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Frequent blood donations alter susceptibility of red blood cells to storage- and stress-induced hemolysis

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Abstract

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AUTHORSHIP CONTRIBUTION

T.K, M.P.B and A.E.M contributed to the design and execution of the study. T.K wrote the manuscript, developed the hemolysis assays, supervised Pittsburgh's testing lab, and performed data analyses and interpretation. A.E.M developed the study protocols, data interpretation and edited and finalized this manuscript. M.P.B and S.K developed the study protocols, supervised donor recruitment and blood center and testing laboratory activities, participated in data analyses and interpretation, edited and finalized this manuscript. G.P.P and Y.G designed and performed the statistical analyses of data from this study. M.C.L and M.S developed the study's protocols including quality assurance of the hemolysis assays, supervised the testing labs at BSRI, and performed data analysis. B.R.S and R.C prepared and coordinated key protocol sections of this study, participated in writing group and reviewed data and manuscript drafts. M.T.G, D.J.T, and J.E.K, participated in the design and execution of the study, data interpretation and editorial input in the manuscript. S.M.E-D assisted with oversight of data coordination from all participating sites. E.L.M supervised collection of data and helped editing the manuscript.

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Background—Frequent whole blood donations increase the prevalence of iron depletion in blood donors, which may subsequently interfere with normal erythropoiesis. The purpose of this study was to evaluate the associations between donation frequency and red blood cell (RBC) storage stability in a racial-ethnically diverse population of blood donors.

Study design—Leukocyte-reduced RBC concentrate-derived samples from 13,403 donors were stored for 39 to 42 days (1–6°C) and then evaluated for storage, osmotic, and oxidative hemolysis. Iron status was evaluated by plasma ferritin measurement and self-reported intake of iron supplements. Donation history in the prior 2 years was obtained for each subject.

Results—Frequent blood donors enrolled in this study were likely to be white, male, and of older age (56.1±5.0 years). Prior donation intensity was negatively associated with oxidative hemolysis ($p<0.0001$) in multivariate analyses correcting for age, sex and race-ethnicity. Increased plasma ferritin concentration was associated with increased RBC susceptibility to each of the three measures of hemolysis ($p<0.0001$ for all), whereas self-reported iron intake was associated with reduced susceptibility to osmotic and oxidative hemolysis ($p<0.0001$ for both).

Conclusions—Frequent blood donations may alter the quality of blood components by modulating RBC predisposition to hemolysis. RBCs collected from frequent donors with low ferritin have altered susceptibility to hemolysis. Thus, frequent donation and associated iron loss may alter the quality of stored RBC components collected from iron deficient donors. Further investigation is necessary to assess post-transfusion safety and efficacy in patients receiving these RBC products.

INTRODUCTION

Biologic heterogeneity in whole blood donors may contribute to differences in the quality of blood components including packed red blood cell (RBC) units for transfusion.[1–3] We have recently demonstrated that genetic and biologic variables, such as race-ethnicity, sex or age, can significantly modulate RBC predisposition to hemolysis during routine blood banking cold storage, and in response to osmotic or oxidative stress.[4] In addition to genetic polymorphisms, behavioral and other non-genetic factors may contribute to donor-specific differences in RBC characteristics and subsequently to variation in storage stability. For example, repeated phlebotomy in frequent blood donors may severely deplete iron stores and subsequently interfere with normal erythropoiesis.[5,6] Recent investigations of frequent blood donors have focused primarily on preventative care against iron deficiency anemia[7–12], with limited understanding regarding the impact of frequent donations on RBC characteristics and resilience to cold storage.

Various lab tests used to diagnose iron deficiency anemia have identified iron depletion in frequent blood donors with substantial changes in RBC phenotype, such as the formation of RBCs with low mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV).[13] However, certain donors exhibit exceptional recovery in response to frequent donations with no apparent impact on RBC indices. These “super donors”[14] may donate whole blood every 56 days, the allowed minimum interval for blood donations in the U.S.A, for several years without deferral for low hemoglobin or hematocrit.[15] Although the impact of intensive donations on RBC storage stability and transfusion efficacy has not been

established in humans, a comparable study in mice has associated frequent phlebotomy with iron-deficient erythropoiesis and reduced 24-h post transfusion recovery of stored murine RBCs.[16]

In the present study, we evaluated the associations between frequent whole-blood donations and RBC predisposition to hemolysis in 13,403 blood donors from the National Heart, Lung and Blood Institute (NHLBI) Recipient Epidemiology Donor Evaluation Study (REDS)-III Red Blood Cell-Omics (RBC-Omics) study. An overarching goal of the RBC-Omics Study was to define genetic, biochemical, behavioral and metabolic bases for donor-specific differences in RBC storage stability and iron metabolism.[4] We report evaluations of donor demographics associated with frequent blood donations, and quantify the impact of prior donations with or without iron intake on three measures of hemolysis, including spontaneous end-of-storage hemolysis and responses of stored RBCs to osmotic and oxidative stress.

MATERIALS AND METHODS

Protection of human subjects:

RBC-Omics was conducted under regulations applicable to all human subject research supported by federal agencies. The Data Coordinating Center (DCC, RTI International, Rockville, MD) of REDS-III supervised the overall compliance of human subjects regulatory protocols including Institutional Review Board approval from all participating blood centers, testing labs and the DCC.

RBC-Omics donor recruitment:

Donor recruitment and testing occurred between December 2013 and December 2015 at four large blood centers: the American Red Cross (ARC, Farmington, CT), the Institute for Transfusion Medicine (ITxM, Pittsburgh, PA), Blood Center of Wisconsin (BCW, Milwaukee, WI), and Blood Centers of the Pacific (BCP, San Francisco, CA). Overall, 97% (13,403) of the whole blood donations provided by 13,770 participant donors ≥18 years of age who provided informed consent were fully evaluable for plasma ferritin levels and measures of hemolysis. Donors were categorized into self-reported racial/ethnic groups: non-Hispanic White, Hispanic White, non-Hispanic African American, non-Hispanic Asian. Donors with multiple races, Hawaiian American, Native American, and other donors were grouped as “Other”. In addition, we recruited a group of 1976 high-intensity donors, who met the specific criteria of 9 or more successful blood donations in the prior 24-months without a low hemoglobin deferral. The overall study design is illustrated in Figure 1.

