

In vitro Analysis of the Hemostatic Properties of Whole Blood Products Prepared with a Platelet-Sparing Leukoreduction Filter

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Abstract

Background: Warm fresh whole blood (WFWB) is an ideal resuscitation fluid for exsanguinating patients but there are myriad logistic and infectious issues associated with its use. Cold whole blood (CWB) may be an acceptable alternative to the reconstituted whole blood (RWB), the current standard of care. A leukoreduction filter has been developed which maintains platelet count while eliminating white blood cells but its effect on platelet function is unknown. We hypothesize that CWB will retain an acceptable functional coagulation profile after filtration and over time.

Study Design and Methods: WFWB and CWB samples were obtained from eight donors and four units of RWB were created. The quantitative and qualitative *in vitro* coagulation profiles of WFWB, RWB, and CWB over time were compared.

Results: Filtration was successful at removing white blood cells (5.5 ± 1.2 vs. $0.3 \pm 0.3 \times 10^6/L$) while retaining an adequate platelet count (172.0 ± 47.0 to $166.0 \pm 42.3 \times 10^9/L$) and hemoglobin concentration (13.7 ± 0.5 vs. 13.0 ± 0.7 g/dL). Rotational Thromboelastography (ROTEM) results revealed a similar clotting time (CT) before and after filtration (64.9 ± 5.1 vs. 64.1 ± 6.8 s) but a decreased maximum clot firmness (MCF) (58.6 ± 4.2 vs. 54.9 ± 4.6 mm). Platelet aggregation decreased substantially (28.8 ± 6.7 vs. 9.3 ± 2.1 ohm) immediately after filtration. CWB function continued to diminish over time.

Conclusion: CWB holds great promise as a surrogate for WFWB, but use of a platelet-sparing LR filter diminishes platelet function almost immediately after filtration.

Keywords: Hemostatic; Thromboelastography; Leukoreduction; Platelet, Coagulation

Introduction

Through experiences gained caring for injured casualties in Operation Iraqi Freedom and Operation Enduring Freedom, there has been a renewed fervor in finding the ideal form of resuscitation, including the use of warm fresh whole blood (WFWB). Lessons learned from reviewing outcomes of critically injured military casualties during these Operations demonstrated substantial improvements in odds of survival when blood components were transfused in nearly equal ratios of red blood cells (RBC), plasma and platelets (1:1:1) [1]. It has been theorized that this method of transfusion of reconstituted whole blood (RWB) leads to improved survival by enhancing oxygen delivery and coagulation factor replacement. However, RWB is at best cold, acidic, contains low number of platelets ($80 \times 10^9/L$) with about half the concentration of coagulation factors as compared to WFWB [2]. Spinella et al. demonstrated a 13% improvement in mortality when WFWB was

transfused [3]. The overwhelming logistics and infectious concerns, however, have virtually eliminated WFWB as an option of colloid in a massively exsanguinating patient.

Cold whole blood (CWB) does not share the logistic and infectious concerns of WFWB as it can be processed in standard fashion including leukoreduction (LR). LR ameliorates the risk of febrile reactions, cytomegalovirus transmission, and many other potential complications [4]. To avoid undesirable elimination of platelets, a platelet-sparing LR filter (Imuflex WB-SP; Terumo Corporation, Tokyo, Japan) was developed. This filter has the capability to create blood products that meet or exceed Food and Drug Administration processing (FDA) regulations and has been validated to preserve platelets while effectively leukoreducing whole blood [5-7]. FDA regulations, however, do not require *in vivo* assessment of coagulation thus the impact of the platelet-sparing LR filter on the coagulation profile and function of the platelets within whole blood products is unclear [8]. Furthermore, maintenance of coagulation activity and platelet function over time post-filtration which would inform optimal CWB lifespan for clinical use has not been demonstrated. This information is critical, as identifying the optimal blood product to

transfuse in exsanguinating patients may lead to reduction of short-term and long-term mortality. To this end, we sought to compare *in vitro* coagulation properties of CWB over time to those of WFWB and RWB in a multimodal fashion. We hypothesized that WFWB would have the optimal coagulation and platelet function profile, followed by CWB as compared to RWB; during the first 21 days of storage and that the filtration step would have minimal effect on platelet count and function.

