Supplemental Materials and Methods

Materials

The following items were purchased: Heparin-coated capillaries (VWR, West Chester, PA), bovine serum albumin (BSA, fraction V, Sigma-Aldrich, St. Louis, MO), clopidogrel (Plavix, Sanofi-Aventis, France), acetylsalicylic acid (Bayer, Whippany, NJ), Antibodies against: mouse GPIbβ-488, activated mouse αIIbβ3 (clone: Jon/A), JAQ-1, mouse CD62P (Emfret Analytics, Wuerzburg, Germany), activated human αIIbβ3 (PAC-1, BD Biosciences, San Jose, CA), and human CD62p (BD Bioscience), platelet agonists: ADP, arachidonic acid, fibrillar collagen type I (Chronolog, Havertown, PA), convulxin (Cayman Chemical, Ann Arbor, MI), VASP-phosphorylation kit (STAGO, Asnières sur Seine Cedex, France), and thromboxane B2 ELISA (Cayman Chemicals, Ann Arbor, MI). Verify Now for aspirin and clopidogrel were completed by University of Washington clinical laboratory Harborview (Seattle, WA).

Methods

Hooke's Law

Hooke's law was used to calculate the force that aggregated platelets produced. $F = k\delta$ where F is force, δ is post deflection, $k = 3\pi Ed^4/64L^3$, where E = 2.26 MPa is the modulus of elasticity of PDMS baked at 110 °C for 18 hours, d = 6.1 μm is the diameter of the post, and L = 25 μm is the length of the post (Figure 2A).

Collection and storage of platelets
a) For autologous transfusion into healthy humans: We collected platelets by apheresis using the Trima Accel Automated Blood Collection System (Terumo BCT, Denver, CO) and stored them in ACD-A and plasma. The target platelet yield was 4.5-6.3 X 10^{11}/unit depending on the donor's baseline platelet count and the target concentration of ~1.5 x 10^6 platelets/μL. Each unit had platelet concentrations and volumes within acceptable bag parameters. CSP were stored without agitation at 1-6°C. RSP were stored at 20-24°C with agitation, following standard practices. Both were stored for 5 days.
b) For in vitro experiments: Apheresis PRP in 100% donor plasma was collected as described above with a maximized target yield. Each unit had platelet concentrations and volumes within acceptable bag parameters. After collection, the platelets were evenly divided into two storage bags and stored in RSP and CSP conditions as described above.

Investigation of washed platelets after storage required developing a new protocol based on previously published methods.[1,2] Platelets were isolated from plasma by centrifugation. Platelets were pelleted at 1000g and resuspended in HEN Buffer[1] supplemented with PGI2. After centrifugation at 800g platelets were resuspended and diluted to 3 x 10^8

platelets/mL with modified Tyrode's buffer[3] supplemented with apyrase. Calcium and fibrinogen were added prior to adding agonists.

For preparation of platelet product platelet rich plasma 300x10^3/uL PRP was prepared by diluting fresh or stored platelets in concomitant plasma obtained from the respective stored platelets. PPP was obtained by pelleting PRP (fresh, RSP, CSP) at 2000g for 10 minutes and removing supernatant plasma. Where indicated, separately 1-6°C stored autologous plasma, that was obtained at the time of apheresis collection, was used to prepare 300x10^3/uL PRP.

Light transmission aggregometry: We performed the experiments at 37 °C under stirring conditions (1000 rpm) on a Chrono-log optical aggregometer (Chrono-log; Havertown, PA). Before agonist addition, light transmission was recorded.