Demographic data, such as ethnicity and sex were collected directly from enrollment interviews and recorded in the RBC-Omics Study Management System (SMS) database. Additional demographic data, including weight, height, and the date of birth, as well as the donation history were derived from the blood centers’ routine donor/donation databases and linked through donor ID, donation date, and donation identification number (DIN). Donor age at time of the enrollment donation was derived by calculating the difference between enrollment date and donor date of birth. A Biological Specimen Inventory (BSI) was used to track biospecimens.

Assessment of iron supplements intake:

Consumption of iron supplements in RBC-Omics was assessed by a questionnaire, for which blood donors were asked to report whether they had taken any multiple vitamins with iron or other iron supplements in the past 30 days, the frequency of iron-containing supplements intake (e.g. daily, weekly), and whether they had increased their iron intake after blood donation.

Blood components:

Whole blood units collected at participating blood centers were processed according to each center's standard operating procedures. An aliquot of whole blood was collected into 10mL EDTA retention tubes for the determination of complete blood count (CBC) using automatic cell counters, and for plasma ferritin levels (ng/mL) determined by batch testing of frozen plasma samples using a quantitative latex agglutination assay on the Beckman Coulter AU680 Chemistry System (Beckman Coulter, Sacramento, CA). Each whole blood unit was filtered to generate a leukocyte-reduced packed RBC (LR-pRBC) unit in additive solution-1 or 3 (AS-1 or AS-3). A representative portion (10–15mL) of RBCs from each LR-pRBC unit was then sterile transferred into a customized transfer bag (Haemonetics, Braintree, MA) created specifically for this study. These transfer bags were made from the same materials as the parent RBC storage bag. Validation studies demonstrated strong correlations in storage outcomes between the parent and the transfer bags.[17] The LR-pRBC parent units were released for distribution for transfusion, whereas the transfer bags were sent to RBC-Omics testing labs (University of Pittsburgh, Pittsburgh, PA, and Blood Systems Research Institute, San Francisco, CA).

RBC hemolysis assays:

All transfer bags were stored under routine blood bank conditions (1–6°C) for 39–42 days, after which the content of each bag was transferred into a 15mL conical tube and two aliquots (1mL) were processed immediately for the hemolytic assays. One aliquot was used for the quantification of spontaneous storage hemolysis and the other for the stress-induced hemolysis assays as described before.[4] Percent end-of-storage hemolysis was determined according to the following equation:

$$\text{Storage hemolysis}(\%) = \frac{(100 - HCT) \times Hb_{supernatant}}{Hb_{total}}$$

Sample hematocrit (HCT) was determined by collecting blood samples into capillary tubes, which were centrifuged in a micro-HCT centrifuge (LW Scientific, Lawrenceville, GA). $Hb_{supernatant}$ refers to the levels of free hemoglobin obtained after centrifugation (1500x g, 10min, 18°C) measured in the supernatant. Hb_{total} refers to the total amount of sample hemoglobin before centrifugation. In the entire study, hemoglobin concentrations (micromolar) were determined by the Drabkin's method.[18]

For the evaluation of stress-induced hemolysis, stored RBCs were washed (1500x g, 10min, 18°C) three times with phosphate-buffered saline (PBS) to remove plasma and additive solution, and immediately subjected to osmotic or oxidative stress assays.

RBC osmotic hemolysis: Washed RBCs were incubated under static conditions (4h at 22°C) in pink test buffer[19] at a final concentration of 1.6%±0.2% after which samples were centrifuged (1500x g, 10min, 18°C), and percent osmotic hemolysis was determined: $\text{Osmotic hemolysis}(\%) = \frac{Hb_{osmotic}}{Hb_{total}} \times 100$, for which $Hb_{osmotic}$ corresponds to supernatant cell-free hemoglobin of pink test-treated RBCs and Hb_{total} refers to the total amount of hemoglobin of each sample.

RBC oxidative hemolysis: RBC susceptibility to oxidative hemolysis was evaluated by incubating RBCs in the presence of 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH, 150mmoL). Thermal (37°C) decomposition of AAPH generates peroxyl radicals leading to lipid peroxidation–mediated hemolysis.[20] AAPH-induced oxidative hemolysis was determined by: $\text{Oxidative hemolysis}(\%) = \frac{Hb_{AAPH} - Hb_{control}}{Hb_{total}} \times 100$, where Hb_{AAPH} corresponds to supernatant cell-free hemoglobin of AAPH-treated RBCs, $Hb_{control}$ corresponds to supernatant cell-free hemoglobin of untreated RBCs, and Hb_{total} refers to the total amount of hemoglobin of each sample.

Statistical analyses:

Descriptive Statistics: To account for center-specific difference in RBC production procedures between hubs, hemolysis measures were adjusted for site specific effects as in the paper in Kanias et al.[4] Mean storage, osmotic, and oxidative hemolysis, along with standard errors of the mean estimations were estimated stratifying by donation history and sex (Figure 3), by donation history and age (Figure 4), by donation history, sex, and iron intake (Figure 5), and by donation history and race-ethnicity group (Supplemental Figure 1) using R statistical software version 3.3.1.[21] Univariate analyses were conducted to compare the mean hemolysis and ferritin differences between first-time/reactivated donors to each of the donation frequency bins for male and female (Figure 3), and for young donors (< 21 years old; Figure 4). Univariate analyses were also conducted to compare the mean hemolysis and ferritin differences affected by iron intake stratified by donation history and sex (Figure 5). Asterisks represent statistically significant differences in mean hemolysis and ferritin ($p < 0.001$). However, since univariate statistical analyses does not account for the effect of other covariates, we report only p-values from the multivariate analysis throughout the manuscript. Visuals and graphs were produced by the ggplot2 package in R.[22]

Impact of frequent blood donations in young donors on hemolysis in stored RBCs: A total of 27 donors younger than 21 years old who donated 5 or more times in the prior 24-months enrolled in the study. This provided the opportunity to evaluate the effect of frequent blood donation in young donors. Differences in hemolysis between young and older frequent donors were compared using the t-test because of the small sample size and consequent limited degrees of freedom in statistical tests.