Methods

Study design

A total of eight subjects' whole blood was collected and analyzed plus 4 units of reconstituted whole blood were created. The *in vitro* coagulation profiles of WFWB, RWB, and CWB were initially compared using quantitative and qualitative methods described below.

Reconstituted whole blood creation

A total of 4 units of RWB were created to approximate a 1:1:1 ratio of red blood cells (RBC), plasma and platelets (1:1:1); 7 day old group O packed red blood cell units (330 mL), group A thawed plasma (220 mL), and one-sixth of a 3 day old apheresis group A platelet component (300/6=50 mL) to total 600 mL for each RWB unit. Within the RWB units, there was a total of 70 mL CP2D (from the FFP), 110 mL AS-3 (from the pRBCs), and 5 mL ACD (from the apheresis platelets) to total 185 mL of preservative solution in the RWB units. Therefore, 185 mL out of the 600 mL (30.5%) was preservative solution.

Whole blood products processing and warm fresh whole blood creation

Standard blood donor consent was obtained which includes consent for donated blood to be used for research purposes. All blood bank guidelines were followed in obtaining blood samples for this study. All donated units were de-identified. Eight healthy, O negative male donors each donated one unit of whole blood per standard blood bank donation procedures but using the Emulex WB-SP filter and corresponding blood bag system. WFWB samples were taken directly from the diversion sampling bag without filtration, placed into 4.5 mL tubes containing sodium citrate anticoagulant (3.2%), and transported to the hematology laboratory for testing within 30 minutes; no additional preservatives or anticoagulants were added.

Cold whole blood (CWB) creation and storage

The remainder of the donated blood immediately entered the collection system and was processed per manufacturer's directions through the platelet-sparing, LR-reduction filter to be stored in Imuflex WB-SP bags that contains 70 mL of CPD anticoagulant/preservative solution with the 500 mL of whole blood bag. The filtration was performed at room temperature. After filtration, aliquots were immediately withdrawn and labeled as CWB0 (cold whole blood day 0) for laboratory analyses. CWB0 was analyzed within 2 hours of filtration. The remaining CWB0 was stored at 1-6°C and aliquots were withdrawn from the WB-SP system on subsequent days 1, 2, 7, 14 and 21 and labeled as CWB2, CWB7, CWB14 and CWB21, respectively.

Laboratory testing

All samples were tested for standard clinical laboratory values including HgB, leukocyte count, platelet count, using the Coulter ACT-10 Hematology Blood Analyzer (Beckman Coulter, Indianapolis, IN). Gentle massage of the bag was performed for 5 minutes prior to drawing blood for analysis using a needle through the collection bag port. A total of 34 cc were collected on each analysis day for the laboratory tests outlined below.

Impedance aggregation and simultaneous ATP release with collagen

Platelet aggregation and ATP secretion in whole blood were measured using the Whole Blood Lumi-aggregometer, Chrono-Log Model 700 (Chrono-Log Corporation, Haverton, PA), according to the manufacturer's recommendations. In brief, for each sample analysis, a 450 µL of citrated (3.2%) whole blood was diluted with 450 µL of 0.9% normal saline. Aggregation and secretion were induced with collagen (5 µg/mL) in a Whole Blood Lumi-aggregometer with AGGRO/LINK8 software and reusable electrode probes. Gain for the impedance was set automatically at 20 ohms (Ω) with maximal platelet aggregation (plateau response) results presented as Ω . An aliquot of 450 µL of the whole blood was pipetted into a prewarmed cuvette with stir bars and combined with 450 µL saline for the luminescence gain setting. After 5 minutes of incubation, 100 µL of CHRONO-LUME reagent was added to the test cuvette with 5 µL of ATP standard (2 µmole) dispensed into the sample. The impedance and luminescence methods for aggregation in whole blood with simultaneous measurement of total adenosine triphosphate (ATP) release are expressed in nanomoles.

Rotational thromboelastometry (ROTEM)

A computerized ROTEM analyzer (Device 3497, TEM Systems Inc, Durham, NC) was used per manufacturer's instructions to measure clot formation as well as clot lysis in the citrated whole blood. The ROTEM channel was programmed for EXTEM. 20 µL of star-tem reagent and 20 µL of ex-tem reagent were added to the ROTEM cup with 300 µL of the whole blood added thereafter. The cup holder was set onto the pin. The control samples (ROTROL N or ROTROL P) were performed according to the manufacturer's instructions by the automated ROTEM pipette. Normal EXTEM ROTEM values were considered as CT 42-74 s; alpha angle 63°-81°, and maximum clot firmness (MCF) 49-71 mm [8].

