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BRIEF REPORT

TRANSFUSION

# Osmotic hemolysis is a donor-specific feature of red blood cells under various storage conditions and genetic backgrounds

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## Abstract

**Background:** Transfusion research has recently focused on the discovery of red blood cell (RBC) storage capacity biomarkers and the elucidation of donor variation effects. This shift of focus can further strengthen personalization of transfusion therapy, by revealing probable links between donor biology, RBC storage lesion profile, and posttransfusion performance.

**Study design and methods:** We performed a paired correlation analysis of osmotic fragility in freshly drawn RBCs and during cold storage in different preservative solutions at weekly intervals until unit's expiration date ($n = 231$), or following 24 h reconstitution in allogeneic plasma ($n = 32$) from healthy controls or transfusion-dependent beta-thalassemia patients.

**Results:** We observed exceptional correlation profiles ($r > 0.700$, $p < 10^{-5}$ in most cases) of RBC osmotic fragility in the ensemble of samples, as well as in subgroups characterized by distinct genetic backgrounds (sex, beta-thalassemia traits, glucose-6-phosphate dehydrogenase deficiency) and storage strategies (additive solutions, whole blood, RBC concentrates). The mean corpuscular fragility (MCF) of fresh and stored RBCs at each storage time significantly correlated with the MCF of stored RBCs measured at all subsequent time points of the storage period (e.g., MCF values of storage day 21 correlated with those of storage days 28, 35 and 42). A similar correlation profile was also observed between the osmotic hemolysis of fresh/stored RBCs before and following *in vitro* reconstitution in plasma from healthy controls or beta-thalassemia patients.

**Conclusion:** Our findings highlighted the potential of osmotic fragility to serve as a donor-signature on RBCs at every step of any individual transfusion chain (donor, blood product, and probably, recipient).

**Abbreviations:** AS, additive solution; CPDA, citrate-phosphate-dextrose-adenine; CPD/SAGM, citrate-phosphate-dextrose/saline-adenine-glucose-mannitol; G6PD, glucose-6-phosphate dehydrogenase; MCF, mean corpuscular fragility; PAGGSM, phosphate-adenine-glucose-guanosine-saline-mannitol; RBC, red blood cell; WBC, white blood cell; WHO, world health organization.

  

KEYWORDS
blood component preparations, donors, RBC Transfusion



## 1 | INTRODUCTION

The recent randomized controlled trials,[1,2] which examined the effect of the age of stored blood upon clinical outcomes, set discussions about time-dependent storage lesions aside. In fact, they shifted the focus of the scientific community on the elucidation of biomarkers of optimum red blood cell (RBC) storage,[3] and consequently blood transfusion, as well as on the effect of blood collection/processing strategies and donor's characteristics on the transfusion chain (donor–blood product–recipient). Such a shift is in line with the observation that not all donors' RBCs are equal regarding their performance *in vitro*, even when stored for the same period.[4] In this context, the effects of both genetic (e.g., ethnicity, sex,[5] beta-thalassemia trait[6]) and nongenetic (smoking,[7] aging[5]) factors have been explored upon several hemolysis, redox, metabolic and physiological parameters of stored RBCs.

The first step toward personalized transfusion medicine was made by Karl Landsteiner with the discovery of the ABO antigens while later, other molecules were also added to the large list of blood groups. Nevertheless, several data indicate that even when compatible blood units are used some transfusion events might not have the desirable Hb increment or lead to undesirable clinical outcomes. Some examples of potentially unsafe donor–recipient pairs are smokers and pediatric patients,[8] rigid RBCs and thalassemia major recipients,[9] G6PD deficient RBCs and neonates or G6PD deficient patients[10] (also indicated at WHO guidelines for safe transfusion), as well as sex-mismatched RBC transfusions.[11] Therefore, transfusion medicine undergoes a period of research for both biomarkers of storage capacity and donor variation effects. Studying the latter might reveal probable links between donor biology, RBC storage lesion profile,[12] and posttransfusion performance[13] to enhance the personalized nature of transfusion. We hereby present a paired study highlighting that osmotic fragility is an intrinsic characteristic of the donor and a potential biomarker of storage quality.