Association between donation history and measures of hemolysis: Evaluation of the association between donation history and each hemolysis measurement was performed by multivariate linear model regression analysis using several covariates. Donation frequency was divided into 4 groups: 0 (first-time donors and reference), 1–4, 5–8, and 9 or more donations 24-months prior to enrollment into this study; age was divided into 5 year bins; self-reported race-ethnicity and sex were indicator variables with white females as reference. Ferritin levels were analyzed as continuous measure. The reference groups were white, female, first-time donors, under 21 years of age, and no self-reported intake of iron supplements. Odds ratios and significances for each factor were calculated in the presence of the other covariates (Table 3).

Multiple comparison adjustment: To account for the fact that we performed multiple statistical tests in the manuscript (Table 3), and to keep the overall Type I error rate below 0.05, we determined tests with p-values <0.001 to be statistically significant.

RESULTS

Race-ethnicity, sex and age associations with donation frequency:

Of the 13,770 consented donors, 13,403 (97%) provided sufficient samples and data for inclusion in the present analysis (further information regarding the study design and population was published by Endres-Dighe et al.[23]). Participant sex and racial-ethnic distributions stratified by donation history are summarized in Table 1. Donors from both sexes were equally represented in RBC-Omics (50.3% females and 49.7% males). White donors constituted the large majority of subjects who donated 9 or more whole blood units in the 24 months prior to study enrollment. Frequent blood donations (≥5 donations in prior 24 months) were more prevalent in male donors across all racial-ethnic groups. For example, 14.6% of African American male donors compared with 8.5% of African American female donors gave ≥5 donations in the prior 24 months. Older male and female donors of all racial-ethnic groups were more likely to be frequent donors than younger donors (Table 2). The average age of all donors with prior history of 9 or more donations was 56.1±5.0 years versus 35.4±3.3 years in first-time donors and 39.4±4.6 years in donors with 1–4 prior donations (Figure 2).

Impact of donation frequency, race-ethnicity, and sex on hemolysis and ferritin in stored RBCs:

Frequent blood donations in male or female donors were associated with enhanced resistance to AAPH-induced oxidative hemolysis in both sexes (Figure 3A). This effect was evident by comparison with the average oxidative hemolysis observed among male and female donors with no prior donations (38.8±9.5%) to those with 9 or more donations (34.1±9.9%; p<0.0001). Conversely, no significant associations were observed between frequent blood donations and the amount of osmotic or storage hemolysis (Figure 3B-C). Donor ferritin levels were inversely correlated with donation frequency in both sexes, with the decline in ferritin greater in male donors (Figure 3D). Frequent blood donations had similar impact on RBC storage or stress hemolysis across all race-ethnicity groups. Thus, no race-specific differences in the response to multiple donations were detected (supplemental

Figure 1). The levels of storage, oxidative and osmotic hemolysis in each donation frequency category are summarized in Table S1.

Impact of frequent blood donations by young donors on hemolysis in stored RBCs:

High intensity donations (5 or more donations in the prior 24 months) in younger donors (<21 years) were associated with increased susceptibility to AAPH-induced oxidative hemolysis (Figure 4A; n=17 donors) and enhanced resistance to osmotic hemolysis (Figure 4B; n=25 donors). These responses to frequent donations were notably different from donors >21 years, whose RBC exhibited an inverse response to 5 or more donations (i.e. decreased levels of oxidative hemolysis and increased levels of osmotic hemolysis). This same analysis did not reveal age-specific differences in spontaneous storage hemolysis (Figure 4C).

Impact of oral iron supplement use in donors on RBC hemolysis:

Consumption of iron supplements in male and female donors was associated with reduced levels of AAPH-induced oxidative (Figure 5A) hemolysis at all donation frequency categories suggesting that the effect of iron supplements on oxidative hemolysis was independent of donation history. Similar trend was observed with osmotic fragility, for which iron consumption was associated with lower levels of osmotic hemolysis in male donors with donation history of 1–4 and 5–8 units, and in female donors with 0–8 prior donations. No effect of iron supplementation was seen on storage hemolysis. Small increments in ferritin levels were observed in male donors at the extremes of donation history categories (i.e. 0 and 9+), and in female donors at all donation history categories (Figure 5D) as reported before.[6]

Multivariate analyses of donor demographics associated with hemolysis:

A multivariate linear model was developed to determine the impact of frequent blood donations on each hemolysis end-point.[4] This model was adjusted for confounding variables including donor sex, age, race-ethnicity, ferritin, and iron supplements (Table 3). The betas in Table 3 represent the change in the hemolysis measure per unit change of the variable in question compared to the reference value. For example, each increment in ferritin concentration was associated with increases in the rates of oxidative, osmotic or storage hemolysis by 0.007%, 0.01%, and 0.0003%, respectively. Among the 3 hemolysis measures, only oxidative hemolysis was associated with frequent donations, as evident by the negative association with increasing number of prior donations in the last 2 years, evident at less frequent donation frequencies but becoming significant at 9 or more donations. Significant negative associations were also observed between oxidative hemolysis and older age (>60 years) and unexpectedly, with multivitamin with iron or other iron supplement intake (all p values<0.0001). Ferritin and male sex were positively associated with all 3 hemolysis endpoints (all p values<0.0001).

The same analyses demonstrated that average osmotic hemolysis in male RBCs was about 4.1% higher than in females, and osmotic hemolysis was negatively correlated with iron intake and with African American or Asian race-ethnicity. Storage hemolysis was significantly (all p values <0.0001) and positively associated with male sex, older age (>65 years), and with Asian or Hispanic race-ethnicity.