Calibrated automated thrombinogram (CAT)

The CAT assay method has previously been described [9]. Thrombin generation was measured using the CAT assay (Thrombinoscope BV, Maastricht, The Netherlands) utilizing a Fluoroskan Ascent plate reader (390 nm excitation, 460 nm emission, Thermo Electron Corp, Vantaa, Finland). Reference plasma CryoCheck™ pooled normal plasma (Precision Biologic, Dartmouth, NS, Canada) was included in each assay. Frozen, citrated, plasma samples were thawed in water bath (37°C for 5 minutes), and corn trypsin inhibitor (CTI, Haematologic Technologies Inc. Essex Junction, VT) was added to each plasma sample (50 µg/mL, final concentration). Assays were performed in triplicate in polypropylene 96-well U bottom micro titer plates (Nunc, Thermo Fischer Scientific, Rochester, NY). The PPP Reagent (5 p.m. tissue factor/4 µM phospholipid, Stago, US) was used as a trigger. Twenty µL of a "trigger" or a known concentration of thrombin- α -2-macroglobulin complex (thrombin

calibrator, Stago, US), the calibrator corrects for inner filter effects and quenching variation among individual plasmas was added to each well, followed by 80 µl of plasma. After an incubation period of 10 minutes at 37°C, thrombin generation was initiated by adding 20 µl of warmed fluorogenic substrate in buffer containing calcium (Stago, US) via an automated dispenser to each well. Progress curves were recorded continuously for 90 minutes at a rate of 3 readings per minute.

Results

Impact of platelets LR filtration

The Imuflex WB-SP was successful at removing WBCs (5.5 ± 1.2 vs. $0.3 \pm 0.3 \times 10^6$ /L) while retaining an adequate platelet count (172.0 ± 47.0 to $166.0 \pm 42.3 \times 10^9$ /L, 96.5% recovery) and Hgb concentration

(13.7 ± 0.5 vs. 13.0 ± 0.7 g/dL). The Hgb values of RWB were lower (9.0 ± 0.7 g/dL) as compared to WFWB (13.7 ± 0.5 g/dL) and CWB. Comparison of ROTEM results revealed a similar clotting time (CT) before and after filtration that remained within normal ranges (64.9 ± 5.1 vs. 64.1 ± 6.8 s). Additionally, MCF decreased minimally after filtration and remained within the normal range (58.6 ± 4.2 vs. 54.9 ± 4.6 mm). There were no appreciable differences after filtration for MCF (9.4 ± 1.6 vs. 9.0 ± 1.6 s). However, the amplitude of platelet aggregation using collagen decreased substantially (28.8 ± 6.7 vs. $9.3 \pm 2.1 \Omega$) as did the Area Under Curve (122.1 ± 29.4 vs. 45.0 ± 15). Peak thrombin generation decreased after filtration in plasma (292.3 ± 18.0 vs. 237.4 ± 17.3). Similarly, the lagtime to initiation of thrombin generation and time to peak thrombin generation was greater prior to filtration (Table 1).