## 2 | MATERIALS AND METHODS

### 2.1 | Blood processing and study planning

We performed a paired correlation analysis of osmotic fragility in freshly drawn and stored RBCs, or stored RBCs reconstituted in allogeneic plasma. For this purpose, blood samples from 231 eligible donors (with Hb >13.5 g/dl for men or >12.5 g/dl for women) collected into citrate vacutainers were analyzed before (freshly drawn RBCs) and during cold storage in the following conditions: non-leukodepleted RBC concentrates in citrate-phosphate-dextrose-adenine (CPDA-1) ($n = 117$), leukoreduced RBC concentrates in citrate-phosphate-dextrose/saline-adenine-glucose-mannitol (CPD/SAGM) ($n = 95$), and whole blood units in CPDA-1 ($n = 19$). In addition, stored RBCs were reconstituted in plasma from 32 subjects to examine fragility in conditions that mimic in part a prospective recipient's plasma, at least in terms of soluble components and body temperature, as previously described.[14] Briefly, RBCs stored for 2, 21, or 42 days in CPD-SAGM were incubated at 37°C, in 5% $CO_2$ for 24 h after reconstitution with allogeneic plasma from healthy controls ($n = 20$) or transfusion-dependent ($\beta$-thalassemia, $n = 12$) subjects. The mean corpuscular fragility (MCF) index (NaCl concentration responsible for 50% of hemolysis) was measured as previously described.[15] The study has been submitted and approved by the Research Bioethics and BioSecure Committee of the Department of Biology and of the Medical School, NKUA. Investigations were carried out in accordance with the principles of the Declaration of Helsinki.

### 2.2 | Statistical analysis

Correlations between MCF values were evaluated by the Pearson's test after testing the variables for normal distribution and the presence of outliers (Shapiro–Wilk and Kolmogorov–Smirnov tests and detrended normal Q–Q plots). Since Pearson's test is sensitive to outliers, such values were excluded and the analysis was performed again, to minimize the false discovery rate associated with the small size of our subgroups. If the outcome was not modified, the outlier was included back to the subgroup. Significance was accepted at $p < .01$ (unless otherwise stated).

## 3 | RESULTS

None of the units exceeded the European or US thresholds of end-of-storage hemolysis (0.8% and 1%, respectively). The osmotic fragility of freshly drawn RBCs demonstrated strong correlation with that of their stored