DISCUSSION

Frequent blood donors are essential to maintain a stable supply of RBCs and other blood components for transfusion therapies.[24] As each blood donation removes about 200–250 mg of iron, frequent donations over short time intervals expose many blood donors to various health risks related to iron deficiency, such as fatigue, anemia, pica, and restless leg syndrome.[5,25] Although ferritin levels progressively decline in response to frequent blood donations, key new findings from this study demonstrated that frequent donations also alter stored RBC susceptibility to oxidative hemolysis, and may have specific impact on young donor RBC susceptibility to osmotic and oxidative hemolysis. Furthermore, we demonstrated direct associations between donor ferritin concentrations and hemolysis, and that predisposition to osmotic or AAPH-induced oxidative hemolysis may be modulated by iron intake.

Frequent donors enrolled in this study were likely to be white, male, and of older age. Female frequent donors were also likely to be of older age and white. These characteristics of frequent donors are similar to recent reports from the Biomedical Excellence for Safer Transfusion (BEST) study of donation patterns in the U.S.[26,27], and an assessment of frequent blood donors in Australia.[24] Of note, the current study included a cohort of 1976 non-Hispanic white donors categorized as high-intensity donors (Figure 1), who comprised the majority of frequent donors with a history of 9 or more donations in the prior 24-months. The sex dichotomy in donation frequency is likely related to differences in susceptibility to donation-induced iron deficiency and subsequent low hemoglobin deferral[28], which is most common in women who have depleted iron stores from menstruation and pregnancy.

The amount of spontaneous storage hemolysis and osmotic hemolysis varied among the four categories of donation frequency, but these differences were not observed in the multivariate model. These observations suggest that frequent blood donations have no apparent impact on RBC membrane integrity or osmotic fragility. By contrast, we found a negative correlation between donation frequency and RBC susceptibility to AAPH-induced oxidative hemolysis. The mechanism behind this phenomenon is not clear and requires further evaluation of the consequences frequent blood donations and iron deficiency impose on RBC antioxidant capacity and rheological properties. Donor age is another factor that can modulate RBC predisposition to AAPH hemolysis.[4] While RBCs from young frequent donors (<21 years, Figure 4A) exhibited increased susceptibility to AAPH hemolysis, older donor RBCs (>60 years in particular, Table 3) were relatively resistant to this stressor. Therefore, further investigations should consider age as a significant modifier of hemolysis in stored RBCs.

We have recently reported sex- and race-specific differences in predisposition to hemolysis in the RBC-Omics cohort. For example, RBCs from African American donors exhibited enhanced resistance to osmotic hemolysis, whereas male sex was associated with increased susceptibility to storage and stress hemolysis.[4] The current analyses suggested that the sex and race-ethnicity differences in hemolysis were independent of prior donation history, as they were observed across all donation categories. In contrast, our evaluations of age-specific alterations in response to frequent donation (5 or more in the prior 24 months) found that RBCs from younger (18–21 years) donors with frequent prior donations exhibit

increased susceptibility to oxidative hemolysis and resistance to osmotic stress. The biochemical mechanisms that underlie such changes in hemolytic profile are unclear but may be related to iron deficiency and stress erythropoiesis in teenage donors, who are at greater risk of adverse reactions in response to frequent blood donations.[29] Of note, these analyses did not reach our stringent definition of statistical significance ($p>0.001$) as they were based on a limited number of young high frequency donors (n=17 in oxidative hemolysis and n=25 in osmotic hemolysis); therefore, further evaluations to assess the impact of frequent blood donations on young adult RBC characteristics and storage stability are needed.

Based on the multivariate analysis, oral iron supplements were associated with reduced oxidative and osmotic hemolysis, an unexpected finding given the positive association between hemolysis and donor ferritin levels reported in Table 3. Iron supplements were also associated with minor increases in plasma ferritin observed primarily in frequent donors. Contrary to intervention studies that demonstrated the effectiveness of oral iron supplements (19 or 38 mg) on donor iron stores and ferritin levels[7,30,31], the minimal effect observed in this multivariable analysis may be related to use of a linear regression model which analyzed ferritin across the entire ferritin range, rather than the logistic models used in the previous analyses, which emphasized the likelihood to have low ferritin levels (<12 ng/mL or <26 ng/mL). It is also possible that these donors had less effective self-directed consumption of iron supplements or, perhaps, incorrectly answered the questions about iron supplement use on the survey. Another possible explanation is that the changes observed in predisposition to hemolysis may have resulted from the action of vitamins or antioxidants in multiple vitamin pills rather than the iron content. Unfortunately, the survey did not allow differentiation between taking multivitamins with iron, or separate iron supplements.

Based on the multivariate analyses, a history of 9 or more donations in the prior 24-months and older age (>60 years) was significantly ($p<0.0001$) and negatively associated with oxidative hemolysis. Conversely, prior donations had no significant impact on storage or osmotic hemolysis suggesting that frequent donations primarily impact the RBC response to oxidative stress. The same analyses revealed positive associations ($p<0.0001$) between storage or stress hemolysis and donor plasma ferritin. These associations suggest that ferritin levels could predict RBC susceptibility to hemolysis in cold storage; however, further evaluations are required to determine the interactions between ferritin, iron, and hemolysis.

The findings presented in this study are limited to *in vitro* measurements of hemolysis in stored RBCs and ferritin measurement in donor plasma. Additional peripheral blood testing to assess donor iron status was not performed, because tests such as soluble transferrin receptor, transferrin saturation and total iron binding capacity add little to ferritin alone for assessment of iron status.[32,33] Further, blood donors are a healthy population with little underlying inflammatory disease that may cause the ferritin test to be an insensitive indicator of iron stores.