Blood Products	WFWB (n=4)	CWB0 (n=4)	CWB2 (n=4)	CWB7 (n=4)	CWB14 (n=4)	CWB21 (n=4)	RWB (n=4)
HgB (g/dL)	13.7 ± 0.5	13.0 ± 0.7	12.9 ± 0.7	13.0 ± 0.6	12.9 ± 0.6	14.9 ± 3.9	9.0 ± 0.7
WBC (× 10 ⁶ /L)	5.5 ± 1.2	0.3 ± 0.3	0.5 ± 0.2	1.0 ± 0.5	0.4 ± 0.3	0.7 ± 0.4	0.13 ± 0.1
Platelets, × 10 ⁹ /L	172.5 ± 47.0	166.0 ± 42.3	119.5 ± 63.7	64.8 ± 19.0	119.8 ± 55.2	101.8 ± 31.9	213.3 ± 36.3
CT, (sec)*	64.9 ± 5.1	64.1 ± 6.8	70.0 ± 7.2	70.6 ± 5.2	78.5 ± 8.0	86.1 ± 7.3	63.8 ± 2.9
MCF, mm*	58.6 ± 4.2	54.9 ± 4.6	51.7 ± 8.8	49.4 ± 6.1	45.9 ± 5.3	40.0 ± 4.1	62.6 ± 1.7
Alpha angle*	70.6 ± 3.4	68.5 ± 3.9	62.7 ± 5.8	55.1 ± 10.9	47.6 ± 13.5	34.9 ± 8.3	74.1 ± 1.0
Platelet aggregation amplitude, (ohm)	28.8 ± 6.7	9.3 ± 2.1	8.0 ± 1.8	2.5 ± 1.7	1.5 ± 0.6	1.0 ± 1.4	10.5 ± 1.9
Platelet aggregation AUC	122.1 ± 29.4	45.0 ± 15.0	30.8 ± 11.5	7.9 ± 6.6	3.6 ± 2.2	3.6 ± 4.9	42.8 ± 12.7
Lagtime (min)	2.6 ± 0.3	2.8 ± 0.6	3.2 ± 0.7	3.7 ± 0.6	4.1 ± 0.6	4.3 ± 0.7	2.3 ± 0.1
Peak Height (nmol)	292.3 ± 18.0	237.4 ± 17.3	244.9 ± 54.3	267.8 ± 59.0	293.6 ± 65.8	297.2 ± 85.6	295.1 ± 7.9
Time to Peak (min)	5.4 ± 0.7	6.4 ± 1.1	7.1 ± 1.0	7.0 ± 1.2	7.1 ± 1.1	7.0 ± 1.7	4.6 ± 0.3

Table 1: Comparisons of Different Blood Products. CWB samples were LR-filtered. *Normal EXTEM ROTEM values: (Clotting time [CT]: 42-74 s; alpha angle 63°-81°; and Maximal clot firmness [MCF] 49-71 mm; HgB: Hemoglobin; g: Grams; dL: Deciliter; WBC: White Blood Cell; s: Seconds; AUC: Area Under Curve; CWB0: Cold Whole Blood Day Zero; RWB: Reconstituted Whole Blood; WFWB: Warm Fresh Whole Blood.

Coagulation profiles of CWB over time as compared to WFWB and RWB

CWB *in vitro* function diminished over time (Table 1). Over time, in CWB, the platelet count continued to decrease starting day 2 of storage (119.5 ± 63.7). The CT of CWB increased over time and became abnormally elevated at day 14 (78.5 ± 8.0 s); however, the kinetics of clot formation as measured by alpha angle was abnormally low by day 2 ($62.7^\circ \pm 5.8$). CT and alpha were normal in WFWB and RWB. Platelet function, as measured by MCF and amplitude of platelet aggregation by collagen, was most robust in WFWB followed by RWB

(Figure 1). However, over time, the MCF begin to decrease and CT increase by day 7 (49.4 ± 6.1 mm) and (70.6 ± 5.2), respectively. The platelet aggregation of CWB decreased immediately, after use of Imuflex WB-SP filter (Table 1) and continues to decrease over time. As noted above, filtration affected aggregation dramatically with a significant decrease of CWB at day 7 over baseline (9.3 ± 2.1 to $2.5 \pm 1.7 \Omega$). Overall, WFWB and RWB performed best; the thrombin generation profile of filtered CWB rapidly deteriorated with the majority of properties abnormal by day 7.