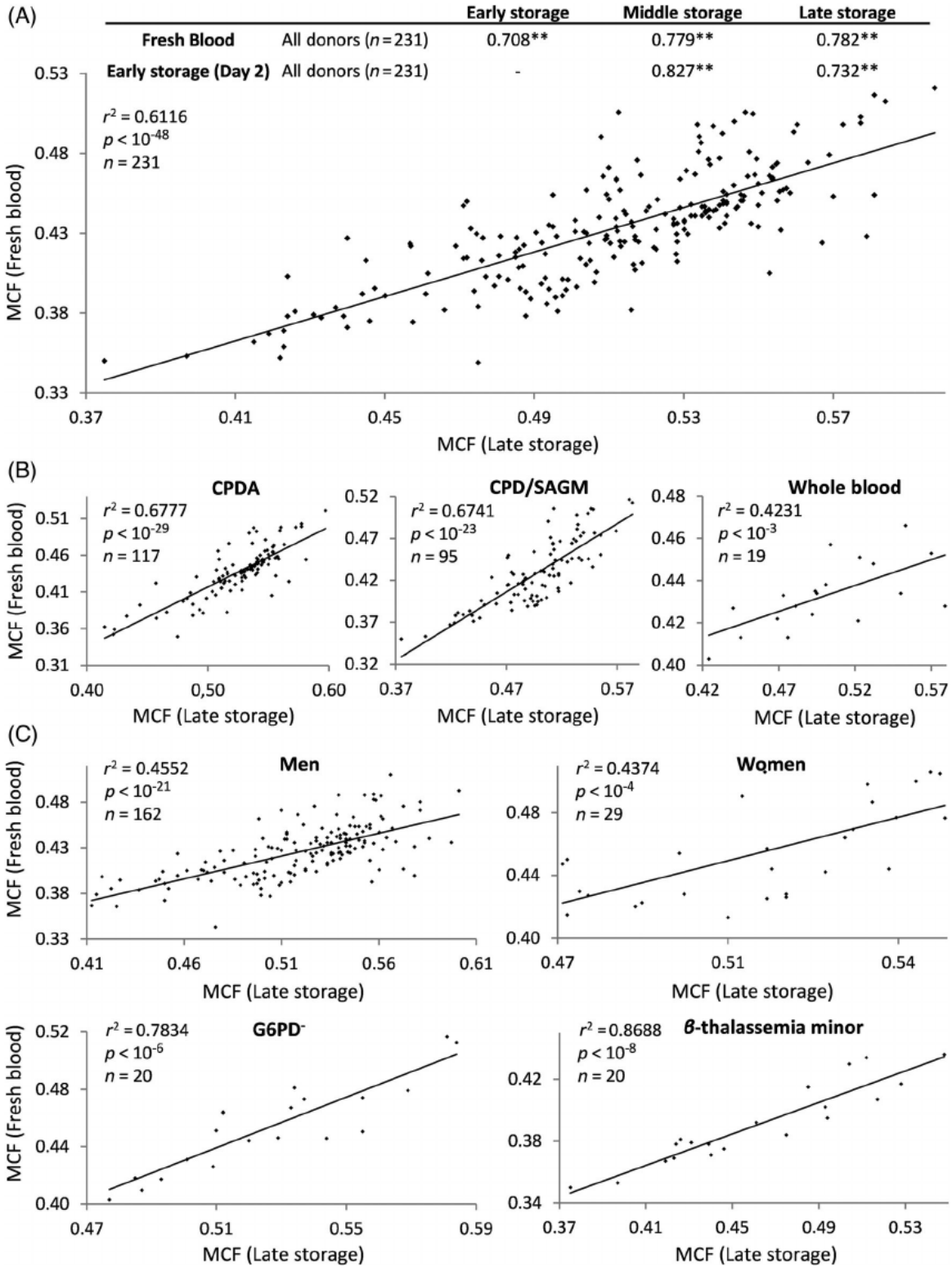


**FIGURE 1** Representative scatterplots for the correlation profile of osmotic fragility in fresh/stored red blood cells (RBCs). Only the correlations between late storage (day 42 for CPD/SAGM and day 35 for CPDA samples) and fresh blood measurements are shown for (A) all examined samples, (B) distinct storage conditions, and (C) donor's genetic background; however, similar significant correlations (with slightly different *r*) were detected throughout the storage period (days 2, 7, 14, 21, 28, 35, and 42, see Table 1). Table insert in panel A shows the repeatable correlation outcome (Pearson's *r* values) of mean corpuscular fragility (MCF) between freshly drawn or early stored RBCs and key time periods of storage. (**) $p \ll .01$

counterparts throughout storage (Figure 1A) in the ensemble of examined samples ($n = 231$). Since the total cohort consisted of distinct subgroups characterized by different storage strategies and donors' genetic background, we further examined if any of these subpopulations influenced the primary result. Secondary correlation analysis proved that this was not the case: MCF levels of fresh and long-stored RBCs significantly correlated with each other in all storage strategies and donor groups (Figure 1B, C). Furthermore, similar significant correlations were detected throughout the storage period, as detailed in Table 1 (part A, B). Of note, as high as 95% of the correlations tested, satisfied the criterion of Bonferroni-like corrections to cap the family wise error at 0.01 across the multiple Pearson analyses.

Considering that most blood bank services have only access to stored units and not to donors *per se*, we checked for the presence of similar MCF correlations during storage. The outcome supported that the osmotic fragility of RBCs at middle/late storage might be also "predicted" by the MCF levels of early stored RBCs (Figure 1A—insert Table). This observation was evident in all subgroups of the study (e.g. CPD/SAGM: day 2–21, $r = 0.870$, $p < 10^{-20}$ and Men: day 2–21, $r = 0.743$, $p < 10^{-20}$). To be more accurate, the MCF of any previous storage time point was directly proportional to that of upcoming ones (e.g. CPD/SAGM: day 14–35, $r = 0.859$; day 21–42, $r = 0.883$, $p < 10^{-20}$ for both).