In the absence of rigorous clinical studies that would evaluate transfusion outcomes, our current understanding of possible patient outcomes is limited to animal studies[34–36] or to retrospective studies that linked donor characteristics with the risk of transfusion-related

mortality.[37–40] With regards to frequent blood donations, a recent study has demonstrated that RBCs produced by mice with mild iron deficiency have greatly decreased survival following storage and transfusion.[16] Several prospective and ongoing studies by this group and others are in progress to investigate the impact of donor characteristics (sex, donation history, iron repletion therapy) on autologous RBC recovery and survival and patient outcomes following allogeneic RBC transfusions. For example, we have identified patients who received RBC units from RBC-Omics donors, and for whom the REDS-III program has recipient outcome data, including pre-/post-transfusion hemoglobin increments, in the linked donor-recipient database.[41–43] In addition, subsequent studies are planned that will recall selected RBC-Omics donors with extremes of hemolysis parameters or identified genetic polymorphisms that were identified in our genome-wide association study to determine *in vivo* RBC survival based on biotin labeling[44–46] and transfusion of autologous stored RBC samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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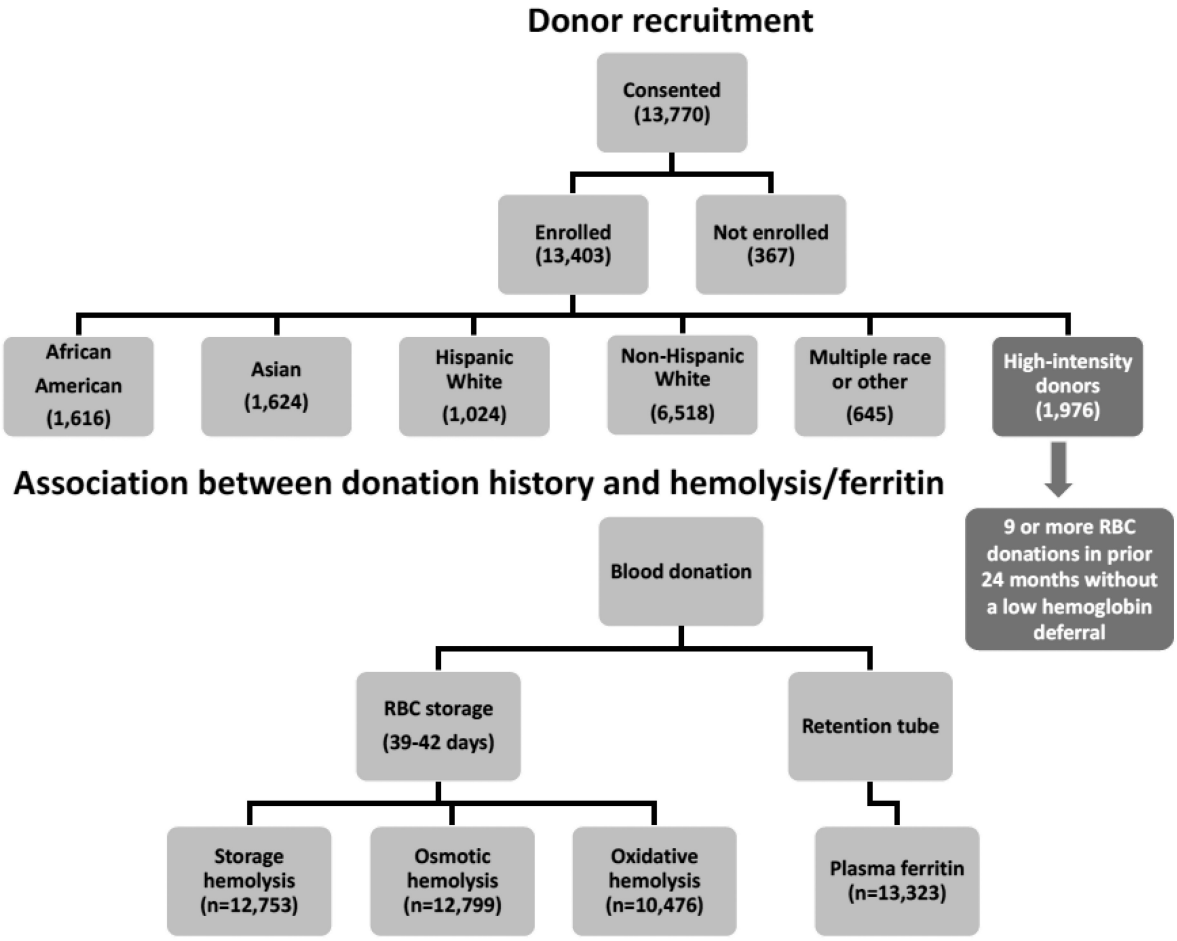

Figure 1:
Flowchart of the RBC-Omics study cohort and donor testing for hemolysis and ferritin.