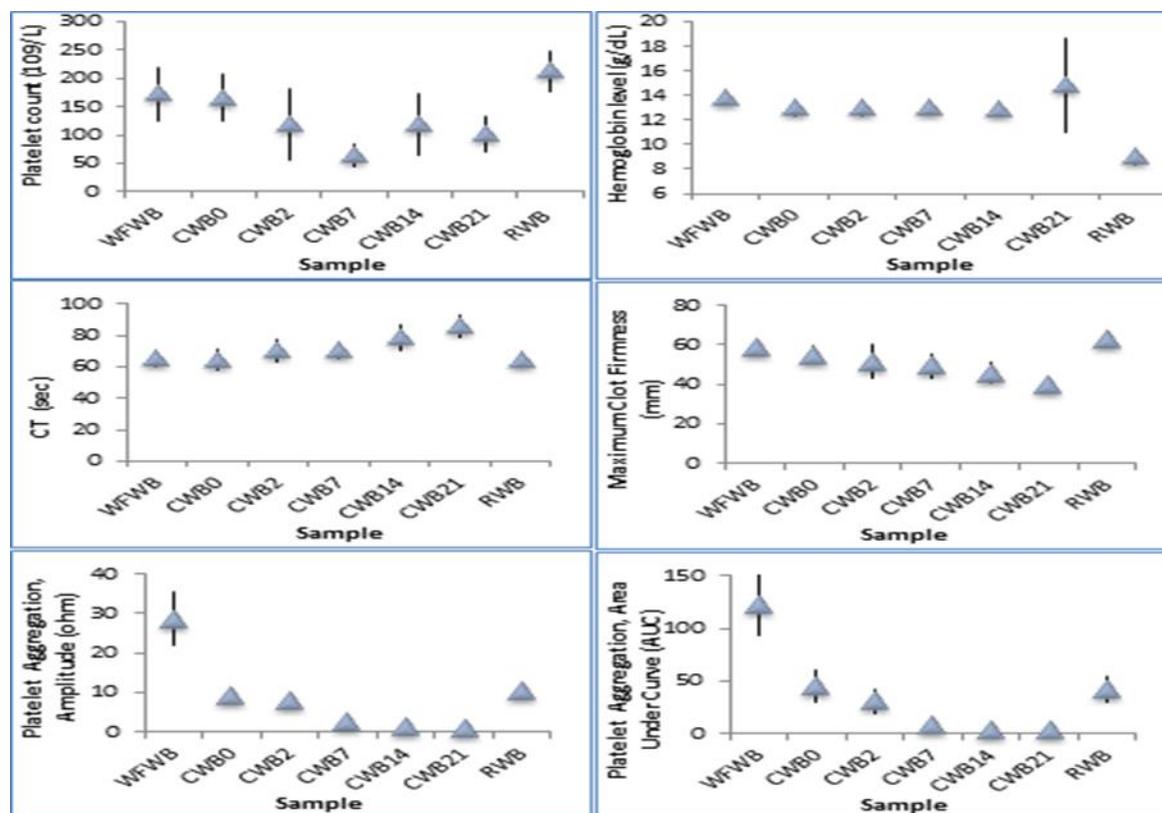


Figure 1: *In vitro* properties of LR-filtered CWB over time as compared to WFWB and RWB

Discussion

This represents the first study to evaluate the impact of a platelet-sparing LR filter on the post-filtration hemostatic status of whole blood components utilizing platelet aggregometry, plasma thrombin generation and whole blood viscoelastic properties. While our study reinforces WFWB as the gold-standard for hemorrhage resuscitation based on hemostatic properties, we have attempted to identify a product with comparable hemostatic properties that avoids the contemporary logistic and infectious concerns of WFWB [10,11]. Our study demonstrates that CWB holds great promise as a surrogate for WFWB, but that use of the platelet-sparing LR filter diminishes platelet function almost immediately after filtration (Table 1). Additionally, LR-filtered CWB should be used within 7 days as platelet function decreases significantly by this date.

Recent military conflicts in the Middle East challenged the notion that blood product constituents are the ideal resuscitative fluid as WFWB was associated with improved mortality compared to balanced ratios of plasma, platelets, and pRBCs [6]. Unfortunately, this practice was responsible for an increased risk of transfusion transmissible infections (TTI) [12]. In response, Cotton et al. created modified whole blood (MWB) processed through a standard LR filter that does not preserve platelets and transfused the product accompanied by apheresis platelets [13]. The authors demonstrated a significant reduction in blood product utilization in a subgroup of patients with traumatic brain injuries.