To further assess the donor biomarker potential of MCF across the transfusion chain, reconstitution experiments were performed in conditions that partly mimic those of a recipient environment, namely interaction of RBCs with plasma soluble factors at body temperature. Despite the fact that heterologous plasma had either neutral or a mild beneficial effect on the osmotic hemolysis of stored RBCs (that equally varies in control vs. thalassemic samples, manuscript in preparation), interestingly, the MCF of freshly drawn, or early stored RBCs correlated well with their MCF measured once reconstituted in heterologous plasma from beta-thalassemia subjects and healthy controls (Table 1, part C). Once more, the MCF of middle or late stored RBCs can be used for predicting osmotic hemolysis post-mixing (e.g. control plasma: day 21 stored-reconstituted RBCs, $r = 0.735$, $p < .001$; $\beta$-thalassemia plasma: day 42 stored-reconstituted RBCs, $r = 0.944$, $p < 10^{-6}$).

**TABLE 1** Statistically significant correlations (Pearson's *r* values) of RBC osmotic fragility between freshly drawn (or early stored RBCs; storage day 2) and stored RBCs of different storage periods in subgroups stratified by storage condition, donor's genetic background, and health status of subjects who donated plasma for the reconstitution experiments

| | Storage day | | | | | | |
|---|---|---|---|---|---|---|---|
| **Subgroups** | **2** | **7** | **14** | **21** | **28** | **35** | **42** |
| A. Storage condition | | | | | | | |
| RBC-CPDA $n = 117$ | 0.846** | ND | ND | 0.911** | ND | 0.823** | ND |
| RBC-CPD/SAGM $n = 95$ | 0.708** | 0.767** | 0.745** | 0.812** | 0.703** | 0.747** | 0.821** |
| Whole blood $n = 19$ | 0.813** | ND | ND | 0.772** | ND | 0.651** | ND |
| B. Genetic background | | | | | | | |
| Men $n = 162$ or 26[a] | 0.709** | 0.562** | 0.623** | 0.893** | 0.632** | 0.466* | 0.675** |
| Women $n = 29$ | 0.703** | 0.717** | 0.749** | 0.732** | 0.603** | 0.647** | 0.661** |
| G6PD$^-$ $n = 20$ | 0.863** | 0.738** | 0.911** | 0.749** | 0.893** | 0.923** | 0.885** |
| -thalassemia minor $n = 20$ | 0.904** | 0.912** | 0.852** | 0.970** | 0.937** | 0.926** | 0.932** |
| C. Plasma from | | | | | | | |
| Control[b] $n = 20$ | 0.737** | ND | ND | 0.741** | ND | ND | 0.740** |
| | 0.686$^{\#\#}$ | ND | ND | 0.683$^{\#\#}$ | ND | ND | 0.692$^{\#\#}$ |
| -thalassemia[b] $n = 12$ | 0.887** | ND | ND | ND | ND | ND | 0.770** |
| | 0.778$^{\#\#}$ | ND | ND | ND | ND | ND | 0.679$^{\#}$ |

Abbreviations: CPDA: citrate-phosphate-dextrose-adenine; CPD/SAGM: citrate-phosphate-dextrose/saline-adenine-glucose-mannitol; ND, not determined; RBC, red blood cell.

**$p < .01$ versus freshly drawn RBCs. *$p < .05$ versus freshly drawn RBCs. $^{\#\#}p < .01$ versus RBCs stored for 2 days.

$^{\#}p < .05$ versus RBCs stored for 2 days.

[a]$n = 162$ samples only for days 2, 21, and 42 of storage.

[b]Stored RBCs reconstituted in plasma from controls or -thalassemia major patients.

Even though a single parameter is difficult to identify good from bad "storers", it is worth mentioning that in this cohort the lowermost MCF cluster of samples (approx. 10% of donors, carriers of beta-thalassemia traits) presented significantly lower ($p < .01$) storage hemolysis[6] compared with the upper high MCF cluster (approx. 10% of the samples, most of them non-leucoreduced RBC units in CPDA-1[15]).