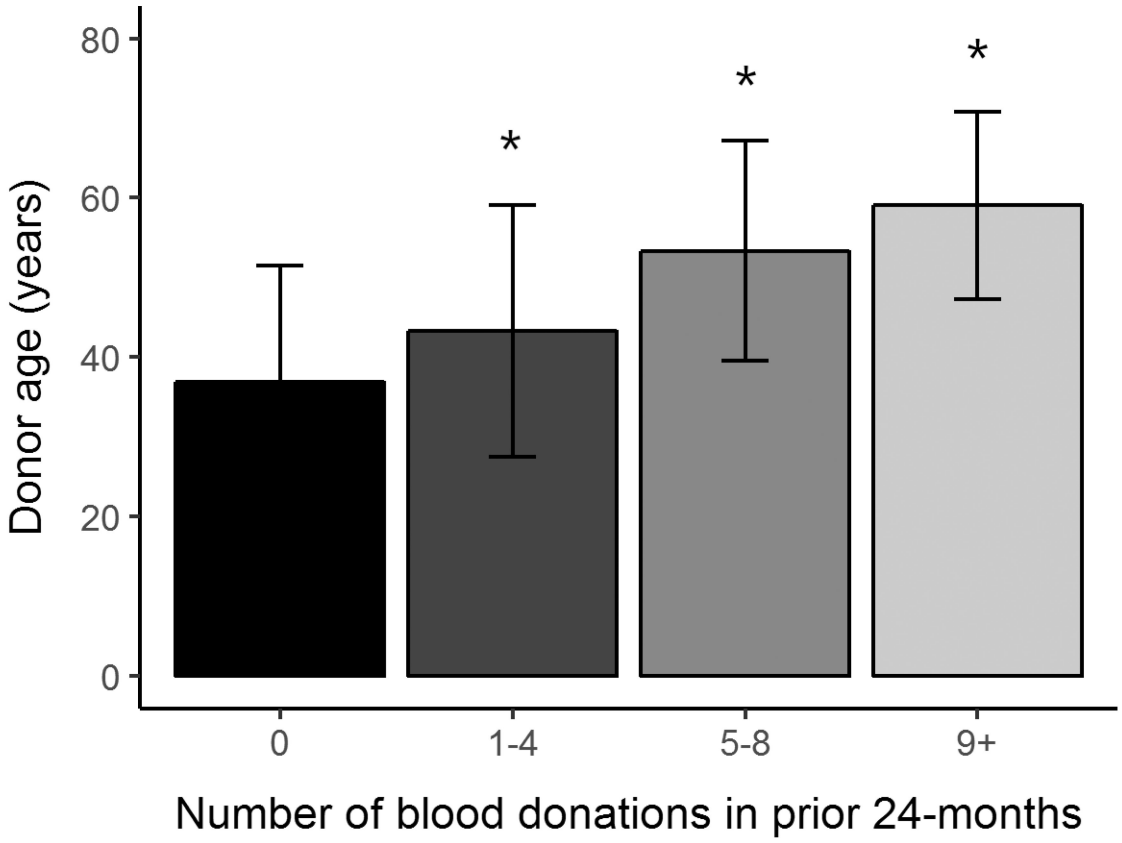

Figure 2: RBC-Omics donor age (years±SD) at selected categories of donation frequency in the 24 months prior to enrollment.

Asterisks represent statistical significant difference in age ($p < 0.001$).

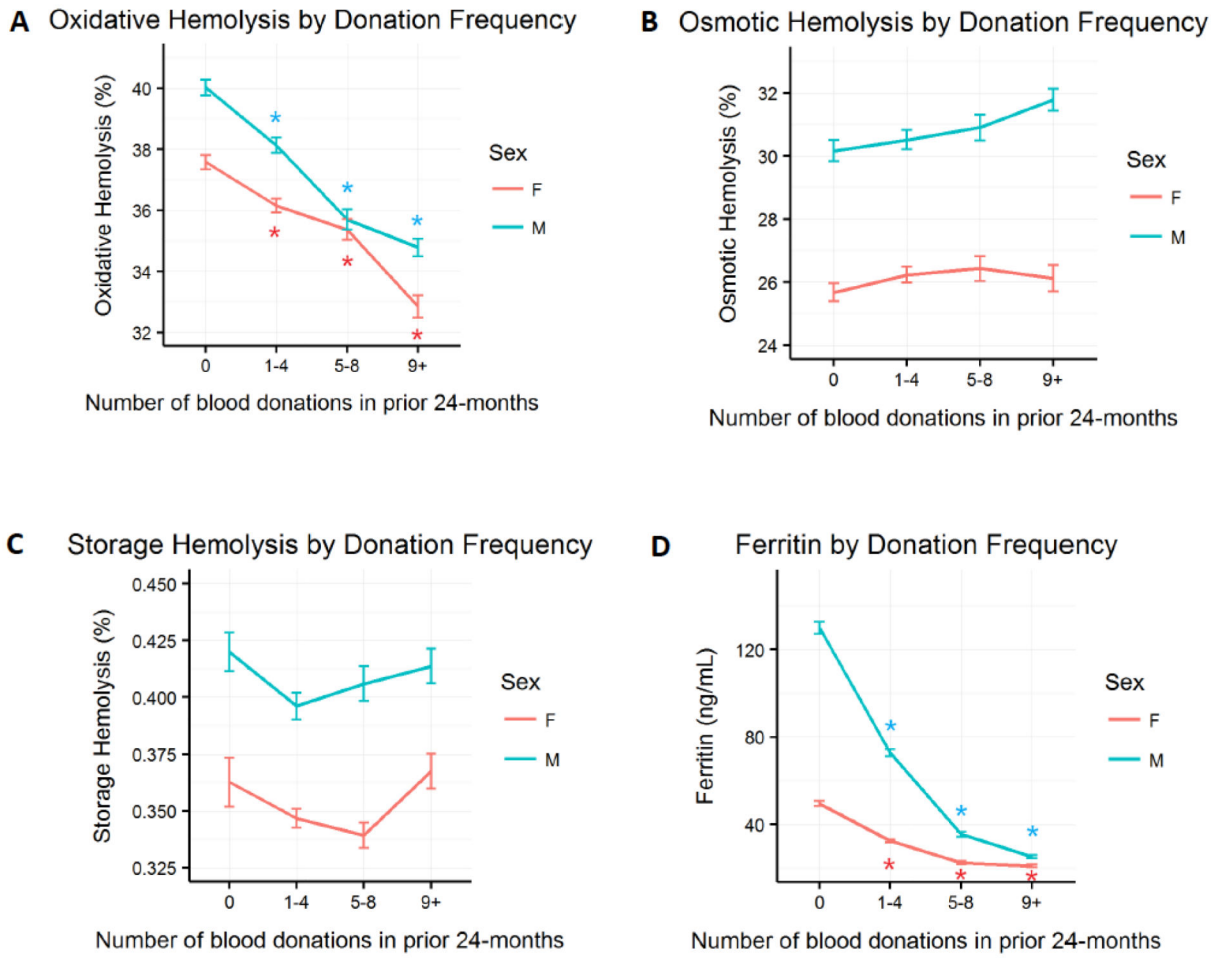

Figure 3: Sex distribution of stress-induced hemolysis, storage hemolysis or donor ferritin levels at selected categories of donation frequency in prior 24 months.

