point of storage (e.g., Day 2 vs. Days 10, 21, 30, and 42). Continuous and dashed lines = positive and negative correlations, respectively.

ACKNOWLEDGMENTS

The authors thank all blood donating volunteers; M.S. Jacovides Hellas S.A. for providing the LTRC blood bags; Prof. R. Prohaska (Institute of Medical Biochemistry, University of Vienna) and Prof. J. Delaunay (Laboratoire d'Hématologie, d'Immunologie et de Cytogénétique, Hopital de Bicetre) for providing antibodies against stomatin and protein 4.1R, respectively; and finally Dr T. Ntouroupi for the thorough editing of the manuscript.

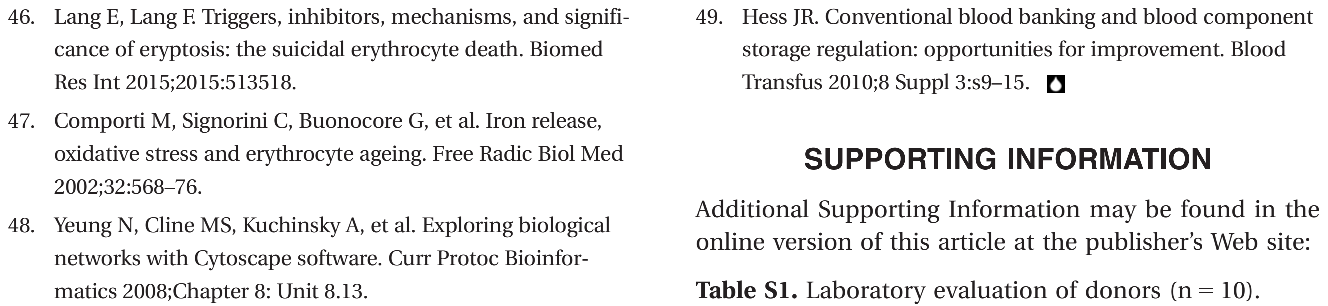
CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

REFERENCES

1. D'Alessandro A, Kriebardis AG, Rinalducci S, et al. An update on red blood cell storage lesions, as gleaned through biochemistry and omics technologies. Transfusion 2015;55: 205–19.
2. Bosman GJ, Werre JM, Willekens FL, et al. Erythrocyte ageing in vivo and in vitro: structural aspects and implications for transfusion. Transfus Med 2008;18:335–47.
3. Hess JR. Scientific problems in the regulation of red blood cell products. Transfusion 2012;52:1827–35.
4. Dern RJ, Gwinn RP, Wiorkowski JJ. Studies on the preservation of human blood. I. Variability in erythrocyte storage characteristics among healthy donors. J Lab Clin Med 1966; 67:955–65.
5. Shalev O, Manny N, Sharon R. Posttransfusional hemolysis in recipients of glucose-6-phosphate dehydrogenase-deficient erythrocytes. Vox Sang 1993;64:94–8.
6. Tarasev M, Alfano K, Chakraborty S, et al. Similar donors—similar blood? Transfusion 2014;54:933–41.
7. Roback JD. Vascular effects of the red blood cell storage lesion. Hematology Am Soc Hematol Educ Program 2011; 2011:475–9.
8. Rubin O, Crettaz D, Canellini G, et al. Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools. Vox Sang 2008;95:288–97.
9. Rinalducci S, D'Amici GM, Blasi B, et al. Peroxiredoxin-2 as a candidate biomarker to test oxidative stress levels of stored red blood cells under blood bank conditions. Transfusion 2011;51:1439–49.
10. Antonelou MH, Tzounakas VL, Velentzas AD, et al. Effects of pre-storage leukoreduction on stored red blood cells signaling: a time-course evaluation from shape to proteome. J Proteomics 2012;76 Spec No:220–38.
11. Agnihotri N, Pal L, Thakur M, et al. The need to label red blood cell units with their haemoglobin content: a single centre study on haemoglobin variations due to donor-related factors. Blood Transfus 2014;12:520–6.
12. Flatt JF, Bawazir WM, Bruce LJ. The involvement of cation leaks in the storage lesion of red blood cells. Front Physiol 2014;5:214.
13. D'Alessandro A, D'Amici GM, Vaglio S, et al. Time-course investigation of SAGM-stored leukocyte-filtered red bood