## 4 | DISCUSSION

These findings suggest that osmotic hemolysis is an innate feature of donor biology under distinct genetic backgrounds that is conserved during storage regardless of the strategy followed. In fact, there was evidence for osmotic fragility as a measurement and predictor of storage lesion since the early 80s,[12] while its donor dependency was recently established by small-[16,17] and large-scale studies performed under the umbrella of REDS-III.[5,18] By using a cohort of different ethnic origin (Mediterranean subjects), donor genetic background (G6PD-deficiency, beta-thalassemia traits), and plasma from potential recipients with transfusion-dependent beta thalassemia (vs. control subjects) compared to the REDS-III, our study expands and validates the preceding findings.

Osmotic hemolysis, in contrast to storage hemolysis, demonstrates good reproducibility when tested in repeated donations of the same donors, highlighting the genetic dependency of RBC behavior in response to osmotic stress.[18] To support this, specific variants in ankyrin and spectrin genes have been found related to osmotic hemolysis *in vitro*.[19] On the opposite side, storage hemolysis, the gold quality standard of hypothermic preservation of RBCs, is a multivariable phenotype, related to a wide array of biochemical, metabolic, physiological, lifestyle, and hormone parameters,[7,20] including osmotic hemolysis.[5] We have previously estimated that osmotic hemolysis contributes by 9%–10% to the total free hemoglobin content of the blood unit,[15] a rather significant proportion considering the multiparametric nature of storage hemolysis. An interesting observation in our results is that, besides having significantly different levels of osmotic fragility, the strongest MCF correlation profiles were found in G6PD-deficient and beta-thalassemia minor donors, suggesting that these genetic factors restrain the storage-driven effects on osmotic hemolysis.[6,21] According to previously reported studies, MCF of stored RBCs constitutes a hub node exhibiting connections with several metabolic and physiological parameters associated with the G6PD-deficiency[20] or beta-thalassemia trait[6] status in freshly drawn RBCs. In the same context, the RBC concentrates exhibited stronger MCF correlation profiles compared with whole blood RBCs. This finding implies that apart from genetic factors, storage strategies and media that are associated with different levels of in-bag hematocrit, donor plasma, or a more heterogeneous cell population (including donor WBCs and platelets) enhance the storage-driven effects on RBC osmotic fragility.

There is little evidence directly linking variation in MCF among donors to the performance of RBCs posttransfusion. On one side, the osmotically resistant sickle-cell trait RBCs exhibit accelerated clearance in animal models of transfusion.[22] On the other side, cells susceptible to osmotic stress may be characterized by low deformability and increased levels of surface phosphatidylserine (a marker of erythrophagocytosis)[23,24] and as such, they might be prone to removal soon after transfusion. In this context, the results of this pilot study, showing that RBCs retain their osmotic hemolysis phenotype when exposed to both control and beta-thalassemic plasma at body temperature, suggest that the donor-related MCF of RBCs might be used to predict at least a part of nonimmune hemolysis in prospective recipients: the one driven by osmotic injuries of transfused RBCs. This possibility might be useful when treating transfusion-dependent individuals or patients with pathophysiological backgrounds related to osmotic stresses. Nonetheless, a limitation of our study is that the *in vitro* model used simulates only a small part of the complex posttransfusion environment, thus *in vivo* studies must be performed to assess the osmotic behavior of transfused RBCs in several recipient settings.

The osmotic fragility test is a technically simple and inexpensive laboratory assay. It provides clinicians with (a) the ability to predict the osmotic behavior of stored (and potentially of transfused) RBCs to choose the best-matched unit in respect to individual patient needs, and (b) the flexibility to do so at any time from blood donation to transfusion. However, since the quality of the RBC unit is affected by blood manufacturing methods,[25] more secondary analyses in other strategies and/or additive solutions (e.g. additive solutions AS1, AS3, phosphate-adenine-glucose-guanosine-saline-mannitol (PAGGSM)) are required to expand the "universal nature" and the biomarker potential of osmotic hemolysis.

Blood units represent the connecting link between blood donors and transfusion recipients in time and space. Although several markers have been proposed,[26-28] it is currently unknown which feature of stored blood (if any) will rise as potential biomarker of transfusion quality and whether we will ever be able to estimate the efficacy and effects of transfusion based on easily

accessible donor characteristics. We hereby present evidence that osmotic fragility represents a donor-signature on RBCs *at every step* of *any* individual transfusion chain, and an old-fashioned but still valuable tool for enhancing knowledge-based personalization of transfusion medicine.

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## CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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