RBC concentrates from male or female donors ages 18–90 years old were stored (1–6°C) for 39–42 days in transfer bags and tested for storage or stress-induced hemolysis as described in Materials and Methods. **A**. Percent AAPH-induced oxidative hemolysis (150mmol/L, 1.5h, 37°C) (n=10,476). **B**. Percent osmotic hemolysis (n=12,799). **C**. Percent spontaneous storage hemolysis (n=12,753). **D**. Donor plasma ferritin (ng/mL) (n=13,323). Error bars represent standard errors of the mean. Asterisks represent statistical significant difference in mean hemolysis or ferritin between first- time/reactivated donors and each of the donation frequency bin for male and female ($p < 0.001$). Hemolysis measures were normalized for blood center differences in blood component manufacturing procedures.

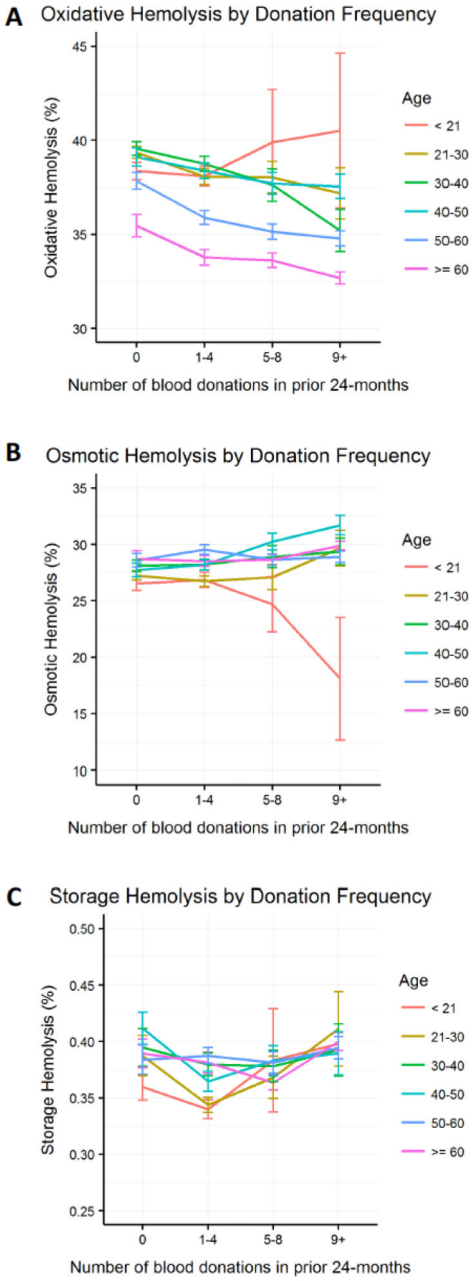

Figure 4: Evaluation of age-specific differences in the rates of stress or storage-induced hemolysis in response to frequent blood donations.

Figure panels represent the amount of **A**. Percent AAPH-induced oxidative hemolysis (150mmol/L, 1.5h, 37°C) (n=10,476). **B**. Percent osmotic hemolysis (n=12,799), and **C**. Percent storage spontaneous hemolysis (n=12,753) at selected age groups (decades) in the 4 categories of donation frequency. Error bars represent standard errors of the mean. A. n=17 and B-C. n=25 young donors (<21 years) at donation categories 5–8 and 9+ combined. Hemolysis measures were normalized for blood center differences in blood component manufacturing procedures.