Analysis of GPVI in platelets by nano flow liquid chromatography-tandem mass spectrometry (nano LC-MS/MS)

We obtained platelets by apheresis from healthy human volunteers as described above and stored for seven days. The relative abundance of GPVI in washed platelets from each group was analyzed by nano LC-MS/MS after trypsin digestion.[4] Briefly, washed platelets were lysed with 1% sodium deoxycholate (SDC) and homogenized with a sonic dismembrator (Fisher scientific). Ten micrograms of total protein from each platelet lysate were reduced by dithiothreitol (DTT), alkylated with iodoacetamide, and then digested with Trypsin/Lys-C mix (Promega) in Tris (pH 8) for 4 hrs at 37°C. Two heavy isotope labelled peptides (LSEAEFEVL*K and EAPDLVL*QR, L*= L-13C615 N, Anaspec) were added as internal standards. The digestion was stopped with trifluoroacetic acid (0.5% final). SDC was extracted with ethyl acetate and the peptides were desalted with C18 cartridges. The tryptic peptides were separated at a flow rate of 300 nL/min on an HSS T3 Column (100 × 0.075 mm, 1.8 μm, Waters), using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Peptides were eluted using a linear gradient of 5%-20% solvent B over 60 min, 20%-30% solvent B over 30 min. The LTQ Orbitrap Velos mass spectrometer was operated in a full scan in Orbitrap, followed by MS/MS acquisition of peptides of interest. The spray voltage was 3.0 kV, and the collision energy for MS/MS was 35%. GPVI abundance was determined by peak area of product ions of tryptic peptides, YGFDQFALYK and CQGPPGVDLYR, from MS/MS acquisition. Normalized results (to day 0, baseline) were used for statistical analysis.

Healthy human subjects study design

Eight healthy human subjects were included in a randomized, crossover study to evaluate the post-transfusion efficacy of CSP relative to the current clinical standard (RSP). We collected apheresis platelets and stored as either RSP or CSP as described above. After four days of platelet storage, participants received a loading dose of ASA

and clopidogrel, and 12–24 hours later were transfused the entire dose of stored platelets. We assessed the efficacy of transfusion with bleeding times, VerifyNOW assay, integrin activation, α-granule secretion, VASP phosphorylation, thromboxane B2 generation, and light transmission aggregometry. We performed tests at baseline, immediately before transfusion (after antiplatelet drug dosing), and three times after transfusion. A wash-out period of at least seven days between the first and the second round of apheresis collection, storage, and transfusion ensured clearance of previously transfused platelets and antiplatelet therapy. After the wash-out period, subjects underwent repeat plateletpheresis, storage at the alternative condition, autologous transfusion, and repeated platelet function assays. To save costs, the Verify NOW assay was performed only at 1h and 24h (no data are available for the 4h time points).

Corrected count increment The corrected count increment was calculated as previously described [5]. CCI= count increment (per μL) × body surface area (m^2) / unit content for all time points.

Unit characteristics and safety assessment. All platelet units passed quality control assessments after storage, except for one RT-stored unit that had to be discarded because of low pH. Transfused platelets were tolerated well by all recipients. One subject developed a headache, which was considered unrelated to transfusion. The absolute amount of platelets transfused did not differ significantly between the two study arms. Still, there was a trend for a higher post-storage platelet count in the cold-storage group (Supplemental Figure 3).

Platelet preparation of whole blood-derived platelets for post-transfusion testing:
Platelets were isolated, washed and re-suspended at a concentration of 3×10^8 platelets/ml in Tyrode's Buffer (137 mM NaCl, 0.3 mM Na2HPO4, 2mM KCl, 12 mM NaHCO3, 5 mM glucose, pH 7.3) containing 0.35% BSA and 1 mM CaCl2.

Bleeding time assay
Bleeding time templates were sterilized before each use. We performed the assay, as described in the original publication by Ivy and Mielke.[6] In brief, disposable surgical blades (#11) made from sterile stainless steel (Medline Industries, Inc., Mundelein, IN) were attached to the sterile template. A sphygmomanometer was inflated to 40 mmHg on the left or right arm. The template and blade were used to perform two controlled scratches on the respective forearm with 1 mm depth and 9 mm length on the volar side. The time required for cessation of bleeding was then measured by blotting with filter

paper (Accriva Diagnostics, Inc, San Diego, CA) every 30 seconds without disturbing the wound. If bleeding persisted, we stopped the test after 30 minutes.