To improve upon MWB, we created a whole blood product that contained all three blood constituents using a newly FDA-approved platelet-sparing LR filter. The FDA requirements for LR is that there should be less than 8.3×10^5 white blood cells per container for platelets derived from whole blood with at least an 85% recovery of platelets [14]. This filter was able to meet these regulations; however, the FDA does not mandate the maintenance of platelet function. Prior to use of this filter in a whole blood product to be used clinically at our institution we wanted to confirm that WBCs were reduced while maintaining platelet count and function. Unfortunately, the latter was not the case as we discovered a dramatic reduction in platelet function immediately after filtration on platelet aggregation. The clinical significance of this observed finding needs to be explored. We speculate that activation of platelets during filtration may lead to the observed reduction in platelet function. Additionally, the ROTEM is a global clotting assay and not a specific test for platelet function as compared to aggregometry. The determinants of MCF can be attributed red blood cells as well as leukocytes. The red blood cells, which also provide a membrane surface for thrombin generation, may contribute to the formation of thrombin, which is necessary for platelet activation and converting soluble fibrinogen into insoluble strands of fibrin strength [15]. Thrombogenic activity of leukocytes has been known for decades. The seminal works done by Niemetz and colleagues demonstrated that leukocytes obtained from rabbits exposed to endotoxin led to intravascular clotting when infused in normal rabbits, and that this procoagulant activity is tissue factor dependent [16,17].

Given the identified detriment to platelet function after LR of CWB, the necessity of LR must be revisited. Numerous studies have demonstrated the safety of transfusing non-LR blood products in trauma patients. The entire CWB experience during the Vietnam War was with non-LR CWB, which had an excellent safety profile [18]. Additionally, a retrospective review by Phelan et al demonstrated similar mortality in 439 patients who received non-LR blood products compared to 240 patients who received LR blood products [19]. A double-blinded, randomized, controlled, clinical trial studying the effects of resuscitation with non-LR pRBCs vs. LR pRBCs in 268 trauma patients confirmed these findings with similar outcomes between cohorts [20].

Elimination of LR may actually hold several benefits for hemorrhaging patients. As shown in our study, the LR filter caused a substantial decrease in platelet function. Given evidence that non-LR products are safe combined with the findings of our study, maintenance of platelet function in CWB without filtration may outweigh the risks of transfusing a whole blood product with leukocytes.

WFWB and RWB had similar thrombin generation kinetics. The CWB initially displayed decreased peak thrombin generation, which progressively increased over time. This observation might be due to development of micro particles (MP) shedding from RBCs and platelets as they age over time in stored CWB. As CWB has undergone the necessary screening for TTI, its risk is equivalent to component blood products and may even be lower since there is only one donor exposure as opposed to at least 3 exposures with RWB.

The *in vitro* effects that storage duration has on CWB have been previously studied, though not with use of a platelet-sparing LR filter. The Norwegian Navy studied the feasibility of a walking blood bank while on active duty during anti-piracy missions as well as the function of a ROTEM machine while at sea [21]. The EXTEM MCF, used as a surrogate for platelet function, decreased over time. Though direct measurement of platelet function using aggregometry was not performed during the missions, the authors had previously reported on platelet aggregation [22]. Platelet function decreased significantly over time in CWB sample with a 50% loss from day 1 to day 5 with another 50% drop between days 5 and 10. Despite use of a platelet-sparing LR filter in our study, we also saw a significant drop in aggregation over time. Furthermore, the greatest decline in function occurred immediately after filtration.

There are several limitations to our study. This work was performed in preparation for introduction of CWB to clinical practice rather than with the goal of publication. Though strong trends were identified in our experiments with respect to platelet function post-filtration that we felt were important to share, the cost constraints and intended small scope of our study precluded rigorous statistical analyses. Additionally, the strong trends we identified convinced us that further utilization of precious blood resources in order to gain statistical significance was unwarranted. A further limitation was the lack of warming CWB units on the day of testing; though it may have decreased platelet quantity, maintaining a stable thermal environment is thought to optimize integrity of the CWB units [23]. We did not address platelet function of CWB processed using non-platelet sparing LR filters. This could be performed in future studies to determine the effect that elimination of platelets has on platelet aggregation.

Due to the demonstrated safety of non-LR blood in trauma patients, and with the discovery that the filter dramatically altered platelet

function, we altered our massive transfusion resuscitation protocol [24]. We believe, based on these results, that non-filtered CWB is at least as efficacious as filtered CWB with the possibility to have a better *in vivo* coagulation profile better. Currently, we have eliminated the LR step from our CWB processing. Whether the *in vitro* benefits of non-LR CWB translates to improved patient outcomes, however, requires further study.

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Conflicts of Interest

The manuscripts contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH, Department of Defense, or NCCR. Each author has read the AABB's policy on Conflict of Interest and has completed the Conflict of Interest form for this manuscript.

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