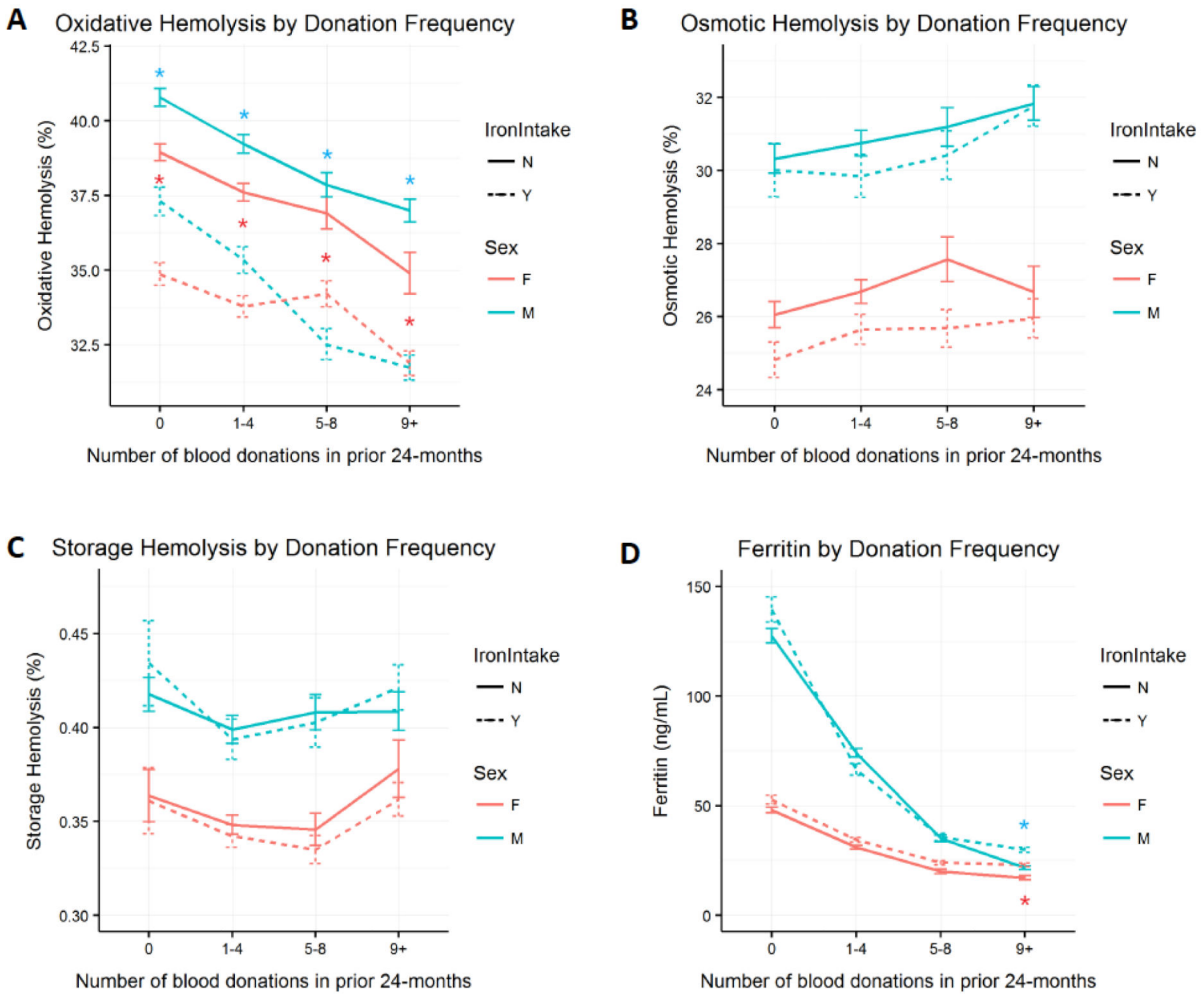

Figure 5: Association of self-reported iron intake with RBC predisposition to stress-induced hemolysis, storage hemolysis or donor ferritin levels.

Data obtained from male (M) and female (F) RBC-Omics donors who responded Yes (Y) or No (N) to the consumption of iron supplements. **A**. Percent AAPH-induced oxidative hemolysis (150mmol/L, 1.5h, 37°C), **B**. Percent osmotic hemolysis, **C**. Percent storage spontaneous hemolysis, and **D**. Donor ferritin levels (ng/mL). Error bars represent standard errors of the mean at the 4 categories of donation frequency. Asterisks represent statistical significant difference in mean hemolysis or ferritin by iron intake status stratified by donation history and sex ($p < 0.001$). Hemolysis measures were normalized for blood center differences in blood component manufacturing procedures.

Table 1:

RBC-Omics blood donor demographics (n=13,403) and donor numbers in each of the 4 categories of donation frequency.

N	African American		Asian		White		Hispanic		Other	
Don Freq	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
0	399	280	406	456	923	751	298	179	172	134
1-4	397	357	273	312	1567	1262	275	172	121	110
5-8	66	80	53	96	890	940	34	45	29	38
9+	8	29	8	20	792	1369	8	13	18	23
Sum	870	746	740	884	4172	4322	615	409	340	305

Table 2:

Distribution of donor age (years±SD) stratified by donation frequency, sex, and race-ethnicity in the RBC-Omics cohort (n=13,403).

Age (years±SD)	African American		Asian		White		Hispanic		Other	
Don Freq	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
0	36.9±14.7	36.0±14.8	32.7±11.9	35.6±12.1	40.1±15.6	41.0±15.8	31.2±12.0	32.5±12.1	32.6±12.5	35.7±12.8
1-4	42.5±16.1	39.9±14.8	36.3±13.6	37.4±13.0	46.5±15.7	47.6±15.4	34.5±13.2	35±14.4	37.1±14.7	37.6±14.8
5-8	51.4±13.7	52.3±13.6	50.2±13.1	44.3±11.2	55.1±13.0	54.2±14.0	48.2±10. 7	42.2±13.5	48.6±16.5	45.5±15.2
9+	62.0±9.3	58.7±11.9	49.8±9.0	58.2±13.2	59.0±11.2	59.4±11.9	58.3±11.6	45.6±15.3	53.8±14.9	56.1±13.6
Average	40.8±15.9	40.5±15.7	35.5±13.5	37.7±13.0	49.3±15.9	51.6±15.6	34.0±13.3	35.0±13.8	36.7±15.0	39.1±15.0

Table 3:

Multivariate linear modeling of hemolysis measures. Differences are considered significant at p<0.001. Reference values were 0 donations, no iron consumption, age < 21, Female, and White race-ethnicity. For continuous measures like ferritin there is no reference value.

	Oxidative Hemolysis		Osmotic Hemolysis		Storage Hemolysis	
	beta	p	beta	p	beta	p
Donation Frequency 1-4	−0.7988	0.0016	0.0998	0.7366	−0.008	0.2723
Donation Frequency 5-8	−0.8267	0.0159	−0.7796	0.0477	6.00E-04	0.9492
Donation Frequency 9+	−1.569	< 0.0001	−1.0589	0.0119	0.0222	0.0315
Ferritin	0.0071	< 0.0001	0.0098	< 0.0001	3.00E-04	< 0.0001
Iron Intake Yes	−3.7556	< 0.0001	−1.1919	< 0.0001	−0.0032	0.5911
Age 21-25	0.9787	0.0535	−0.6215	0.3017	0.0237	0.1069
Age 25-30	0.4887	0.3216	−0.6827	0.2414	0.0051	0.7203
Age 30-35	1.0576	0.0361	−0.2312	0.6981	0.0164	0.2608
Age 35-40	1.0243	0.0475	0.3122	0.6105	0.046	0.0021
Age 40-45	1.5454	0.0026	0.285	0.6422	0.0253	0.0917
Age 45-50	0.7283	0.1516	0.9106	0.1282	0.0386	0.0084
Age 50-55	−0.4248	0.3823	0.2013	0.7267	0.0417	0.003
Age 55-60	−1.4726	0.0023	0.3644	0.5223	0.0456	0.0011
Age 60-65	−2.3678	< 0.0001	0.1337	0.8218	0.0261	0.072
Age >= 65	−3.3611	< 0.0001	−0.2263	0.6964	0.0564	< 0.0001
Race African American	−0.0816	0.783	−13.028	< 0.0001	0.021	0.0176
Race Asian	−0.3059	0.3513	−3.2147	< 0.0001	0.0579	< 0.0001
Race Hispanic	0.74	0.0453	−1.228	0.0051	0.0535	< 0.0001
Sex M	1.0175	< 0.0001	4.1337	< 0.0001	0.0435	< 0.0001