αIIbβ3 activation / α-granule secretion Platelets in plasma or whole blood was activated with ADP, arachidonic acid, or convulxin at the indicated concentration for 5 minutes, then stained with PAC-1 antibody conjugated to FITC, CD62P (clone AK2) antibody conjugated to PE, and CD61 conjugated to PerCP (all from BD Biosciences) for 15 minutes and studied immediately by flow cytometry. Platelets were identified by forward/side scatter characteristics and as CD61+ events.

Thrombin Generation Assay

Apheresis platelets were obtained and stored as outlined above. Platelets were diluted from storage concentration ($1{,}500x10^3/\mu L$) with either storage supernatant plasma, separately-stored, 4°C autologous plasma, or fresh frozen plasma (stored at -80°C on the day of collection) from the same donor to dilute platelets to a target concentration of $1x10^5/\mu L$. The diluted platelets were tested in a commercially available fluorogenic Stago Thrombinoscope (Stago, Parsippany, NJ, USA). Peak thrombin concentration, time to thrombin peak, endogenous thrombin potential (AUC), and lag time (time to first thrombin detection) were read as reported by the instrument.

Mice

UBiC-GFP mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and crossed with C57BL6/J to generate f1 offspring with GFP+ platelets. C57BL6/J were purchased from Jackson Laboratories. The Animal Care and Use Committee of Bloodworks Northwest Research Institute approved all experimental procedures.

Supplemental Figure Legends

Supplemental Figure 1: Enrollment and study flow chart

Healthy human subjects were screened, enrolled, and randomized according to the flow diagram. Abbreviations: PLT (platelet), HCT (hematocrit), QC (quality control).

Supplemental Figure 2. Platelet unit characteristics

(A) Graphical representation of the transfusion sequence with study arm assignment and duration of wash-out period in between transfusion arms. The left columns represent the first (randomized) group assignment (blue bars: 4°C-stored, red bars: RT-stored), the middle column shows the duration of the wash-out period (in days), and the right column represents the second study assignment ("alternative study product"). One subject could not complete the second (RT-Stored) arm because of quality control

failure (blank second arm) ***(B)*** Number of platelets before (pre) and after (post) storage. Post storage data indicate the number of platelets transfused. Dot plots for RT-stored (red circles) and 4°C-stored (blue squares), n=8, **p=0.0034 for RT pre vs. post, *p=0.01 for RT post vs. 4°C post, *p=0.0275 (for pre RT vs. pre 4°C), n.s. = not significant.

Supplemental Figure 3. Platelet function characterization after transfusion in healthy subjects

BL: Baseline, *LD:* after loading dose, *1h, 4h, 24h post transfusion:* time points after transfusions ***(A)*** We stimulated platelet-rich plasma with arachidonic acid (1mM), and low dose (LD, 5mM), and high dose (HD, 20mM) ADP for 5 minutes and stained with either PAC-1 antibody for integrin activation or an antibody against, P-selectin (Anti-CD62P), and CD61 antibodies at the indicated time points. ***(B)*** Platelet-rich plasma was utilized to assess VASP-phosphorylation with a commercially available assay by flow cytometry. Results were calculated as the platelet reactivity index (PRI) based on manufacturer's instructions. The dotted line represents the defined cut off for P2Y12-inhibition at 0.5 PRI. ***(C)*** Thromboxane B2 generation measured by ELISA after stimulation with 0.5mM arachidonic acid. Data are shown as mean ± SEM, n=6-7. ***(D)*** Platelet reactivity tested by Verify NOW for clopidogrel (PRU, P2Y12 reaction units). Dashed line indicates cut off for P2Y12 inhibition at 210 PRUs. ***(E)*** Absolute bleeding time was determined as the mean of two separate forearm cuts at indicated time points. Transfusion of RSP (red circles) and transfusion of 4°C-stored platelets (blue squares). Shown as mean ± SEM, n=7-8.