cell concentrates: from metabolism to proteomics. Haematologica 2012;97:107–15.
14. Harboe M. A method for determination of hemoglobin in plasma by near-ultraviolet spectrophotometry. Scand J Clin Lab Invest 1959;11:66–70.
15. Han V, Serrano K, Devine DV. A comparative study of common techniques used to measure haemolysis in stored red cell concentrates. Vox Sang 2010;98:116–23.
16. Kraus A, Roth HP, Kirchgessner M. Supplementation with vitamin C, vitamin E or beta-carotene influences osmotic fragility and oxidative damage of erythrocytes of zinc-deficient rats. J Nutr 1997;127:1290–6.
17. Raval JS, Waters JH, Seltsam A, et al. The use of the mechanical fragility test in evaluating sublethal RBC injury during storage. Vox Sang 2010;99:325–31.
18. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 1996;239:70–6.
19. Duplancic D, Kukoc-Modun L, Modun D, et al. Simple and rapid method for the determination of uric acid-independent antioxidant capacity. Molecules 2011;16:7058–68.
20. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 1969;27:502–22.
21. Lawrie AS, Harrison P, Cardigan RA, et al. The characterization and impact of microparticles on haemostasis within fresh-frozen plasma. Vox Sang 2008;95:197–204.
22. Lacroix R, Robert S, Poncelet P, et al. Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. J Thromb Haemost 2010;8:2571–4.
23. Reinhart WH, Chien S. Red cell rheology in stomatocyte-echinocyte transformation: roles of cell geometry and cell shape. Blood 1986;67:1110–8.
24. Berezina TL, Zaets SB, Morgan C, et al. Influence of storage on red blood cell rheological properties. J Surg Res 2002;102:6–12.
25. Beutler E, West C, Blume KG. The removal of leukocytes and platelets from whole blood. J Lab Clin Med 1976;88:328–33.
26. Assenov Y, Ramirez F, Schelhorn SE, et al. Computing topological parameters of biological networks. Bioinformatics 2008;24:282–4.
27. Burger P, Hilarius-Stokman P, de Korte D, et al. CD47 functions as a molecular switch for erythrocyte phagocytosis. Blood 2012;119:5512–21.
28. Dumont LJ, AuBuchon JP. Evaluation of proposed FDA criteria for the evaluation of radiolabeled red cell recovery trials. Transfusion 2008;48:1053–60.
29. Hess JR, Sparrow RL, van der Meer PF, et al. Red blood cell hemolysis during blood bank storage: using national quality management data to answer basic scientific questions. Transfusion 2009;49:2599–603.
30. Buchwald H, Menchaca HJ, Michalek VN, et al. Pilot study of oxygen transport rate of banked red blood cells. Vox Sang 2009;96:44–8.
31. Tzounakas VL, Georgatzakou HT, Kriebardis AG, et al. Uric acid variation among regular blood donors is indicative of red blood cell susceptibility to storage lesion markers: a new hypothesis tested. Transfusion 2015;55:2659–71.
32. Högman CF, Meryman HT. Storage parameters affecting red blood cell survival and function after transfusion. Transfus Med Rev 1999;13:275–96.
33. Eber SW, Pekrun A, Neufeldt A, et al. Prevalence of increased osmotic fragility of erythrocytes in German blood donors: screening using a modified glycerol lysis test. Ann Hematol 1992;64:88–92.
34. Almizraq R, Tchir JD, Holovati JL, et al. Storage of red blood cells affects membrane composition, microvesiculation, and In vitro quality. Transfusion 2013;53:2258–67.
35. Antonelou MH, Kriebardis AG, Stamoulis KE, et al. Red blood cell aging markers during storage in citrate-phosphate-dextrose-saline-adenine-glucose-mannitol. Transfusion 2010;50:376–89.
36. Józwik M, Szczypka M, Gajewska J, et al. Antioxidant defence of red blood cells and plasma in stored human blood. Clin Chim Acta 1997;267:129–42.
37. Donadee C, Raat NJ, Kanias T, et al. Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. Circulation 2011;124:465–76.
38. Bastos-Amador P, Royo F, Gonzalez E, et al. Proteomic analysis of microvesicles from plasma of healthy donors reveals high individual variability. J Proteomics 2012;75:3574–84.
39. Bosman GJ, Stappers M, Novotny VM. Changes in band 3 structure as determinants of erythrocyte integrity during storage and survival after transfusion. Blood Transfus 2010;8 Suppl 3:s48–52.
40. van 't Erve TJ, Doskey CM, Wagner BA, et al. Heritability of glutathione and related metabolites in stored red blood cells. Free Radic Biol Med 2014;76:107–13.
41. Dinkla S, Peppelman M, Van Der Raadt J, et al. Phosphatidylserine exposure on stored red blood cells as a parameter for donor-dependent variation in product quality. Blood Transfus 2014;12:204–9.
42. Salzer U, Zhu R, Luten M, et al. Vesicles generated during storage of red cells are rich in the lipid raft marker stomatin. Transfusion 2008;48:451–62.
43. Antonelou MH, Kriebardis AG, Stamoulis KE, et al. Apolipoprotein J/clusterin in human erythrocytes is involved in the molecular process of defected material disposal during vesiculation. PLoS One 2011;6:e26033.
44. Rochette J, Craig JE, Thein SL. Fetal hemoglobin levels in adults. Blood Rev 1994;8:213–24.
45. Gunn RB, Silvers DN, Rosse WF. Potassium permeability in β-thalassemia minor red blood cells. J Clin Invest 1972;51:1043–50.



46. Lang E, Lang F. Triggers, inhibitors, mechanisms, and significance of eryptosis: the suicidal erythrocyte death. Biomed Res Int 2015;2015:513518.
47. Comporti M, Signorini C, Buonocore G, et al. Iron release, oxidative stress and erythrocyte ageing. Free Radic Biol Med 2002;32:568–76.
48. Yeung N, Cline MS, Kuchinsky A, et al. Exploring biological networks with Cytoscape software. Curr Protoc Bioinformatics 2008;Chapter 8: Unit 8.13.
49. Hess JR. Conventional blood banking and blood component storage regulation: opportunities for improvement. Blood Transfus 2010;8 Suppl 3:s9–15.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's Web site:

Table S1. Laboratory evaluation of donors (n = 10).