Supplemental Figure 4. Thrombin generation assay in CSP vs. RSP

Fresh and stored platelet-rich plasma was diluted to reach target concentration with either ***(A)*** stored supernatant plasma, ***(B)*** separate, 4°C-stored concomitant plasma, or ***(C)*** separate, fresh frozen plasma from the same donor. Peak: Peak thrombin concentration, ttPeak: time to peak thrombin concentration, ETP: Endogenous Thrombin Potential, Lag time: Time for thrombin generation to first occur. Data are shown as mean ± SEM, n=3-6, *p≤0.05, **p<0.01

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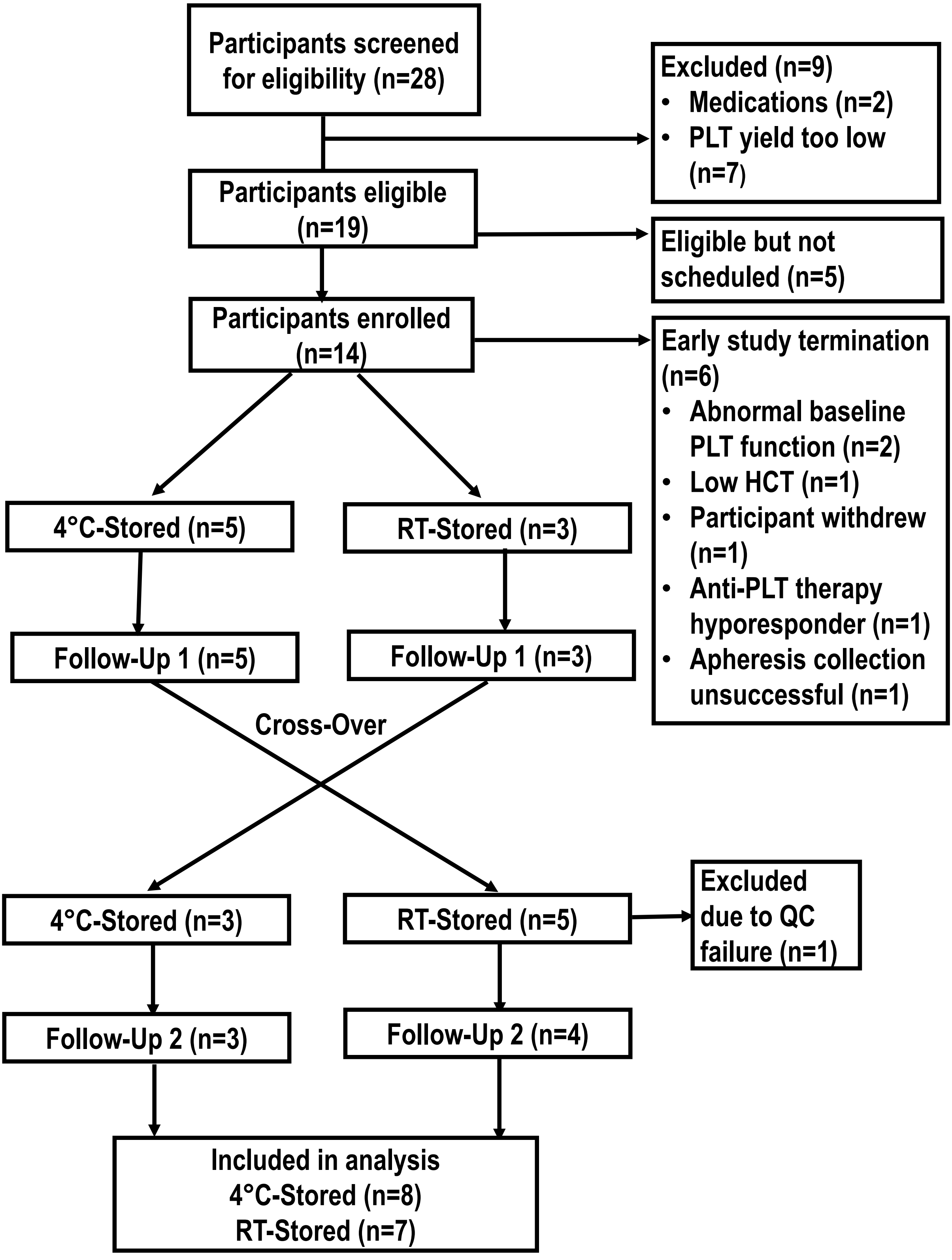
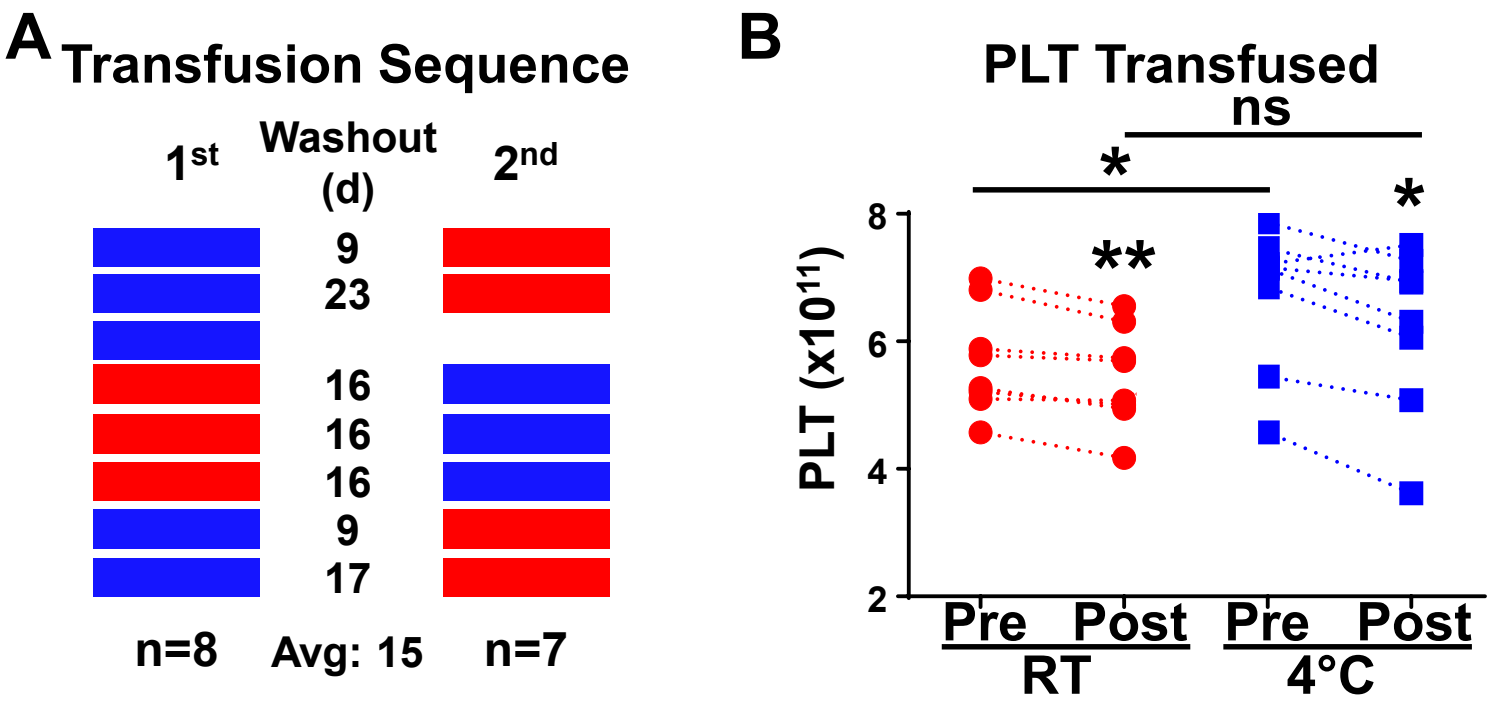
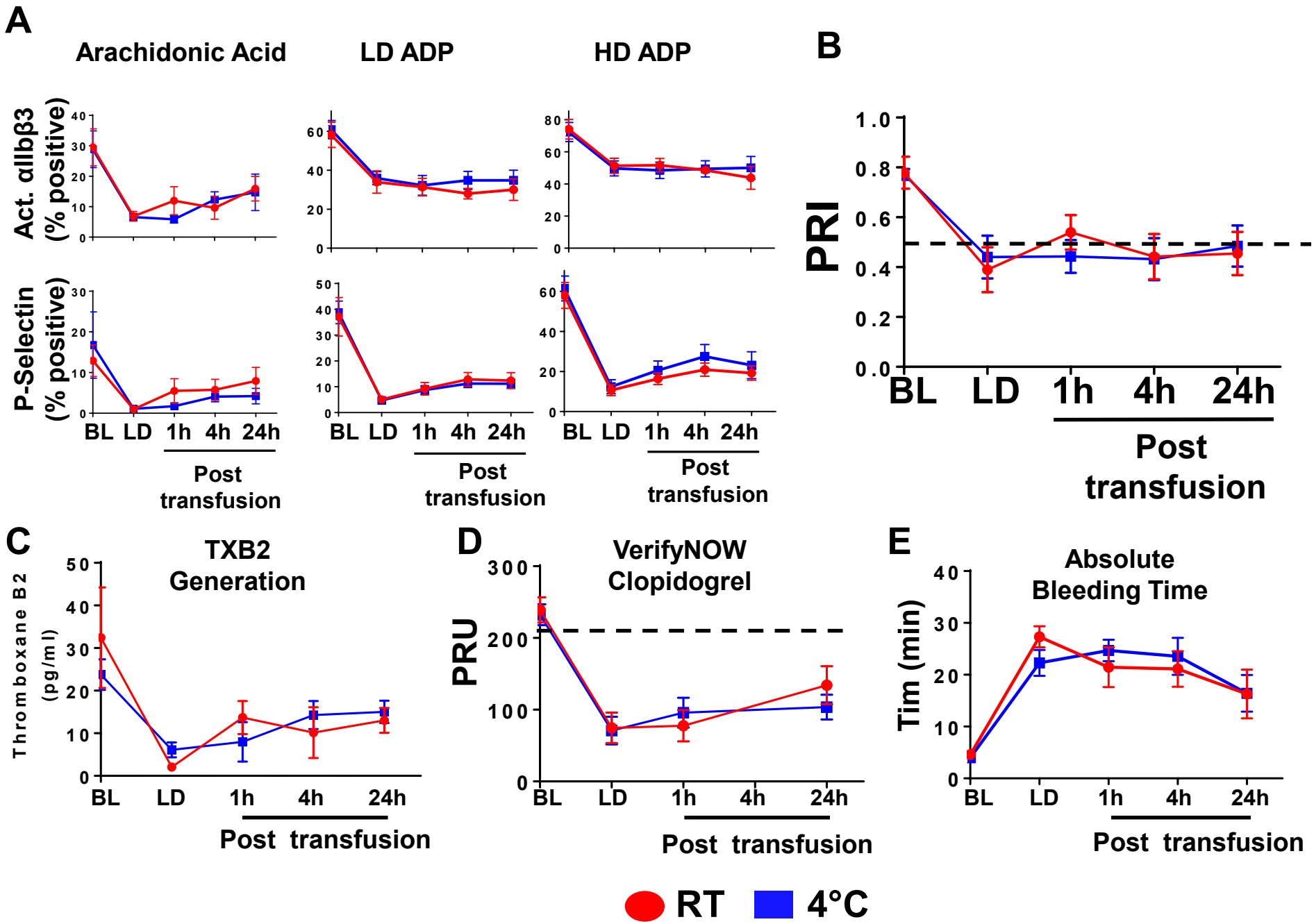
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4